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PURIFICATION AND CHARACTERIZATION OF A β-GLUCOSIDASE SPECIFIC FOR ISOFLAVONE CONJUGATES FROM SOYBEAN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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*****

The Ohio State University
1997

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ABSTRACT

The objective of this research project is to purify and characterize an isoflavone-conjugate-specific β-glucosidase, which is a key enzyme hydrolysing the constitutive isoflavone conjugates in soybean to release their aglycones, genistein and daidzein. Genistein was found to be toxic to the soybean pathogen, Phytophthora sojae, the causal agent of soybean root rot, one of the most devastating diseases of soybean. On the other hand, daidzein is the precursor of the soybean phytoalexin, glyceollin, which is more toxic and believed to play an important role as a second line of host defense. It is proposed that the isoflavone-conjugate-specific β-glucosidase is involved in broad spectrum resistance or tolerance since the quick buildup of toxic substances in proximal cell populations hampers invading pathogens.

The isoflavone-conjugate-specific β-glucosidase was found mainly in soybean roots, especially in root tips, which is consistent with the fact that the malonylated conjugate of daidzein reaches the highest levels in root tips. In addition, this enzyme is found to exist apoplastically, and can be washed off partially from the root cell wall fraction by high concentrations of sodium chloride. The isoflavone-conjugate-specific β-glucosidase was purified by means of ammonium sulfate fractionation and DEAE Sephadex and CM-Sephadex ion exchanges to near homogeneity. The isoflavone-
conjugate-specific β-glucosidase exhibited high sensitivity to silver (Ag⁺) as well as mercury (Hg²⁺) ions, which permanently and covalently block free SH groups, indicating that a free sulfhydryl group is essential to enzyme activity. The presence of free sulfhydryl group protectants such as β-mercaptoethanol protected the enzyme activity from inhibition, confirming the importance of the sulfhydryl group in the activity of this enzyme. The common β-glucosidase inhibitor, glucono-δ-lactone, did not suppress 50% of the enzyme activity until the concentration was as high as 24 mM. With respect to substrate specificities, the isoflavone-conjugate-specific β-glucosidase showed very high specificity with its own natural substrates, including the malonyl glucosyl conjugates and glucosyl conjugates, but low specificity toward other glucosides. Surprisingly, this enzyme appears to cleave the malonylated glucosides without prior demalonylation. In contrast, other β-glucosidases only hydrolyze terminal unmodified glucose moieties from their respective structures. A working model is proposed to illustrate the possible functions of the isoflavone-conjugate-specific β-glucosidase in plant defense and the interactions between microbes and soybean.
Dedicated to Shu-Kang, Shinru, and my parents
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Physiology and Biochemistry of Disease Resistance – An Overview

The occurrence of plant disease is not a common feature in nature, although it happens frequently in a uniform agricultural environment. There are more than a thousand plant diseases caused by fungi, bacteria, viruses, and nematodes, but much more microbes serve as nonpathogens to plants even under optimal conditions for disease development. These non-pathogens do not trigger incompatible responses when they encounter plant hosts. This kind of resistance is called non-host resistance. However, even though some potentially pathogenic microbes, for instance, fungi, can invade plants successfully, disease on a particular plant species may still not appear. This phenomenon is called a basic incompatibility at the species level (Callow, 1987). On the other hand, within a plant species, some strains or races of a known pathogen cannot cause disease on certain cultivars, but some others can. These are incompatibility and compatibility at the cultivar level, respectively (Callow, 1987). Although non-host resistance is more prevalent in the nature, the mechanism of non-host resistance still remains unclear (Staskawicz et al., 1995). In contrast, at either the species or cultivar level, disease resistance is carried out by an array of defense strategies, including passive/active and physical/chemical ones (Johal et al., 1995). The induced or active responses of host plants to infection by pathogens has been more attractive and studied more extensively to date.

The active defense mechanisms of plants include cell wall reinforcement by means of phenolic polymer deposition (Graham and Graham, 1991a), leading to lignification.
(Walter, 1992), and the cross-linking of hydroproline-rich proteins. Also included are the synthesis of phytoalexins, the induction of pathogenesis-related proteins (including the hydrolytic enzymes, chitinase and glucanase), the production of active oxygen species (Low and Merida, 1996), and ultimately, a form of programmed cell death termed the hypersensitive response (HR) at the location where pathogens invade (Dixon et al., 1994; Lamb et al., 1989). To deploy defense mechanisms successfully, host plants need to recognize the pathogens, in a way grossly similar to the immune system in mammals. The recognition, which usually occurs after pathogen penetration and colonization of plant tissues, may result from the interactions between either specific or general elicitors and their receptors, or by means of cell membrane depolarization.

**Gene-for-Gene Hypothesis**

The proposed gene-for-gene hypothesis and its modifications partially interpret the plant-microbe recognition. The principle of the gene-for-gene concept, proposed by Flor (1956), is that for every gene that confers avirulence in the pathogen, there is a corresponding gene in the host conferring resistance (Flor, 1971). In the host, the resistance gene (R) is usually dominant to susceptibility (r), and virulence (a) is usually recessive to avirulence (A) in the pathogen. The avirulence gene of a given pathogen and the resistance gene of a given host are usually dominant. Only the interaction of the products of an avirulence gene with the corresponding resistance gene will result in the resistant or incompatible response. On the basis of this hypothesis, a modified model was proposed by Callow (1984), which depicts the possible relationships between elicitors, nonspecific suppressers and the host in the evolution of race- and cultivar-specificity. The prevailing view of coevolution between host and pathogen is driven by natural selection in the local plant population. This point of view was widely used to explain the rapid emergence (or selection) of new races that break the resistance in the field. However, recent data has challenged the natural selection theory. Several lines of evidence have demonstrated that gene flow and genetic drift may be at least as important as true natural selection in the
genetic dynamics to build new races (Thompson and Burdon, 1992). Furthermore, an interesting observation in populations of host and pathogen is that each host usually carries few resistance factors, but each pathogen has many virulence factors (Frank, 1992).

**Resistance Genes in Plants**

In Callow's model (Callow, 1984), the sensor recognizing the elicitor was thought to be a receptor encoded by the corresponding resistance gene, and the receptor is not necessarily solely on the membrane. The putative receptor could also be intracellular as shown in several polypeptides deduced from cloned resistance genes. Several lines of evidence from recent molecular analysis of the cloned resistance genes support the idea that resistance genes encode receptors. According to the cloned gene sequences, there are at least four classes of gene products differentiated by the structures of the deduced products (Dangl, 1995; Staskawicz et al., 1995; Song et al., 1995). The first class includes the *R* genes from *Arabidopsis* (*RPS2* and *RPM1*; Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995), tobacco (*N*; Whitham et al., 1994), and flax (*L*; Ellis et al., 1995). The common features of these gene products are the imperfect leucine-rich repeats (LRRs), which are involved in protein-protein interactions (Kobe and Deisenhofer, 1993), and a conserved nucleotide-binding site. These gene products may be cytoplasmic, and the molecular recognition of these proteins and their respective elicitors occurs inside plant cells (Leister et al., 1996). The second class of *R* gene products is represented by the tomato Cf-9 protein encoded by the *Cf-9* gene against the fungal pathogen, *Cladosporium fulvum*, with express the AVR9 peptide elicitor (Jones et al., 1994). The Cf-9 protein also contains leucine-rich repeats, but distinctively, the LRRs are extracytoplasmic. In addition, followed the LRRs there is a C-terminal membrane anchor, indicating that Cf-9 protein is a receptor binding to the putative ligand, AVR9 peptide outside the cell (Jones et al., 1994). The first cloned nematode resistance gene, *Hslpro-1*, belongs to this class as well. *Hslpro-1* was cloned from wild beet to resist the feeding of beet cyst nematode (*Heterodera schachtii*), and confers resistance in cultivated sugar beets that are highly susceptible to the cyst
nematode. Intriguingly, this resistance gene encodes a putative signal peptide, an imperfect LRR, a hydrophobic transmembrane region, and a charged amino acid C-terminus, indicating that the Hs1 \textsuperscript{pro-l} protein is also a receptor residing on the plasmalemma. However, the elicitor remains unclear to date (Cai et al., 1997). The third class of \textit{R} gene is represented by the \textit{Pto} \textit{R} gene from tomato that resists \textit{Pseudomonas syringae} expressing the \textit{avrPto} avirulence gene (Martin et al., 1993). The Pto protein is a cytoplasmic serine/threonine kinase with a potential myristolation site, which makes Pto a potential membrane-associated protein. It has been suggested that the Pto protein interacts with some other gene products including Fen, controlling the sensitivity to the pesticide fenthion and its gene sharing 80\% homology with \textit{Pto} (Martin et al., 1994), Prf, that is required for \textit{Pto}-mediated resistance and fenthion sensitivity (Salmeron et al., 1994), and Pt\textit{i} protein, which is another serine/threonine kinase phosphorylated by the Pto protein (Zhou et al., 1995). Very recent data using the yeast two hybrid system demonstrated that \textit{avrPto} directly interacts with Pto, but not with Fen (Tang et al., 1996; Scofield et al., 1996). The last class includes the rice resistance gene \textit{Xa21} against the pathogen \textit{Xanthomonas oryzae pv. oryzae} race 6. The \textit{Xa21} gene encodes a receptor kinase-like protein consisting of an N-terminal signal peptide, LRRs, a hydrophobic membrane-spanning helix, and an intracellular protein kinase domain with serine/threonine specificity (Song et al., 1995). A new receptor-like kinase encoded at the \textit{Lr10} disease resistance locus of wheat, conferring disease resistance against leaf rust, has been isolated. The gene of this receptor-like protein kinase, \textit{Lrk10}, and \textit{Lr10} belong to the same genetic locus, providing, therefore, a good candidate for disease resistance genes in the complex wheat genome (Feuillet et al., 1997).

Usually, a resistance gene cannot confer resistance alone. Some other genes may be also involved in disease resistance to play the roles in signal transduction and regulation. As mentioned above, for instance, Pt\textit{i} protein is the substrate of Pto kinase, and once it is active, it phosphorylates the next protein to further transduce the signal. According to newly released data, a new model was proposed to describe the interactions between Pto and \textit{AvrPto} proteins (Lamb, 1996). In this model, \textit{AvrPto} is introduced into tomato cytoplasm.
by means of a type III secretion apparatus encoded by the hrp genes, and then interacts with Pto that is associated with Prf, a transmembrane protein. The phosphorylation cascade is transduced to trigger either the oxidative burst and hypersensitive cell death or other gene activations (Lamb, 1996). It is interesting that most of the cloned R genes appear to have alleles in susceptible cultivars of the same plant species as seen by hybridization experiments. For instance, pto and fen alleles are found in susceptible hosts and the predicted proteins are 87 and 98% identical to the Pto and Fen protein kinases, respectively. Interestingly, these two proteins are also active protein kinases and pto is able to phosphorylate the Pt1 protein kinase similar to Pto protein. However, pto interacts weakly with Pto-interacting proteins including Pt1, 2, 4, and 6, suggesting that several amino acid substitutions in pto impair the recognition and cannot, thus, offer resistance (Jia et al., 1997). On the other hand, AVR9 binding analysis showed that this specific elicitor did bind to the membrane preparation from the cultivars with Cf-9 and Cf-0 genes. The latter cultivar, however, does not respond to the AVR9 peptide, suggesting the loss of other gene products playing roles downstream of signal perception (Honee et al., 1994). Additionally, two distinct mutants not in the Cf-9 locus, show that other genes are required for the fully resistant response, designated as Rcr-1 and Rcr-2 (Required for Cladosporium resistance; Hammond-Kosack and Jones, 1995). Similarly, nar-1 and nar-2 genes affect resistance mediated by Mla12 in barley against powdery mildew Erysiphe graminis f. sp. hordei (Freialdenhoven et al., 1994). The function of these genes remains unclear.

Another disease resistance gene from maize, Hml, which acts by a non-gene-for-gene mechanism has also been cloned too. In fact, it was the first resistance gene cloned by gene tagging strategy (Johal and Briggs, 1992). Hml encodes an HC toxin reductase that hydrolyzes the cyclic tetrapeptide HC toxin produced by the fungal pathogen Cochliobolus carbonum (Johal and Briggs, 1992). Briggs and Johal (1994) proposed a new concept of “compatible interaction”, instead of incompatible interaction, which is the traditional gene-for-gene interaction, to describe this kind of resistance. In compatible interactions, resistance results from an interaction in which the compatible factor is destroyed by a
resistance factor of the plant. This kind of resistance is also very stable compared to the resistance conferred by gene-for-gene interactions.

A handful of other resistance genes are current targets for isolation. They are, for example, I2, Lv, and Mi in tomato against *Fusarium oxysporum f. sp. lycopersici*, *Leveillula taurica* and *Meloidogyne* spp., respectively, *RP1* in maize against *Puccinia sorghi*, *Rpg4* in soybean against *Pseudomonas syringae* pv. *lycopersici*, *Leveillula taurica* and *Meloidogyne* spp., respectively, *RP1* in maize against *Puccinia sorghi*, *Rpg4* in soybean against *Pseudomonas syringae* pv. *lycopersici*, *RPP5* in *Arabidopsis* against *Peronospora parasitica*, and some viral resistance genes such as *Tm2a* and *Ts-5* in tomato against TMV and tomato spotted wilt virus, respectively (Martin, 1996).

**The Recognition of Elicitors**

**A. Race-Specific Elicitors**

AVR proteins have been considered as specific elicitors (race-specific elicitors) to trigger a battery of defense responses in plants with corresponding resistance genes. The first avirulent gene cloned was the *avrA* gene from *Pseudomonas syringae* pv. *glycinea* race 6 (Staskawicz et al., 1984), and the first fungal avirulent gene cloned was *avr9* from *Cladosporium fulvum*, a biotrophic pathogen of tomato (van Kan et al., 1991). The AVR9 protein is a 28-amino acid polypeptide which elicits necrosis in hosts carrying the *Cf9* gene (Schottens-Toma and de Wit, 1988). Another avirulence gene product from *C. fulvum*, AVR4, corresponding to the *Cf4* resistance gene of tomato, is a 106-amino acid protein (Joosten et al., 1994). Other fungal avirulence genes include *PWL2* and *AVR2-YAMO* of the rice blast fungus, *Magnaporthe grisea*, *avrRs1* of the barley pathogen, *Rhynchosporium secales*, and the putative avirulence gene *parA1* from *Phytophthora parasitica* (De Wit, 1995). It is worth noting that fungal avirulence genes encode not only race-specific elicitors, but also species-specific elicitors such as *PWL2* and *parA1* genes (De Wit, 1995). *PWL2* and *AVR2-YAMO* genes encode glycine-rich proteins of 145 amino acids with a signal peptide and 223 amino acids, respectively. Both proteins have no significant homology to other proteins with known functions. A necrosis-inducing protein, NIP1, was found to elicit disease resistance other than the hypersensitive response in resistant barley carrying *Rrs1*.
By reverse genetics, the *nip1* gene has been cloned and encodes an 82-amino acid pre-protein with a 22-amino acid secretory signal sequence. This *nip1* gene has been demonstrated to be the *AvrRrs1* avirulence gene (Rohe et al., 1995), and therefore NIP1 is the product of the fungal avirulence gene, *AvrRrs1*. NIP1 is a bifunctional signal molecule that elicits disease resistance in *Rrs1* barley and acts as a virulence factor in *rrs1* barley (Fiegen et al., 1996). In the incompatible reaction, NIP1 elicits specifically the accumulation of mRNA encoding the barley PR protein PRHv-1, whereas in susceptible cultivars, the elicitor acts as an non-specific toxin that stimulates plasma membrane-localized H^+-ATPase to kill the host and to obtain nutrients (Fiegen et al., 1996). Parasiticein, produced by *Phytophthora parasitica*, is one of the α-elicitins which are acidic proteins in contrast to the basic hydrophilic β-elicitins such as cryptogein (Yu, 1995). Parasiticein producers are all non-pathogenic, and non-producers are all pathogenic with various degrees of aggressiveness. From this and some other genetic studies, parasiticein can be considered as a species-specific avirulence factor in the *P. parasitica*-tobacco interaction (Ricci et al., 1992). Based on the amino acid sequence of parasiticein, the *parAl* gene has been cloned and it encodes a pre-protein of 118 amino acids with a 20-amino acid signal peptide. Similar to NIP1, parasiticein may be not only a avirulence factor, but also a factor in the parasitic and saprophytic phases of the pathogen (Kamoun et al, 1994). For viruses, the first proven viral avirulence gene product causing HR in tobacco with the *N* resistance gene is the coat protein of tobacco mosaic virus (TMV) (Culver and Dawson, 1991). The understanding of avirulence genes and their products provides insight into function of the gene-for-gene systems (Keen, 1992). The interaction of AVR gene product and its corresponding resistance gene product is very specific. A single base-pair change in an avirulence gene can cause the loss of resistance. For instance, in TMV, there is only one amino acid difference, due to a point mutation, between an avirulent strain and the virulent one (Knorr and Dawson, 1988). Similarly, one point mutation in the *avr4* gene of *Cladosporium fulvum*, replacing a cysteine residue by a tyrosine residue at the three position, caused a total loss of resistance (Joosten et al., 1994). The *Arabidopsis thaliana*
disease resistance genes RPS2 and RPM1 confer disease resistance to *Pseudomonas syringae* carrying *avrRpt2* and *avrRpm1* (and *avrB*), respectively. Expression of these avirulence genes in each corresponding host's cells by a quantitative transient expression assay revealed the fact that no other bacterial factors but the avirulence gene products are required for the specific resistance response (Leister et al., 1996). In addition, gene transfer and disruption experiments also demonstrated that AVR4 and AVR9 are the molecular determinants of avirulence for their corresponding hosts (Marmeisse et al., 1993).

Some AVR proteins are located apparently in the cytoplasm of the microorganisms, particularly bacterial avirulent genes such as *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria* (Brown et al., 1993), *avrRpt2*, *avrB* and so on, without known physiological or biochemical functions. Three models have been proposed to illustrate the binding and interaction between AVR proteins and host cells (Leach and White, 1996). In model 1, AVR protein is the elicitor *per se* and recognized by host receptor anchored on plasmalemma. AVR9 and Cf-9 belong to this class. In model 2, the avirulent gene encodes an enzyme which is involved in the elicitor biosynthesis, and then the elicitor is bound by the host specific receptor on the membrane. An example is the *avrD* gene product from *Pseudomonas syringae* pv. *tomato*, which is an enzyme in the biosynthetic pathway for the specific elicitors, syringolide 1 and 2 (Keen et al., 1990), which trigger HR in soybean containing the *Rpg4* resistance gene. Model 3 suggests that the elicitor (AVR) is secreted into the host cell through several *hrp* gene products which form a Type III secretion apparatus (He, 1996), similar to the mechanisms used by the animal pathogen, *Shigella*, to enter epithelial cells (Ménard et al., 1996). Harpin<sub>poss</sub> was the first proteinaceous elicitor suggested to be secreted via this Type III apparatus (He et al., 1993). In addition to being the specific ligand to elicit host responses, AVR proteins may benefit pathogenicity, from the evolutionary point of view as well, although they are not critical for the survival of the pathogen (Leach and White, 1996).
B. General Elicitors

In other cases, the perception of signals may be more general, instead of occurring in the gene-for-gene manner. General elicitors may result from specific structural or chemical features of the pathogens or from the general stress and damage associated with the invasion. General elicitors, also called nonhost elicitors (Atkinson, 1993) or non-race-specific elicitors (Lamb et al., 1989), can be of either pathogen or plant origin. Well defined bacterial nonhost elicitors are encoded by the *hrp* genes (hypersensitive reaction and pathogenicity). The *hrp* gene products, derived from *Pseudomonas*, *Xanthomonas*, and *Erwinia* spp., are required for pathogenicity on susceptible host plants and for induction of local resistance on resistance host and nonhost plants (Willis et al., 1991). A 44 kD protein, harpin, encoded by the *Erwinia amylovora hrpN* gene, was demonstrated to elicit HR in tobacco, tomato, and *Arabidopsis thaliana* (Wei et al., 1992). Some *hrp* genes are regulated or activated by plant signals or environmental factors in a manner like the two-component signaling systems in *Agrobacterium* and *Rhizobium* (Clarke et al., 1992; Rahme et al., 1991).

Fungal elicitors include oligosaccharides such as the 1,3-1,6-β-D-glucans and chitosan, galactose- and mannose-rich glycoproteins, and long chain fatty acids, such as arachidonic acid (Darvill and Albersheim, 1984; Lamb et al., 1989). Among these elicitors, the fungal glucan elicitors have been studied most extensively, and mainly for their induction of phytoalexin responses in soybean cotyledon bioassays (Darvill and Albersheim, 1984; Ebel and Cosio, 1994). The best-characterized one is a 1,3-1,6-β-linked glucan from *Phytophthora sojae* cell wall, the causal agent of soybean root rot (Sharp et al., 1984). The smallest active molecule purified from this fungus has a degree of polymerization (DP) of seven, and can elicit phytoalexins in soybean tissues when present at about 1 pg/g tissue (Sharp et al., 1984a). Structure-function studies showed that the biological activity of the oligo-β-glucosides requires a structural backbone (as below) with or without a reducing end (Sharp et al., 1984b; Hahn and Cheong, 1991). However, it is not necessary that the
Glu-1,3-β-Glu-1,3-β-Glu-1,3-β-Glu-1,3-β-Glu (reducing end or not)

\[
\begin{array}{c}
| & | \\
1,6-β & 1,6-β \\
| & |
\end{array}
\]

Glu Glu

naturally occurring glucan elicitor should be as small as DP 7. In fact, the release of elicitor-active carbohydrates from fungal cell walls by β-1,3-endoglucanase rather than acid extract resulted in larger fragments (Okinaka et al., 1995). In addition, the release of fungal cell wall fragments occurs not only from the hydrolysis of mycelial walls of *P. sojae* by β-1,3-endoglucanase (Keen and Yoshikawa, 1983), but also spontaneously during the normal process of cyst germination of *P. sojae* (Waldmüller et al., 1992).

Chitin- and chitosan- (de-N-acetylated chitin) derived fragments, released from fungal cell walls by plant chitinases and chitosanases, are another group of oligosaccharides that elicit plant lignification, phytoalexin synthesis, callose deposition, and the induction of proteinase inhibitors (Hahn et al., 1993). Structure activity studies revealed that the oligomers having a DP of less than four are not active, while oligomers with a DP of six or more are active, although the activity was also dependent on the plant species (Hahn et al., 1993). The basic structures of an oligochitin and an oligochitosan are illustrated as follows, where GlcNAc is N-acetylglucosamine and GlcN is glucosamine.

\[
\begin{array}{c}
\beta\text{GlcNAc}(1\rightarrow[4]\text{GlcNAc}(1\rightarrow],n4)\text{GlcNAc} & \text{Oligochitin} \\
\beta\text{GlcN}(1\rightarrow[4]\text{GlcN}(1\rightarrow],n4)\text{GlcN} & \text{Oligochitosan}
\end{array}
\]

The last type of oligosaccharide, oligogalacturonides, is of plant origin. Oligogalacturonide elicitors are released by treating the primary cell walls containing homogalacturonans with acid or pectic degrading enzymes. The elicitors stimulate the accumulation of phytoalexins, the induction of PR-proteins such as β-glucanase and chitinase, the deposition of lignin and the accumulation of proteinase inhibitors (Hahn et al., 1993). The basic structure is \(\alpha\text{GalUA}(1\rightarrow[4]\alpha\text{GalUA}(1\rightarrow],n4)\text{GalUA}\), where GalUA
represents α-galacturonic acid (Mohnen and Hahn, 1993; Côté and Hahn, 1994). The size range of active oligagalacturonides is quite narrow, and usually a DP between 10-15 is required to elicit most of the plant defense responses. It also stimulates defense gene transcription with a DP greater than 9 in appropriate conditions (Messiaen, and Van Custem, 1993). In addition to oligogalacturonides, xyloglucan oligosaccharins are also of plant origin. This class of oligosaccharides was thought to regulate plant hormones by suppressing auxin-stimulated growth (Côté and Hahn, 1994). However, a recent report uncovered that pretreatment with pentaxyloglucan (XG5) and nonaxyloglucan (XG9) on wheat kernels reduced the number of infected plants which were inoculated with *Fusarium culmorum* spores. Moreover, they also promoted phytoalexin accumulation in soybean cotyledons (Pavlova et al., 1996).

Other than oligosaccharides, proteinaceous fungal elicitors are the second large group for triggering plant responses. A 67 kD glycoprotein elicitor from cell walls of *Puccinia graminis* f. sp. *tritici* was purified, and the carbohydrate portion of the molecule was shown to be responsible for its activity, which is triggering of the hypersensitive lignification response in wheat (Kogel et al., 1988). The fungal pathogen, *Phytophthora sojae*, produces not only cell wall glucan as elicitor, it also yields a glycoprotein elicitor with a *Mr* of 42 kD that triggers defense responses, including H^+/Ca^{2+} influxes, K^+/Cl^- effluxes, an oxidative burst, defense-related gene activation, and phytoalexin formation in parsley. The elicitor activity resides in the polypeptide portion, and more precisely a 13-amino acid oligopeptide located in the C-terminal third of the protein (Sacks et al., 1995). A class of small protein elicitors, termed elicins, was identified from *Phytophthora parasitica* var. *nicotianae*, *P. capsici* and *P. cryptogea* (Ricci et al., 1989; Yu, 1995). Elicitins induce a hypersensitive necrosis in tobacco and systemic acquired resistance (Kamoun et al., 1994). A 10 kD elicitin, cryptogein, the best characterized one, induces the production of the sesquiterpenoid phytoalexin capsidiol at nM levels and the expression of defense-related genes (Suty et al., 1995). Another class of potent elicitors active in tomato suspension cultures are high mannnose-containing glycopeptides from yeast, which induce ethylene
biosynthesis and phenylalanine ammonia lyase (Basse and Boller, 1992). Interestingly, the elicitor can also be obtained by cleavage of yeast invertase with chymotrypsin. The most active glycopeptides are those that contain 10-12 mannosyl residues, and at least 9 mannosyl residues are needed to maintain good elicitor activity (Basse et al., 1992).

Two novel classes of elicitors, fungal sterol and cutin monomers, were reported recently (Grando et al., 1995; Schweizer et al., 1996). An abundant fungal sterol, ergosterol, purified from Cladosporium fulvum spore exudates, was found to elicit extracellular alkalization in tomato cell cultures. Other plant sterols and cholesterol were not active, except stigmasterol, but it was 1 million fold less active than ergosterol (Grando et al., 1995). The plant cuticle consists of hydroxylated and epoxydized fatty acids, and can be hydrolyzed by cutinase from plant pathogenic fungi to release cutin monomers, which are recognized by a number of fungal pathogens as chemical signals resulting in activation of the cutinase gene and enhancing cutinase activity (Kolattukudy, 1985). In addition to being a signal for fungi, cutin monomers also serve as endogenous elicitors of defense reactions such as induction of a transient alkalization of potato cell culture medium, activity similar to the reactions to chitin and chitotetraose. Due to the heterogeneity of cutin monomers, the effect of cutin monomers appeared to be dependent on their structure. For instance, cis-9,10-epoxy-18-hydroxystearic acid and palmitic acid are the most and least active compounds, respectively (Schweizer et al., 1996).

C. The Receptors for Specific Elicitors

The receptors for specific elicitors are believed to be the resistance gene products, although no direct evidence to support the assumption has been derived from the deduced amino acid sequences. The resistance genes and their products have been described earlier, so only one more result is described in this section. The race-specific elicitor AVR9 from Cladosporium fulvum was labeled with $^{125}$I at the N-terminal tyrosine residue for binding studies. The plasma membranes were isolated from tomato cultivars with and without the Cf9 resistance gene (MM-Cf9 and MM-Cf0, respectively). Specific, saturable, and
reversible binding between the labeled elicitor and these two plasma membranes was observed. The kinetics and binding capacity were similar for both membranes with dissociation constants of 0.07 nM. Moreover, some other solanaceous plants also contained AVR9 binding sites, but the AVR9 protein did not cause observable necrosis on these plants. These results suggests that other factors in addition to the AVR elicitor-binding proteins are required to complete the resistance. However, it is not demonstrated that the binding site on the MM-Cf9 is encoded by the resistance gene, Cf9, whose product was predicted to be a transmembrane receptor (Kooman-Gersmann et al., 1996).

**D. The Receptors for General Elicitors**

A recent review has described elicitors and their receptors in detail (Hahn, 1996). The search for the receptor for the well-studied hepta-β-glucoside had begun as early as 1987, using 3H-labeled glucans with a DP of 18-22 as ligands (Schmidt and Ebel, 1987). Evidence was shown that the receptor for the β-glucan elicitor was present on soybean plasma membrane and possessed a high affinity of Kd of 10-40 nM, irrespective of using 3H-labeled glucans with DP of 18-22 or 125I-labeled tyramine derivatives at the reducing end of the same glucans (Cosio et al., 1988). The putative receptor showed saturable and reversible characteristics by ligand-binding studies (Hahn and Cheong, 1991). The receptor was solubilized by detergents, Zwittergent 3-12 (ZW 3-12) and a lysolecithin analog (ES12H), and showed a pI close to neutral (Cosio et al., 1990a). Further study revealed that the 125I-labeled tyramine derivatives of heptaglucoside were able to replace the large glucan used previously. This indicated that the binding sites of the large glucans were identical to those binding to hepta-β-glucosides (Cosio et al., 1990b). Another label, a APEP conjugate (a photolabile derivative), was used to identify the receptor as a 70 kD protein by detergent solublization and subsequent column chromatography, but a significant loss of the binding activity following solublization was also reported (Cosio et al., 1992). Due to the loss of activity, other detergents, n-dodecylsucrose, n-dodecylmaltoside, and Triton X-114 were used (Cheong et al., 1993). In this study, the hepta-β-glucoside binding protein comigrated
with a plasma membrane marker enzyme, the vanadate-sensitive H\(^+\)-ATPase, on linear sucrose density gradient, and other results were similar to previous data (Cheong et al., 1993). This low abundance β-glucan elicitor binding protein was solubilized and purified up to 9000-fold by a rapid one-step purification method. By this method, a major protein in SDS-PAGE with *M* \(_r\) of 75 kDa was shown. The native form of this protein was an oligomer with *M* \(_r\) of 240 kDa (Mithöfer et al., 1996). The cDNA cloning of the 75-kDa binding protein is underway (Antelo et al., 1996). Although a lot of efforts have tried to purify the binding protein, the pure protein and more characteristics still await further studies.

Using an \(^{125}\text{I}\)-labeled tyramine conjugate of N-acetylchitooctaose as the ligand, incubated with microsomal membrane preparations from suspension-cultured rice cells, the high-affinity binding site for this oligochitin was found and had a *K* \(_d\) of 5.4 nM. Competitive studies showed that this binding site is specific for oligochitin and did not bind oligochitosan, which coincided with the fact that oligochitosans are not elicitors of phytoalexins in rice (Shibuya et al., 1993). The binding protein was solubilized by Triton X-100, and the solubilized protein showed a saturable mode of binding with radiolabeled ligand with a *K* \(_d\) of 90 nM. The oligochitin binding protein was purified to apparent homogeneity by affinity column chromatography and showed a molecular weight of 70 kDa (Shibuya et al., 1996). Similarly, the oligochitin binding site was also demonstrated to be on the plasma membrane of tomato cultured cells. By different labeling methods, with derivatization of t-butoxycarbonyl-l-[\(^{35}\text{S}\)]methionine, the specific binding sites on purified membranes were identified and showed a *K* \(_d\) of 23 nM. Binding on intact tomato cells showed a *K* \(_d\) of 1.4 nM. Again, the oligochitosan could not compete for binding with oligochitins, whereas oligochitins of DP 4 and 5 and a lipochitooligosaccharide from *Rhizobium leguminosarum* were effective competitors (Baureithel et al., 1994).

The binding proteins for several proteinaceous elicitors were also identified and some of them were characterized as well. The glycoprotein from the wheat leaf rust fungus, *Puccinia graminis*, was conjugated with ethyl \([1-\text{C}]\)acetimidate which did not reduce its activity. A high-affinity binding site, with a *K* \(_d\) of 2 \(\mu\)M, was demonstrated on wheat plasma
membranes. The binding protein seemed to have two subunits or there were two membrane proteins associated with this radiolabeled ligand (Kogel et al., 1991). Unfortunately, no further characteristics have been identified. Since the 13-amino acid oligopeptide (Pep-13) derived from the 42-kD glycoprotein produced by Phytophthora sojae is an active elicitor, binding studies using this peptide were carried out. The tyrosine in this peptide was radiolabeled with $^{125}$I and bound to microsomal membranes and intact parsley protoplasts in a specific, reversible, and saturable manner, indicating the presence of a specific binding protein. The $K_d$ values of the specific binding to microsomal membranes and the intact protoplast were 2.4 nM and 11.4 nM, respectively. An excellent correspondence between elicitor binding and elicitor-induced plant responses strongly supports that this binding site serves as a receptor of this Pep-13 elicitor (Nümberger et al., 1994). Moreover, through the use of covalent cross linking by using the radiolabeled ligand interacting with total membranes and intact protoplasts, a 91 kD protein was identified and competitive studies confirmed that this protein was the receptor (Nümberger et al., 1995).

A purified glycoprotein, derived from yeast invertase and containing 10 mannosyl residues (gP$_{\text{Man}}^10$) was derivatized with t-butoxycarbonyl-L-$^{35}$S]Methionine N-hydroxysuccinimidy l ester, and used as a ligand. A single class of reversible, high-affinity binding sites with $K_d$s of 0.7 and 3.3 nM were detected in cells and microsomal membranes of tomato, respectively (Basse et al., 1993). This elicitor binding site was solubilized from microsomal membranes, using neutral detergents (n-docecylmaltoside and n-dodecanoylsucrose), in an active form with $K_d$ of 1-4 nM. It was consequently purified 67-fold by DEAE-Sephacel anion-exchange chromatography. The partially purified binding protein selectively bound to N-linked glycans with nine mannosyl residues from fungal glycoproteins, but not with the typical mammalian glycans with nine mannosyl residues (Fath and Boller, 1996).

**Physiology and Biochemistry of Signal Transduction in Plant Defense Mechanisms**

After colonization by pathogens or perception of elicitors, a variety of incompatible
reactions occur in plant cells to ensure the final outcome, the restriction of disease development. The signals from outside are perceived by their specific receptors on the membrane or inside the cell, and then transduced as a cascade to evoke a series of reactions, and finally reach the nucleus where transcription of specific genes is initiated. Usually the incompatible reaction and disease resistance are associated with the hypersensitive reaction (HR), sometimes referred to as programmed cell death (PCD), which leads to membrane damage, necrosis, and cell collapse, and localizes pathogens (Goodman and Novacky, 1994). Biochemical responses correlated with the HR include electrolyte loss from plant cells, activation of $K^+$/H$^+$ exchange (XR), calcium influx, increased lipoxygenase activity, transient oxidative bursts, activation of specific defense-related genes, accumulation of phytoalexins, and alteration of plant cell walls (Atkinson, 1993). Therefore, the physiology and biochemistry involved in signal transduction and in the hypersensitive reaction are briefly described in this section.

An Overview of Signal Transduction

Signals transmitted from the extracellular spaces into the cytoplasm via the plasmalemma have been studied extensively in animal systems. Fortunately, many of the components involved in signal transduction of animal systems are also present in plant systems with some small differences. In general, the first step is always the binding of the ligands, such as neurotransmitters or hormones, with their specific receptors, leading to a conformation change and initiating the signal transduction cascade. There are three types of receptors involved in signal transduction: cytoplasmic receptors, e.g. steroid hormone receptors (Knowland, 1984), tyrosine kinase receptors, e.g. insulin and growth factor receptors, and G-protein-mediated receptors, e.g. the β-adrenergic receptors (Gennis, 1989). The last two are membrane bound receptors. The tyrosine kinase receptor has one transmembrane segment and the cytoplasmic domain is a tyrosine-specific protein kinase. Usually, the target of this kinase is the receptor per se, called autophosphorylation. The G-protein-mediated receptor, on the other hand, has seven transmembrane segments. On the
cytoplasmic side, the receptor is associated with a GTP binding protein which has three subunits: $\alpha$, where the GTP is bound, $\beta$, and $\gamma$. When the extracellular ligand (agonist) is bound by the receptor, a conformational change activates the $\alpha$ subunit to bind a GTP molecule and then the $\alpha$ subunit is dissociated from the $\beta$ and $\gamma$ subunits to move toward an effector, another membrane associated protein, which is then activated. The effectors, including adenylate cyclase, phospholipase C, ion channels, and so on, trigger subsequent reactions. The default GTPase activity of the $\alpha$ subunit hydrolyzes GTP to GDP, and then re-associates with the $\beta$ and $\gamma$ subunits to await the next stimulus (Gillman, 1987). Although cAMP, the product of adenylate cyclase, is a well-known secondary messenger in animal systems, invoking an cAMP-dependent protein kinase which phosphorylates other proteins downstream to regulate cell functions, its role in plant systems still remains equivocal. Another effector, phospholipase C (PLC), plays an essential role in both systems. Activated phospholipase C cleaves the membrane component, phosphatidylinositol 4,5-biphosphate (PIP$_2$), into two sub-components, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$). Both of them are messengers: DAG triggers the activity of protein kinase C (PKC), which is also membrane-bound and then phosphorylates other downstream proteins; IP$_3$, on the other hand, binds to a specific receptor on the organelle membrane, for instance, endoplasmic reticulum or vacuole, where calcium ion (Ca$^{2+}$) is sequestered in the lumen, to release Ca$^{2+}$. Calcium ion has many physiological functions including the activation of PKC and Ca$^{2+}$-calmodulin-dependent protein kinase. Another G-protein associated effector is the calcium channel on the plasma membrane, which also regulates the Ca$^{2+}$ homostasis in the cell (Schroeder and Thuleau, 1991).

There are several pharmacological agents or inhibitors used to facilitate study of signal transduction in animal systems. Fortunately, they can also be applied on plant systems. For example, GTP$_\gamma$S and GDP$_\beta$S, non-hydrolyzed analogs of GTP and GDP, stimulate and inhibit G-proteins, respectively. Some G-protein $\alpha$ subunits can be inhibited by two bacterial toxins, cholera toxin and pertussis toxin, through ADP-ribosylation (Gillman, 1987). Several calcium ionophores such as ionomycin or A23187 can stimulate
Ca^{2+} influx across the plasma membrane, and phorbol esters can mimic the action of DAG. Moreover, kinase inhibitors, such as staurosporine and K-252a, and phosphatase inhibitors, such as okadaic acid, are often used to study the regulation of protein phosphorylation and dephosphorylation (Verhey and Lomax, 1993). Collectively, these chemicals provide excellent tools to study signal transduction in cells.

**GTP-Binding Proteins (G-Proteins) in the Defense Mechanism**

G-proteins have been demonstrated to be present in plants since 1987, and their roles in plant signal transduction have been established (Ma, 1993). G-proteins were found to be involved in elicitation of the rapid oxidative burst in soybean (Legendre et al., 1992). A protein with molecular weight of 45 kDa was visualized in immunoblots of soybean extracts by antibodies raised against a conserved sequence in both animal and plant Gα subunits. This Gα was involved in attenuating the oxidative burst, because an internalized antigen-binding fragment of anti-Gα antibody enhanced up to 10-fold the oxidative burst induced by oligogalacturonide elicitor. In addition, mastoparan, a wasp venom peptide that activates multiple Gα proteins, mimicked the action of elicitor to induce the oxidative burst, and cholera toxin, which ADP-ribosylates Gα and activates G-proteins, also weakly enhanced the induction of active oxygen species. These data suggested the involvement of G-proteins in elicitor signal transduction (Legendre et al., 1992). Cholera toxin is not very effective on stimulating plant defense responses. It enhanced elicitor-induced PAL (phenylalanine ammonia lyase) activity two- to three-fold in bean suspension cells, and induced a two-fold increase in accumulation of phytoalexin in carrot cells (Kurosaki et al., 1987; Bolwell et al., 1991). The oligogalacturonide caused homologous desensitization (reducing or diminishing responses of a cell following a second exposure to the same stimulus) of soybean cells, while the G protein activator, mastoparan, was a very weak elicitor of the oxidative burst in soybean cells, but rapidly desensitized these cells to elicitation by elicitor (heterologous desensitization) (Legendre et al., 1993). Treatment of potato tuber tissue with fungal elicitor from *Phytophthora infestans* hyphal wall.
components caused an increase of GTP$_S$ binding to microsomal fractions obtained from
tuber tissues, and this binding was inhibited by GDP, suggesting that a G-protein was
involved in signal transduction following the elicitor stimulus (Kawakita and Doke, 1994).
Moreover, mastoparan and GTP$_S$, activators of G-proteins, induced an increase in NADH
oxidase activity and ferricyanide reduction and an inhibition of ascorbate peroxidase
activity in tomato Cf5 cells in the presence of a specific elicitor from Cladosporium fulvum
race 4 containing the avr5 gene. Conversely, GDP$_S$, an inactivator of G-proteins, abolished
the increase in NADH oxidase activity and the reduction of ferricyanide induced by race 4
elicitor (Vera-Estrella et al., 1994b).

Phospholipid Metabolism and Ca$^{2+}$ in the Defense Mechanism

Three kinds of phospholipases are involved in signal transduction. Phospholipase A
serves as an effector upon activation by G proteins, phosphorylation, or Ca$^{2+}$ binding. This
enzyme hydrolyzes the ester bond at the sn-2 position of phospholipids to release a free
fatty acid and lysophospholipid. Phospholipase C is specific for phosphatidylinositol 4,5-
bisphosphate, and releases secondary messengers as mentioned earlier. Phospholipase D, on
the other hand, hydrolyzes phospholipids to generate phosphatidic acid (PA) and a free head
group, such as choline. PA can be further metabolized by PA phosphohydrolase to form
diacylglycerol, a secondary messenger. Some lines of indirect evidence showed that
phospholipase A$_2$ and D were involved in HR-induced plant responses (Adam et al., 1989;
Keppler and Novacky, 1989). For PLC activity, on the other hand, Atkinson et al. (1993)
found it played a role in XR (K$^+$/H$^+$ exchange response) when a tobacco resistant cultivar
was treated with an avirulent strain of Pseudomonas syringae pv. syringae. When the
increase of phosphatidylinositol was blocked, by the phospholipase C inhibitors, bromo-
phenacylbromide and neomycin, the XR reaction also disappeared, implicating that PLC
was activated by the incompatible interaction and PLC in turn promoted XR (Atkinson et
al., 1993). In addition, calcium influx, XR, and HR were found to be prevented by La$^{3+}$, a
blocker of nonvoltage-regulated calcium channels (Hille, 1984), and by the treatment with
phospholipase inhibitors (Atkinson et al., 1993), suggesting that IP$_3$ might play a regulatory role in HR cells. Indeed, IP$_3$ also accumulated in tobacco cells after onset of the XR (Atkinson, 1993). A pure oligogalacturonide elicitor stimulated an oxidative burst in soybean cells via the binding receptor on the surface, and the elicitor promoted a transient increase in IP$_3$ to 2.6 fold from basal levels with a rapid decrease in phosphatidylinositol mono-phosphate and phosphatidylinositol 4,5-bis-phosphate prior to the oxidative burst (Legendre et al., 1993). Neomycin sulfate also prevented 60% of the accumulation of IP$_3$ and the oxidative burst, suggesting that PLC plays a role in the activation of defense systems in soybean cells. However, the oxidative burst might come from another PLC-independent pathway which contributed 40% of AOS production. Moreover, this activation was mediated by a heterotrimeric GTP-binding protein (G-protein), because mastoparan, a G-protein activator, mimicked the action of this plant elicitor (Legendre et al., 1993). The roles and the signal transduction of the oxidative burst in HR will be described below. By measuring the soybean oxidative burst, three different elicitors were tested to demonstrate the requirement of phospholipase A in the defense mechanism. The plant elicitor, oligogalacturonic acid, evoked an oxidative burst as usual and did not increase the phospholipase A activity. In contrast, bacterial harpin and an undefined elicitor in *Verticillium dahliae* extract, stimulated both the oxidative burst and enzyme activity. However, the inductive ability of harpin for the oxidative burst did not require phospholipase A, since the application of the phospholipase A inhibitor, chlorpromazine-HCl did not alter the oxidative burst. On the other hand, the oxidative burst induced by the elicitor from *V. dahliae* was mediated by phospholipase A, indicating that plant systems use various signaling intermediates to activate the same defense responses convergently (Chandra et al., 1996). In the rice-*Xanthomonas oryzae* pv. *oryzae* system, phospholipase D (PLD) was examined in susceptible and resistant cultivars. There were three forms of PLD found in both cultivars and the duration of activation of these enzymes were similar in both cultivars too. In addition, PLD was always associated with the inner membrane and was distributed evenly in susceptible cultivars during the first 24 hours after inoculation.
However, PLD was clustered preferentially in membranes adjacent to bacterial cells in resistant interactions after 12 hours of challenge, which was consistent with a role of PLD in membrane damage for making the membrane more permeable (Young et al., 1996).

A 42-kDa glycoprotein isolated from Phytophthora sojae culture filtrate triggered phytoalexin biosynthesis in parsley cells (Parker et al., 1991). The application of this glycoprotein elicitor lead to transient influxes of Ca^{2+} and H^{+} and effluxes of K^{+} and Cl^{-} within 30 min after the addition of elicitor (Scheel et al., 1991). The influx of Ca^{2+} was through the Ca^{2+} channel on the plasmalemma of parsley cells and was essential for the accumulation of phytoalexin, because blockage of this channel by several specific channel blockers lead to inhibition of phytoalexin biosynthesis (Sacks et al., 1993). The utilization of calmodulin antagonists demonstrated the involvement of calmodulin in this particular case, implying that calmodulin-dependent kinases may also play a role in signal transduction (Sacks et al., 1993). Similarly, the branched β1,3-β1,6 glucan elicitor from Phytophthora sojae cell wall invoked the same influxes of Ca^{2+} and H^{+} and effluxes of K^{+} and Cl^{-} in soybean cells, and the same Ca^{2+} channel blockers used in the parsley case also inhibited the activation of chalcone synthase (CHS), a branched key enzyme in phenylpropanoid pathway (Ebel et al., 1993). Furthermore, La^{3+} also inhibited both Ca^{2+} influxes and CHS activity (Ebel et al., 1993). Syringolide 1, an avrD gene specified elicitor, also promoted extracellular alkalization, K^{+} efflux, and Ca^{2+} influx in soybean cell culture lines with the resistance gene, Rpg4, but not in susceptible lines. Again, the Ca^{2+} channel blocker, La^{3+}, suppressed all responses, indicating that syringolide 1 activated a Ca^{2+} influx-dependent signaling pathway in Rpg4 soybean cells (Atkinson et al., 1996). Cryptogein-induced reactions in tobacco cells were also dependent on Ca^{2+} influx, which was found in the first minute after the treatment of suspension-cultured tobacco cells with cryptogein. Once initiated, the Ca^{2+} influx was needed at least for the first hour to sustain the defense responses. The addition of ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid and La^{3+}, both blocking Ca^{2+} channels, to the cells suppressed defense-related responses including extracellular alkalization, active oxygen species, and phytoalexin production.

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Moreover, a Ca\(^{2+}\) ionophore, A23187, was only able to trigger one of defense responses, extracellular alkalization. The activation of Ca\(^{2+}\) channels was not triggered by cryptogein, since staurosporine, a protein kinase inhibitor (see below), inhibited the Ca\(^{2+}\) influx induced by cryptogein, suggesting that a phosphorylation reaction occurred upstream of Ca\(^{2+}\) influx (Tavernier et al., 1995). Several defense-related genes encoding basic chitinase, acidic chitinase, and basic β-1,3-glucanase were also regulated by Ca\(^{2+}\). For example, a Ca\(^{2+}\) ionophore induced acidic chitinase mRNA, and calcium channel blockers prevented elicitor-induced increases in levels of basic chitinase and β-1,3-glucanase mRNAs (Suzuki et al., 1995). The increase of intracellular Ca\(^{2+}\) levels was also found to play a role in regulating AOS (active oxygen species, see below) production. In spruce cultured cells, cell wall components from the ectomycorrhizal fungi *Amanita muscaria* and *Hebeloma crustuliniforme* and from the pathogen *Heterobasidion annosum* were able to elicit a transient AOS, although a larger amount of elicitors from mycorrhizal fungi were needed to trigger the same level of AOS production. The induction of AOS was Ca\(^{2+}\) dependent since, in Ca\(^{2+}\)-depleted medium, the induced AOS was suppressed and ionophore A23187 also triggered the AOS production in the presence of Ca\(^{2+}\) (Schwacke and Hager, 1992). Similarly, La\(^{3+}\) also inhibited AOS production in tobacco cultured cells (Baker et al., 1993).

**Phosphorylation and Dephosphorylation in the Defense Mechanisms**

Phosphorylation and dephosphorylation of proteins by kinases and phosphatases are known to be responsible for the regulation of enzyme activities and to serve as a cascade system for the amplification of signals (Ranjeva and Boudet, 1987). Several types of protein kinases are found in plant cells, including calcium-dependent protein kinases (Roberts, 1993), receptor-like protein kinases, cyclin-dependent kinases, and MAP (mitogen-activated protein) kinases (Ma, 1993). Most of them have been demonstrated to play a role in housekeeping functions. For example, cyclin-dependent protein kinases may be the regulators of the cell cycle (Ma, 1993). Although some metabolic enzymes were found to be regulated by phosphorylation, such as pyruvate dehydrogenase complex and nitrate reductase (Ma,
1993), several studies suggest that protein phosphorylation and/or dephosphorylation also play roles in defense responses after treatment with elicitors. In the earlier studies, research only demonstrated that phosphorylation/dephosphorylation occurred on some uncertain proteins without known functions (Grab et al., 1989; Dietrich et al., 1990). The phosphorylation/dephosphorylation of proteins may be regulated by some endogenous factors other than elicitors as well. For example, a 69-kDa protein (pp69) was phosphorylated in vivo without elicitor presence, and this phosphorylation was enhanced by a very small (≤ 1,000) factor from soybean cell culture, which was insensitive to alkaline phosphatase. The phosphorylated pp69 was dephosphorylated in response to β-glucan elicitor treatment of soybean culture cells (Grab et al., 1989). A avrD derived elicitor, syringolide 1, from Pseudomonas syringae pv. tomato dephosphorylated a 55 kDa plasma membrane protein from soybean, irrespective of the plants harboring the resistance gene, Rpg4, or the susceptible gene, rpg4, although the latter required higher elicitor concentration (Stayton et al., 1993). In cultured parsley cells, the P. sojae glycoprotein elicitor stimulated phytoalexin biosynthesis accompanied with the phosphorylation of a neutral 45-kDa protein, found in cytoplasmic and microsomal fractions, in a very short time (Dietrich et al., 1990).

By using protein kinase and protein phosphatase inhibitors, the role of phosphorylation or dephosphorylation have been established. In spruce cells, the AOS induced by elicitors from fungi was inhibited by protein kinase inhibitor staurosporine (Schwacke and Hager, 1992). In contrast, the protein kinase inhibitors, staurosporine and K-252a, could not shutdown the accumulation of phytoalexin in parsley, but a phosphatase inhibitor, okadaic acid, completely abolished it (Renelt et al., 1993), suggesting that the phosphorylation of 45-kDa protein may not be involved in signal transduction from elicitor to phytoalexin synthesis but the dephosphorylation of an unknown substrate was involved in this transmission. Similarly, a synergistic effect was shown that little amount of glucan elicitors, which were not able to elicit the activity of chalcone synthase alone, were markedly enhanced by the presence of K-252a in soybean cells (Ebel et al., 1993). A
plasma membrane protein, pp34, was the target of the phosphorylation evoked by the addition of defined oligogalacturonides. This phosphorylated protein was purified, but the function of it remained unclear (Jacinto et al., 1993). In addition, the purified oligogalacturonides, 13 to at least 26 residues long, could stimulate pp34 phosphorylation (Reymond et al., 1995). Another type of elicitor, cryptogein, a proteinaceous elicitor from Phytophthora cryptogea, caused a strong alkalinization of the culture medium and a transient production of activated oxygen species in cultural tobacco cells. These physiological responses were completely sensitive to staurosporine, suggesting that some phosphorylated proteins were essential for the transduction of elicitor signals (Viard et al., 1994). K-252a and staurosporine at concentrations of 1 μM and 2 μM, respectively, completely inhibited H₂O₂ production induced by P. sojae wall glucan in soybean cv. Williams 82 cells. On the other hand, the protein phosphatase 2A inhibitor, cantharidin, stimulated H₂O₂ production in the absence of fungal elicitor, while the protein phosphatase 2B inhibitor, cypermethrin, had no effect (Levine et al., 1994). Likewise, the induction of H₂O₂ and O₂⁻ by oligogalactoturonide in soybean suspension cells was suppressed by K-252a and staurosporine and induced by calyculin A and okadaic acid even without the presence of elicitor (Chandra and Low, 1995).

Ethylene is a well-known plant hormone involved in the physiological responses to environmental stress. This plant hormone is capable of inducing pathogenesis-related proteins (PR proteins) in tobacco cells. Intriguingly, this ethylene signal is transduced via protein phosphorylation, because the application of ethylene leads to the induction of PR proteins and a transient protein phosphorylation, which were blocked by the presence of protein kinase inhibitors (Raz and Fluhr, 1993). However, the phosphatase inhibitor, okadaic acid, was able to mimic the action of ethylene without the application of ethylene in the normal culture cells. On the other hand, a fungal elicitor, xylanase, which also induces the PR protein, was not affected by protein kinase inhibitors, indicating that xylanase-induced pathway is an ethylene-independent pathway (Raz and Fluhr, 1993). A similar experiment showed that xylanase caused the rapid medium alkalization and the
accumulation of ethylene in tomato cells, and another phosphatase inhibitor, calyculin A, totally mimicked the elicitor action. The actions of both xylanase and calyculin A were prevented by protein kinase inhibitors (Felix et al., 1994). Taken together, the fungal elicitor, xylanase, is able to induce the biosynthesis of ethylene in the cell, and then ethylene stimulates the accumulation of PR proteins by means of phosphorylation of specific proteins in cultured cells. Besides, xylanase can provoke PR proteins directly via an ethylene-independent pathway, which is not affected by protein kinase inhibitors and only the ethylene-dependent pathway can be mimicked by phosphatase inhibitors, either okadaic acid or calyculin.

Reciprocally, the race-specific elicitor, avr5 gene product from Cladosporium fulvum race 4, caused 4-fold increase in H⁺-ATPase activity, coincided with the acidification of growth medium of resistant tomato cultivar containing Cf5 resistance gene (Vera-Esrella et al., 1994a). This phenomenon was blocked by okadaic acid but not by staurosporine, implying that dephosphorylation was essential in this case. Moreover, the effects of non-hydrolyzable GTP analog and mastoparan, which activated G-proteins, showed that the involvement of GTP-binding protein lead to activating a phosphatase, which in turn stimulated the H⁺-ATPase activity (Vera-Estrella et al., 1994a). In an incompatible combination, intercellular fluid (IF) from Cladosporium fulvum race 4 and race 2.3 (both containing avr5 gene product) caused a marked dephosphorylation of plasma membrane H⁺-ATPase isolated from Cf-5 cells within 30 min, while the dephosphorylation did not occur in the compatible combination. However, the dephosphorylated H⁺-ATPase on the membrane of intact tomato cells was rephosphorylated from 30 min to 1 h (phase 1) and from 1 h to 2 h (phase 2), but the rephosphorylation did not happen in the isolated plasma membrane. The phase 1 phosphorylation was dependent on Ca²⁺-dependent protein kinase C (PKC) by using specific PKC inhibitors including bisindolylmaleimide, calphostin C, chelerythrine, and N-(2-guanidinoethyl)-5-isoquinolinesul-fonamide. On the other hand, the phase 2 phosphorylation was caused by another protein kinases since bisindolylmaleimide could not inhibit the enzyme activity but staurosporine could. In
addition, the activity of the second protein kinase required Ca\(^{2+}\) and calmodulin. Interestingly, the second Ca\(^{2+}\)/calmodulin dependent protein kinase was also dependent on the first Ca\(^{2+}\)-dependent protein kinase activity, indicating a phosphorylation cascade (Xing et al., 1996).

An elicitor derived from the cell walls of Phytophthora infestans rapidly and transiently activated a 47-kDa protein kinase which phosphorylated serine/threonine residues of the myelin basic protein (MBP), an in vitro kinase substrate, along with defense genes activation. The defense gene activation and the activation of MBP kinase were suppressed by staurosporine and Gd\(^{3+}\), which blocks Ca\(^{2+}\) channel. The phosphorylation of MBP was inhibited by staursporine as well. Interestingly, the MBP kinase was also phosphorylated at tyrosine position in a conserved sequence by an unknown protein kinase which was stimulated by fungal elicitor. Due to the characteristics of phosphorylation of tyrosine in a conserved sequence, the MBP kinase was very likely to be a mitogen-activating protein (MAP) kinase. According to these data, a phosphorylation cascade was transduced as following sequence: the perception of elicitor, activation of a staurosporine-sensitive upstream kinase, activation of 47-kD MBP kinase by phosphorylation on tyrosine residue, and the phosphorylation of some other substrate in vivo (Suzuki and Shinshi, 1995).

**Lipoxygenase Activity in the Defense Mechanism**

Lipoxygenases (LOXs) catalyze the hydroperoxidation of fatty acids containing an unsaturated 1,4, cis-pentadiene configuration, such as linoleic, linolenic, and arachidonic acids to their hydroperoxides. These products may serve as calcium ionophores, signals for inducing proteinase inhibitors, e.g. methyl jasmonate, and wound hormone, e.g. traumatin (Hildebrand, 1989). In addition, the production of free radicals has also drawn a lot of attention. Most of the lipoxygenases have Mr between 90 and 100 kDa and mainly in cytosol with some exceptions associated with membranes. For instance, the LOX in tomato was demonstrated to be a 100-kDa protein with optimum activity at pH 7.0 by western blot
with the antibody against pea LOX (Koch et al., 1992). LOX was also found in both microsomal (1%) and soluble fractions from potato, both were 95 kDa and the optimum activity of the microsomal one was pH 5.5. This controversy may be due to the different isoforms present in plants (Bostock et al., 1992). In fact, LOX can be classified according to the site of oxygen insertion on substrates. For example, there are 5-, 8-, 11-, 12-, and 15-LOX for arachidonic acid as substrate (Bostock et al., 1992). LOX may have three distinct ways to play a role in host-pathogen interactions (Slusarenko et al., 1993); that is 1) causing membrane damage, 2) providing signal molecules (Melan et al., 1993), and 3) producing substances that are chemically or enzymatically modified to result in toxic metabolites (Slusarenko et al., 1993).

According to the observation that LOX activity increased during the HR in bean leaves after the inoculation of Pseudomonas syringae pv. phaseolicola, LOX activity was proposed to contribute directly to the membrane damage in HR (Croft et al., 1990). A study illustrated the relationship between HR and LOX in tomato revealed that LOX mRNA was induced by Pseudomonas syringae pv. syringae within 3 hours, and the enzyme activity began to increase between 6 and 12 hours and then to reach the maximum in 24 to 48 hours (Koch et al., 1992). The timing of induction of LOX is coincident with the development of HR (Goodman and Novacky, 1994). In addition, LOX is considered to be involved in XR activation of tobacco which was inoculated with Pseudomonas syringae pv. syringae, because the XR activation was inhibited by LOX inhibitors, eicosatetraynoic and eicosatriynoic acids, after the fatty acids were released by the addition of this pathogen (Atkinson, 1993). These inhibitors did not block calcium influx, implying that the LOX acts downstream of calcium influx and PLC activity (Atkinson, 1993). The race-specific elicitor isolated from Cladosporium fulvum induced LOX activity and lipid peroxidation in tomato leaves under either the dark or the light, but necrosis only developed in the light, implying the LOX response and necrosis may be two different pathways (Peever and Higgins, 1989). By treating with intercellular fluid (IF) from C. fulvum race 0, the lipid peroxidation and electrolyte leakage from tomato cells were Cf genes-, avirulence elicitor dose-, and relative.
humidity (RH)-dependent. At ambient RH, lipid peroxidation occurred in cultivar carrying Cf9 gene was faster than in Cf2 cultivar. In high RH and low elicitor dose conditions, Cf-Avr-dependent lipid peroxidation was markedly attenuated. On the other hand, significant electrolyte leakage was found after 18 h post treatment only at low RH conditions. The LOX activity was induced by avirulent elicitor after 8 h treatment in tomato having Cf genes (Cf9 and Cf2), while no significant increase was found in the Cf0 cultivar. In addition, the LOX induction in the Cf2 cultivar seemed to be dependent on the dose of avr2 elicitor, whereas LOX induction in Cf9-avr9 system was independent (May et al., 1996).

As mentioned earlier, linoleic acid (18:3), one of the most abundant fatty acids in plants, is also the target of LOX in plants. After the application of the bacterial pathogen, Pseudomonas syringae pv. phaseolicola, several volatile products were evolved from 13-hydroperoxylinolenic acid, the first product of LOX in the lipoxygenase pathway, in an incompatible cultivar of bean (Croft et al., 1993). Surprisingly, two of these volatile metabolites, i.e. low concentration of trans-2 hexenal and high concentration of cis-3-hexenol, showed bactericidal effects. Owing to the presence of two possible products of LOX activity, either 9- or 13 hydroperoxylinolenic acid, it seems likely that only the 13-LOX was activated by this pathogen in the HR (Croft et al., 1993). Additionally, it is believed that these bactericidal compounds play a role in pathogen localization (Atkinson, 1993).

Two fatty acids, eicosapentaenoic acid (EPA) and arachidonic acid (AA), from the fungal pathogen Phytophthora infestans, have been identified to be the elicitors for inducing HR and subsequent sesquiterpene phytoalexin accumulation in resistant potatoes (Bostock et al., 1981). The use of radioactive AA to monitor this particular type of elicitor revealed an observation that AA was rapidly released from sporangia of Phytophthora infestans and then metabolized in its host (Ricker and Bostock, 1992). On the other hand, a LOX inhibitor, salicylhydroxamic acid (SHAM) prevented the HR induced by EPA and AA (Preisig and Kuc, 1987), suggesting that LOX was involved in the signaling pathway en route the HR and the elicitation of phytoalexin. Moreover, Bostock et al. (1992) found that
abscisic acid (ABA), SHAM, and n-propyl gallate (PG), three inhibitors preventing the induction of HR in potato discs treated with AA, suppressed the AA-induced increment of LOX activity. Interestingly, however, the antibiotic cycloheximide, which was able to abolish the induction of phytoalexin, did not inhibit LOX activity but enhanced it like the action of AA (Bostock et al., 1992). Because PG is a free radical scavenger and SHAM is the competitor of reducing substrate sites (Preisig and Kuc, 1987), other oxidative agents, such as activated oxygen species, may be also involved in the induction of HR and phytoalexin, and the metabolites in “lipoxygenase pathway” may be sensitive to these chemicals as well (Bostock et al., 1992). The enhancement of cycloheximide, in addition, suggests that there may be some proteinaceous negative regulators involved in the AA-induced signaling pathway (Bostock et al., 1992). By using a transgenic plant where a chloroplast lipoxygenase, LOX2, was suppressed, Bell et al. (1995) found that the lipoxygenase was required for wound-induced jasmonic acid accumulation in Arabidopsis. In addition, lipoxygenase genes in tobacco were not constitutively expressed to a detectable level in healthy plants, while a rapid and transient accumulation of transcripts was found after the treatment with elicitor or with zoospores of Phytophthora parasitica f. sp. nicotianae (Véronési et al., 1996).

**Active Oxygen Species (AOS) in the Defense Mechanism**

*The Basic Understandings of AOS*

One rapid process tightly associated with the early events during the HR, is the striking release of AOS, termed oxidative burst (Dixon et al., 1994). Basically, the AOS result from the acceptance of one electron to reduce molecular O$_2$. The predominant species are superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH·) (Baker and Orlandi, 1995; Mehdy, 1994). Due to the correlation between the oxidative burst and HR in a number of host-pathogen interactions, the AOS, therefore, is considered to be one of the important elements responsible for disease resistance (Sutherland, 1991; Baker and Orlandi, 1995; Hammond-Kosack and Jones, 1996; Low and Merida, 1996).
The first reaction during the pathogen-induced oxidative burst is the one-electron reduction of molecular $O_2$, resulting in the formation of superoxide anion ($O_2^-$). This toxic species is further converted to other AOS species by means of Haber-Weiss reaction described as follows (Halliwell and Gutteridge, 1984).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (2)$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^- \quad (3)$$

The superoxide anion can be dismutated spontaneously or catalyzed by superoxide dismutase (SOD), and the rate of this reaction with enzyme is $10^{10}$ times more rapid than that of the spontaneous one. In addition, the SOD activity is dependent on the pH of the medium, and SOD is favored by acidic conditions, such as in the apoplasm (Mehdy, 1994; Sutherland, 1991). In equation (2), the cupric ion ($Cu^{2+}$) can also be reduced by superoxide anion, and both of these ions can be the cofactors or in the prothetic group of holoenzymes or they can be bound by low molecular weight chelators, including citrate, ATP, GTP, and other phosphate esters (Halliwell and Gutteridge, 1984; Mehdy, 1994).

Among the AOS, hydrogen peroxide ($H_2O_2$) diffuses freely across plasma membrane while superoxide anions ($O_2^-$) diffuse very slowly. Both hydrogen peroxide and superoxide anion are less reactive than hydroxyl radicals ($OH^*$) and hydroperoxyl radicals ($HO_2^*$), which are able to initiate lipid peroxidation (Mehdy, 1994). The targets of AOS include DNA, enzymes, and membrane lipids. For example, DNA is vulnerable to AOS, leading to strand breakage, particularly at the guanine residues. On the other hand, His, Met, and Trp residues are the most susceptible sites in proteins, and the outcome is usually the loss of enzyme activity. AOS attacks membrane lipids, especially the polyunsaturated fatty acids, resulting in peroxidation of lipid and membrane damage (Tzeng and DeVay, 1993). Because of the natural generation of AOS in living cells under the aerobic conditions, plant cells per se have evolved mechanisms to reduce these toxic materials (Foyer et al., 1994). The toxicity of AOS can be reduced by enzymes or some reducing chemicals. Several enzymes including superoxide dismutase (Bowler et al., 1992), catalase, peroxidase, and
glutathione reductase, and reducing substances, such as ascorbate, glutathion, and thiol compounds (Elstner, 1991) can efficiently remove the AOS generated in the cells. In addition, one of the peroxidases, ascorbate-specific peroxidase, and other two enzymes, glutathione reductase and dehydroascorbate reductase, remove $\text{H}_2\text{O}_2$ through a pathway called ascorbate-GSH pathway, which plays a central role in protecting the chloroplasts and other cellular components from oxidative damage (Nakano and Asada, 1981; Foyer et al., 1994).

The Occurrence of AOS in HR and Other Defense Responses

The discovery of the involvement of AOS in resistance responses was reported by Doke (1983a). In this study, nitroblue tetrazolium and cytochrome c was reduced in the incompatible reaction between \textit{Phytophthora infestans} and potato, indicating that the superoxide anion was involved in this early response (within an hour) after infection (Doke, 1983a, b). The inhibitors, which blocked the rapid HR death and reduced phytoalexin accumulation, also suppressed $\text{O}_2^-$ generation, mainly from NADPH oxidase on the plasma membrane. On the other hand, the addition of superoxide dismutase which dismutates the $\text{O}_2^-$ to hydrogen peroxide (equation 1) prevented the HR and phytoalexin synthesis as well (Doke, 1985). The findings suggested that $\text{O}_2^-$ generation may be directly involved in host cell hypersensitivity.

The occurrence of oxidative burst may be one of the earliest events during the HR. The race-specific elicitor from an incompatible race of \textit{Cladosporium fulvum} elicited the AOS in a tomato cell suspension within 2 minutes. In this case, only the incompatible reaction showed the reduction of cytochrome c, the production of malonaldehyde, a product of lipid peroxidation, and luminol-dependent chemiluminescence, indicating the involvement of AOS (Vera-Estrella et al., 1992). One nonspecific fungal elicitor from \textit{Verticillium dahliae} also promoted the oxidative burst in soybean cell culture within 1 to 2 minutes (Apostol et al., 1989). Similarly, HR accompanied by increased $\text{O}_2^-$ generation and lipid peroxidation was induced by bacterial race-specific elicitor (Adam et al., 1989).
Moreover, the avirulence gene product of *Pseudomonas syringae* pv. *glycinea* race 4 triggered active oxygen production concomitant with K⁺/H⁺ response (XR) (Orlandi et al., 1992). Interestingly, the generation of active oxygen species in potato treated with *Phytophthora infestans* and in tobacco treated with *Pseudomonas syringae* could be divided into two stages. The first rapid oxidative burst was found in 0-1 hour and second one was prolonged in 3-6 hours. In addition, incompatible pathogens initiate both phases of AOS production, while compatible pathogens induce only the first phase (Chai and Doke, 1987; Keppler et al., 1989). This phenomenon implied an idea that the first oxidative burst might be involved in directly reducing pathogen viability, and the second one might act as secondary messengers, especially hydrogen peroxide, to evoke other defense responses (Apostol et al., 1989). Alternatively, only the second phase is involved in killing pathogens and disease resistance, and this point of view is supported by recent reports (Chandra et al., 1996; Mehdy et al., 1996). Transgenic tomatoes with or without the disease resistance gene, *Pto*, were challenged by *Pseudomonas syringae* pv. *tomato* harboring *avrPto* gene and only the incompatible reaction resulted in two phases of AOS production. In contrast, the tomato without the *Pto* gene, which was inoculated with bacteria only showed the first burst. However, both plants with or without the *Pto* gene produced first and second oxidative burst when challenged with *P. syringae* pv. *tabaci*, a nonhost pathogen, which elicited the hypersensitive response on both plants as well (Chandra et al., 1996).

The possible sources of AOS production during plant-pathogen interactions include NAD(P)H oxidases, peroxidases, and lipoxygenases (Baker and Orlandi, 1995). A study using a race-specific elicitor from *Cladosporium fulvum* (avr5) and a tomato plant with the *Cf5* gene has shown that the elicitor induced an increase in NADH oxidase and NADH-dependent cytochrome c reductase activities, but ascorbate peroxidase activity was decreased. These data suggested that the change of redox potential on the host plasma membrane, causing the electron transfer from reducing agents to oxygen, may be involved in the production of AOS, which in turn is responsible for the race-specific interaction (Vera-Estrella et al., 1994b). The NADH oxidase is indeed a candidate to produce
superoxide anions in plants, since the use of the specific inhibitor, diphenylene iodonium (DPI), blocks AOS production (Jones, 1994; Levine et al., 1994). Moreover, there is another mechanism to produce AOS in barley. The germin-like oxalate oxidase protein in barley containing the Mla1 resistance gene against the powdery mildew fungus, Erysiphe graminis f.sp. hordei, was identified as well. This enzyme also generated H$_2$O$_2$. The enzyme activity was increased after 24 h post-inoculation and reached 10-fold higher after 48 h inoculation compared to the control plant (Zhang et al., 1995). In addition, the AOS are not only induced by pathogens or their elicitors but also produced constitutively from oxidative phosphorylation in mitochondria, photosynthesis in chloroplast, and enzymes involved in reduction-oxidation processes including photo-respiration and fatty acid β-oxidation (Tzeng and DeVay, 1993).

**The Functions of AOS in Plant Defense**

Although the exact roles of oxidative burst still remain to be established, the possible functions of the production of AOS are the inhibition of the growth of microorganisms and the stimulation of other defense mechanisms either through direct involvement or indirect as secondary messengers (Mehdy, 1994; Tenhaken et al., 1995; Low and Merida, 1996; Mehdy et al., 1996).

**Inhibition of the growth of pathogens**

The scavengers of O$_2^-$, which impeded the HR in tobacco treated with an incompatible race of P. syringae pv. tabaci, increased bacterial growth *in planta* (Keppler et al, 1989). H$_2$O$_2$ was also found to inhibit the germination of several fungal spores (Peng and Kuc, 1992). However, the inhibition of pathogens is highly dependent on the concentration of AOS presented to pathogens and the antioxidant mechanisms of the pathogens (Mehdy et al., 1996). Micromolar range of H$_2$O$_2$ concentrations at the infection site seemed to be effective in inhibiting the pathogens growth. For instance, H$_2$O$_2$ of 0.1 mM, generated by a glucose oxidase, completely inhibited the growth of Erwinia carotovora pv. carotovora,
and caused greater than 95% inhibition of *Phytophthora infestans* growth *in vitro*. The development of lesions caused by *P. infestans* was significantly delayed in the transgenic potato encoding this fungal glucose oxidase as well (Wu et al., 1995).

**Modification of the Host Cell Wall**

The cell wall serves as a structural barrier and is usually the first line of defense against plant pathogens. In addition, plants are also equipped to modify the cell wall by forming callose, papillae, depositing phenolic substances (Matern et al., 1995), and cross-linking hydroxyproline-rich glycoproteins in response to wounding, infection, and elicitation (Wycoff et al., 1992). The oxidative cross-linking of cell wall proteins caused by AOS has been brought a lot of attention, since this is one of the earliest detectable effects of elicitor-induced responses. In soybean, a 33-kDa SDS-extractable protein from cell walls was insoluble after elicitation by *Phytophthora sojae* wall glucan or the addition of glutathione, and this 33-kDa protein (p33) was found to be retained in the cell wall instead of degraded or translocated (Bradley et al., 1992). Similarly, another 100-kDa protein was also made insoluble upon the elicitation. The involvement of AOS was demonstrated by addition of catalase and ascorbic acid which prevented the action of H$_2$O$_2$ in the cell wall and simultaneously blocked the p33 insolubilization induced by fungal elicitor. The p33 is a highly repetitive proline-rich protein containing many tyrosine residues. It was suggested that the cross-links responsible for the insolubilization of p33 was through the formation of isodityrosine cross-links catalyzed by peroxidases in the presence of H$_2$O$_2$ (Bradley et al., 1992). This cross-link reaction was very fast. The reaction was initiated within 2 min by adding glutathione, and complete within 10 min. Likewise, the response to fungal elicitor was complete within 20 min (Bradley et al., 1992). The oxidative cross-linking of (hydroxy)proline-rich structural proteins was also observed in the incompatible interaction between soybean cv. Harosoy and *Pseudomonas syringae* pv. *glycinea* race 4, containing *avrD* avirulence gene. After treatment with Psg race 4 (*avrD*), the oxidative cross-linking reaction was complete within 24 hours, concomitant with visible HR lesions (Brisson et al., 34)
1994). The oxidative cross-linking of the plant cell wall increases resistance to cell wall-degrading enzymes, suggesting that oxidative cross-linking protects plant cells from the pathogens equipped with cell wall degrading enzymes (Brisson et al., 1994). In addition to protein-protein cross-linking, oxidative cross-linking of proteins to other cell wall components such as pectins and lignins may also be involved in protection from pathogens. However, further studies are needed to clarify the function of AOS in other cross-linking protection.

H₂O₂ is also required for lignification and indeed the correlation between the site of H₂O₂ biosynthesis and the site of active lignin deposition was also found (Olson and Varner, 1996). In the bean cv. Red Mexican, the increase in activities of an acidic peroxidase in zone 1 (the closest area to the treatment with an avirulent strain of *Pseudomonas syringae pv. phaseolicola*) was correlated with lignification in inoculated tissues. In zone 1, the activities of other enzymes, including xanthine oxidase and glutathione reductase, were increased while the activity of catalase was reduced, suggesting that the increase of AOS by inducing the peroxidase and xanthine oxidase activities and reducing catalase activity is associated with HR in incompatible interaction (Milosevic and Slusarenko, 1996). The elicitor which induced oxidative burst was also able to induce lignification in spruce (Lange et al., 1995), suggesting that the cross-linking of phenols in the cell wall was enhanced in response to pathogen attack.

**AOS in Phytoalexin Biosynthesis and other Protectant Genes Activation**

The induction of phytoalexin biosynthesis by oxidative burst is still controversial. By using a crude fungal elicitor, a correlation between AOS production and glyceollin synthesis was found. Relatively high amounts of exogenous H₂O₂ (0.5-1.0 mM) induced the production of phytoalexin in soybean suspension culture, and the addition of catalase suppressed the elicitor-induced phytoalexin, suggesting that H₂O₂ was a potential regulator of phytoalexin biosynthesis (Apostol et al., 1989). In addition, H₂O₂, hydroperoxides and oxidative stress also directly stimulated the production of phytoalexin (Guo et al., 1993).
The treatment of sulfhydryl reagents and hydroperoxide with soybean suspension cells triggered a significant induction of phytoalexin. Both treatments caused a rise of cellular active oxygen species and lipid peroxidation, which were sufficient to initiate glyceollin accumulation in soybean (Degousée et al., 1994). In contrast, the use of 1 mM H₂O₂ alone only resulted in little induction of PAL and chalcone synthase (CHS) mRNA transcripts (Levine et al., 1994). The crude fungal elicitor showing the correlation between AOS and phytoalexin production (Apostol et al., 1989) was further separated into a protein moiety which was responsible for glyceollin induction and a carbohydrate portion that stimulated AOS production (Davis et al., 1993). However, due to the heterogeneity of AOS, the negative result of one species can not rule out the involvement of other species.

Other than genes involved in biosynthesis of phytoalexin, several genes functioning in cellular protection, such as glutathione S-transferase (GSH), glutathione peroxidase and polyubiquitin, were found to be also activated by H₂O₂ (Levine et al., 1994). Glutathione S-transferase plays roles in both normal cellular metabolism and in the detoxification of a wide range of xenobiotic compounds. GSH is also involved in stress responses including pathogen attack, oxidative stress and heavy metal toxicity (Marrs, 1996). The GSH induction was suppressed by the addition of catalase, DPI, and other scavengers, suggesting the involvement of AOS in GST induction (Levine et al., 1994). The induced GST may in turn detoxify lipid peroxides by conjugation with glutathione (GSH). Glutathione peroxidase also catalyzes the GSH-dependent reduction of H₂O₂ to form glutathione disulfide and induce more glutathione synthesis (Marrs, 1996). Due to the protective function of GST, the induction of GST and glutathione peroxidase likely occurs in the adjacent cells of HR (Tenhaken et al., 1995). The H₂O₂ has been demonstrated to be a diffusible signal across a dialysis membrane from one side where the soybean cells were treated with an avirulent bacterial strain to the other side where the cells were not treated. In the non-treated soybean cells, GST and glutathione peroxidase mRNA transcripts were induced although there were no bacteria in this cell batch (Levine et al., 1994).
AOS As a Causal Agent of Hypersensitive Cell Death

Hypersensitive responses (HR) are found to be associated with disease resistance. The rapid cell death in HR is thought to be involved in defense mechanism to inhibit pathogen growth to the local site of invasion. There are several lines of evidence suggest that the death of plant cells during the HR process results from the activation of a programmed cell death pathway, although it is not completely clear whether plant cells undergoing HR exhibit apoptotic cell death similar to animal cell apoptosis or necrotic cell death (Dangl et al., 1996; Greenberg, 1996; Jones and Dangl, 1996; Levine et al., 1996; Mittler and Lam, 1996). The characteristics of apoptotic cell death include cytoskeletal disruption, membrane blebbing, cell shrinkage, nuclear condensation, and DNA fragmentation, while the necrotic cell death is characterized by rapid cell swelling, lysis and the early loss of membrane integrity, leading to cytoplasm leakage (Thompson, 1995).

AOS from the oxidative burst have been suggested to be one of the factors contributing to host cell death as part of the HR (Adam et al., 1989; Levine et al., 1994). Induction cell death by H$_2$O$_2$ seems to be a threshold phenomenon (Tenhaken et al., 1995). Treatment of soybean cells with a high concentration of exogenous H$_2$O$_2$ (6-8 mM) resulted in cell death, similar to the cell death triggered by an avirulent bacterial strain. The exogenous concentration of H$_2$O$_2$ has a short half life of about 5 minutes, and thus, the actual concentration for eliciting cell death may be lower than 6-8 mM. On the other hand, to induce protectant gene transcription, a relatively small amount of H$_2$O$_2$ was sufficient (Levine et al., 1994). Soybean cv. Williams 82 was inoculated with Pseudomonas syringae pv. glycinea carrying avrA (avirulent strain) and avrC (virulent strain), and only the avirulent strain caused oxidative burst and cell death. H$_2$O$_2$ stimulated a rapid influx of Ca$^{2+}$ into soybean cells, which activated an apoptotic cell death with morphological changes that define apoptosis. Interestingly, the induction of GST gene was found to be Ca$^{2+}$ independent. Apoptosis was always correlated with hypersensitive responses found not only in soybean- $Ps g$ (avrA) system but also in Arabidopsis and P. syringae pv. tomato and tobacco and cryptogein which was isolated from Phytophthora cryptogea and induced non-
host resistance in tobacco (Levine et al., 1996). The requirement of AOS in HR was indirectly demonstrated by using tobacco (genotype NN) and tobacco mosaic virus (TMV) under low oxygen conditions. Tobacco (genotype NN) containing the resistance gene (N') resists TMV and produces HR lesions at ambient O₂ conditions. However, the death of tobacco cells infected with TMV was suppressed at low oxygen pressure. Contrary to cell death, virus replication and the activation of PR protein (PR-1a) were not inhibited at low oxygen levels. Pseudomonas syringae pv. phaseolicola, a bean pathogen that causes an HR in tobacco, was inoculated on tobacco at low oxygen conditions. Again, the programmed cell death was inhibited. The uncouple phenomenon between HR cell death and PR-1a production may be due to either two different pathways for these two responses or to the requirement of large amounts of H₂O₂ for triggering cell death and small amount for induction of PR proteins (Mittler et al., 1996). In the cultured parsley-Phytophthora infestans system, the activated mitochondrial activity and active oxygen species were found prior to the occurrence of programmed cell death. Treatment of parsley cells with salicylic acid, an inhibitor of catalase, induced a rapid cell death, which was inhibited by the presence of n-propyl gallate, a scavenger of AOS and inhibitor of lipoxygenase. The intracellular peroxides were monitored and a strong correlation between intracellular peroxides and membrane damage resulting in cell death was obtained as well (Naton et al., 1996). On the other hand, bacterial mutants that failed to induce cell death in tobacco cell were still able to elicit oxidative burst. Usually, the hrp/hrm genes of Pseudomonas syringae pv. syringae trigger the HR and XR in tobacco cells as well as two phases of oxidative burst (Glazener et al., 1996). A mutant in the hrp cluster inhibited the second phase of active oxygen response, while the mutant in hrmA locus did not affect phase II AOS production. However, the mutant in hrmA locus could not stimulate the hypersensitive reaction in tobacco leaves nor cell death in suspension culture, indicating that the AOS, mainly H₂O₂, in the second phase was not sufficient to trigger hypersensitive cell death (Glazener et al., 1996).
CHAPTER 1

GENERAL INFORMATION ON β-GLUCOSIDASES IN PLANTS

INTRODUCTION

The Function of β-Glucosidases in Plants

β-Glucosidases (EC 3.2.1.21) are a class of enzyme hydrolyzing the glucosidic linkage between glucose moieties and their aglycones. This kind of enzymes is distributed widely in the living world including animals, plants, fungi, and bacteria (Esen, 1993). In fungi and bacteria, β-glucosidases are mainly involved in the process of cellulose degradation in which β-glucosidases hydrolyze cellobiose as part of the cellulase complex (Wood and Bhat, 1988). In plants, β-glucosidase activity was found to be involved in regulation of plant hormones, the release of secondary metabolites to play roles in defense mechanisms (Bell, 1981), as signals to microorganisms in soil (Phillips and Streit, 1996), in floral pigmentation (Harborne and Mabry, 1982), and in lignification (Hösel et al., 1978; Marcinowski and Grisebach, 1978). The diverse functions of β-glucosidases are reviewed as follows.

Hormone Regulation

Most plant hormones form conjugates to remain inactive in storage until they are needed (Sembdner et al., 1994; Kleczkowski and Schell, 1995). The plant hormones such as auxins, gibberellins, cytokinins, ABAs, jasmonates, and salicylic acid are found as glucosyl
conjugates, and some of them are quantitatively important (Sembdner et al., 1994). Auxin and cytokinin glucosides in maize have been shown to be transported from the endosperm to the embryo, where they are hydrolyzed by a β-glucosidase (Nowacki and Bandurski, 1980). Another β-glucosidase, p60, has been purified from maize and liberates cytokinin (Campos et al., 1992). Therefore, β-glucosidases regulating the concentration of active form in the phytohormone pools may play an important role in the control of plant development.

**The Release of Secondary Metabolites**

**Protective Functions**

An overwhelming number of secondary metabolites are accumulated and stored as glycosides within plants (Hösel, 1981). The release of secondary metabolites of plants from their glycosides are considered to play various roles in plants including protecting themselves from herbivores, pathogens, and animals. The release of cyanogenic compounds (which evolves toxic hydrogen cyanide) from their glycosides, termed cyanogenesis, is a classical case in plant defense to herbivores (Jones, 1988; Nahrstedt, 1985). Apart from protection, cyanogenic glucosides may also serve as storage forms for reduced nitrogen (Poulton, 1990). In addition, a β-glucosidase was indirectly involved in cabbage resistance to its herbivorous enemies by releasing a mixture of volatiles that attract parasitic wasps to attack the caterpillars (Mattiacci et al., 1995). Salicylic acid (SA) was demonstrated to be a key component in systemic acquired resistance in plants (Enyedi et al., 1992; Klessig and Malamy, 1992; Gaffney et al., 1993). SA occurs mainly as a glucoside in plants (Klick and Herrmann, 1988), and a tobacco β-glucosidase in intercellular spaces was induced by exogenous SA along with the increase of free SA in the same compartment (Seo et al., 1995). DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazine-3[4]-one), the most important preformed toxic substance in maize and wheat, is also found as a glucoside in intact maize. DIMBOA inhibits electron transport and phosphorylation reactions in bovine mitochondria, spinach chloroplast, and maize mitochondria and chloroplast, but the DIMBOA-glucoside does not (Massardo et al., 1994). DIMBOA also has bacteriostatic and fungitoxic activities against fungal pathogens such as *Fusarium nivale*, *Puccinia graminis*
var. tritici, and Helminthosporium turcicum (Mace, 1973). Additionally, DIMBOA can block the induction of vir genes of Agrobacterium by acetosyringone and was suggested to be the reason that Agrobacterium fails to infect maize (Salir et al., 1990). One β-glucosidase was partially purified and shown to be specific for DIMBOA glucoside (Cuevas et al., 1992). A gingiberaceous tropical plant, Costus speciosus, contains spirostanol glycoside (a C-glycoside) which shows saponin functions such as antifungal and haemolytic activities (Osbourn, 1996). The spirostanol glycoside is derived from furostanol glucoside via a β-glucosidase activity (Inoue and Ebizuka, 1996). In addition, avenacosides, oat saponins, are also preformed toxins, and they are not toxic until the glucose at C-26 position is hydrolyzed by a specific β-glucosidase (oat avenacosidase) (Gus-Mayer et al., 1994; Osbourn, 1996). Several phytoalexins and coumestans are also present as glucoside conjugates, such as medicarpin-3-O-glycoside in alfalfa (Sakagami et al., 1974) and coumestrol glycosides in soybean (Le-Van, 1984) and in alfalfa (Tiller et al., 1994). Both of the aglycones showed toxicity to microbes. Phytoalexins are toxic to fungal pathogens, while coumestrol is harmful to bacteria and nematodes (Lyon and Woods, 1975; Rich et al., 1977).

The Release of Flavonoids and Isoflavones

Flavonoids and isoflavonoids are constitutive secondary metabolites in plants, and their functions are fairly diverse, including plant-microbe interactions, plant sexual reproduction, and pigmentation (Heller and Forkmann, 1994; Koes et al., 1994; Dakora and Phillips, 1996).

A. Symbiotic Plant-Microbe Interactions

In the early stages of plant-symbiont interactions, there is signal exchange involved between hosts and bacterial symbionts (Promé and Demont, 1996). In the case of nodulating bacteria, the plant produces signals, mainly flavonoids liberated from their conjugates by β-glucosidase activity, to activate the nod genes in bacteria, and then the bacteria subsequently synthesize the Nod factors to trigger early nodule development on the
host (Long, 1996). The nod-inducing compounds regulating the transcription of microbial genes include isoflavones such as genistein and daidzein (Kossak et al., 1987; Siqueira et al., 1991), flavones such as chrysin, apigenin and luteolin, flavanones such as naringenin, flavonols such as kaempferol and quercetin, and chalcones such as 4,4'-dihydroxy-2'-methoxychalcone and isoliquiritigenin (Redmond et al., 1986; Recourt et al., 1991). In contrast, several isoflavones serve as nod gene inhibitors instead of inducers, for instance daidzein, biochanin A, and formononetin in white clover (Djordjevic et al., 1987), and the latter two in soybean (Kossak et al., 1990). All of these flavonoids and chalcones have their corresponding glycosides in plants.

B. Pathogenic Plant-Microbe Interactions

On the other hand, phenolic signals also play an important role in plant-pathogen interactions (Lynn and Chang, 1990; Siqueira et al., 1991; Dixon and Paiva, 1995). Agrobacterium-infected plants show the crown gall symptoms, indicating cell proliferation. It has been demonstrated that auxin and cytokinin-biosynthetic enzymes, encoded by T-DNA, contribute to this symptom. A class of phenylpropanoid compounds, dehydrodiconiferyl glucosides (DCGs), inducers of plant cell division, was identified to be activated by the cytokinins in tobacco (Binns et al., 1987). However, it remains unclear whether there is a β-glucosidase to regulate this interaction. Besides the three most active inducers of Agrobacterium vir genes, acetosyringone, syringaldehyde, and 3,5-dimethoxyacetophenone, coniferin also activates vir genes. Thus, β-glucosidase activity was proposed to determine the virulence and host range of different Agrobacterium isolates (Morris et al., 1991). Isoflavones can not only be the regulators of nod genes, but also serve as chemoattractants of Phytophthora sojae (Morris and Ward, 1992). The simple isoflavones found in soybean exudates were active as the attractants at 10 nM to P. sojae, but were inactive to non-pathogens of soybean (Morris and Ward, 1992), indicating that the specific interaction between soybean and P. sojae is of particular importance (Deacon and Donaldson, 1993). Another isoflavone, cochliophilin A, from spinach roots, also attracts Aphanomyces cochlioides at 1 nM (Horio et al., 1992).
C. Plant Sexual Reproduction and Pigmentation

A number of plants accumulate flavonoids, including anthocyanins, flavonols, and chalcones, in the anthers and the pistil, the male and female sex organs, respectively. By studying mutants, it was found that flavonoids play an essential role in pollen development (Koes et al., 1994). A majority of pollen flavonols are present as glucoside conjugates in petunia anthers, and petunia pollen lacking flavonols is not able to germinate and sustain tube growth (Taylor and Jorgensen, 1992). The defect can be restored by adding kaempferol, but not kaempferol-3-O-glycoside (Vogt and Taylor, 1995). Bright color is essential for insect-pollinated plants. Most of these flower pigments belong to the class of anthocyanins (red and purple) and chalcones (yellow). Flavonols and flavanones are also present in the petals and form colored complexes with anthocyanin and metal ions (Koes et al., 1994). Dihydroflavonols are the direct precursors for anthocyanins in flowers of Petunia hybrida, and the dihydroflavonol glucosides are accumulated in anthocyanin-synthesis negative mutants. However, the dihydroflavonol β-glucosidase seems unlikely to be involved in anthocyanin synthesis, because there is no difference in β-glucosidase activity between the mutants able to convert dihydro-flavonols into anthocyanins and the mutants unable to do so (Schram et al., 1982).

Lignification

Coniferin and syringin, the cinnamyl alcohol glucosides, have been suggested to be involved in lignification. In gymnosperms, coniferyl alcohol is the monomeric precursor of lignin, and in angiosperms, coniferyl and sinapyl alcohol are the precursors. Coniferin specific β-glucosidases were found in spruce (Picea abies) (Marcinowski and Grisebach, 1978), chickpea (Hösel et al., 1978), and lodgepole pine (Dharmawardhana et al., 1995); and another coniferin and syringin specific enzyme was purified from soybean (Hösel and Todenhagen, 1980). Although the role of cinnamyl alcohol glucosides is not clear, the functions of storage and transport from cytoplasm to cell wall are suggested, since the glucosides are not the substrates of peroxidase which polymerizes the monomer into cell
wall polymers, and thus there is no nonspecific polymerization occurring in the cytoplasm (Hösel and Todenhagen, 1980).

**Substrate Specificity of β-Glucosidases**

Due to their ubiquitous presence, similar structures and catalytic properties of β-glucosidases are expected, and a viewpoint that β-glucosidases only recognize the glucose moiety has been prevalent as well (Esen, 1993). For the sake of convenience and lack of availability of physiologically natural substrates, the chromogenic and fluorogenic substrates such as p-nitrophenyl β-glucoside and 4-methylumbelliferyl β-glucoside had been used extensively. By using these synthetic substrates, some β-glucosidases with lower affinity to them could not be identified, thus leading workers to erroneous conclusions (Hösel and Conn, 1982). The other reason that β-glucosidases lack specificity to the aglycones is the use of enzyme mixtures such as emulsin from almond instead of homogeneous enzymes (Hösel and Conn, 1982). Many β-glucosidases specific for the aglycone moiety of their substrates have been recently purified and characterized (Conn, 1993), and they will be described bellow.

The β-glucosidases specific for the cyanogenic di- and monoglucosides, amygdalin and prunsain from black cherry seeds, and linustatin and linamarin from flax seeds were purified to homogeneity (Fan and Conn, 1985; Kuroki and Poulton, 1986). The β-glucosidases which cleave the terminal glucose of diglucosides are inactive for removing the monoglucosides, and vise versa. The β-glucosidase purified from one plant is specific to its own cyanogenic glucoside, but not to the substrates from other plants (Hösel and Conn, 1982). The syringin and coniferin specific β-glucosidase from soybean showed 100% and 110% relative activity to these phenolic glucosides, respectively, but only 0.5% to isoflavone glucoside and 0.4% to 4-methylumbelliferyl β-glucoside (Hösel and Todenhagen, 1980). The Michaelis constant of the spruce coniferin β-glucosidase was 0.38 mM and showed highest velocity with coniferin (Marcinowski and Grisebach, 1978). The lodgepole pine coniferin β-glucosidase preferred coniferin ($K_m = 0.18$ mM) and syringin ($K_m = 0.29$ mM) as substrates (Dharmawardhana et al., 1995). In healthy, intact sweet clover
(Melilotus alba) leaves, all the coumarins are in the glucoside form, and upon disruption of tissue, the bound coumarins are converted to free coumarins by a β-glucosidase specific for cis-o-coumaric acid β-glucoside with a \( K_m \) of 2.2 mM (Hösel, 1981). A partially purified β-glucosidase more specific for hydroxamic acid glucoside was shown to have different characteristics from the one specific for p-NPG, and the \( K_m \) values for two hydroxamic acids, DIMBOA- and DIBOA-glucosides are 0.11 and 0.17 mM, respectively (Cuevas et al., 1992). Two β-glucosidases involved in indole alkaloid biosynthesis in Catharanthus showed a \( K_m \) of 0.2 mM and 0.1 mM to their endogenous substrate, strictosidine glucoside, but no activity to p-nitrophenyl β-glucoside (Hamscheidt and Zenk, 1980). The furostanol glucoside 26-O-β-glucosidase is highly specific for cleavage of the C-26-bound glucose moiety of furostanol glucosides with a \( K_m \) of 50 mM (Inoue and Ebizuka, 1996). The isoflavone specific β-glucosidase in chickpea favored isoflavone-7-O-glucoside with a \( K_m \) of 0.02 mM, whereas the \( K_m \) with aromatic glucosides are 100 times larger (Hösel and Barz, 1975). A flavone glucoside specific β-glucosidase from Chamomilla recutita shows a \( K_m \) for apigenin 7-O-glucoside of 0.25mM and of 0.29 mM for luteolin 7-O-glucoside (Maier et al., 1991, 1993). Another β-glucosidase from almond cleaves apigenin 7-O-glucoside with a \( K_m \) of 0.76 mM (Pekic et al., 1994). Taken together, for purifying the isoflavone-conjugate-specific β-glucosidase in soybean, the use of isoflavone conjugates as substrates is thus essential to monitor the enzyme activity.

The Localization of β-Glucosidases in Plants

In general, the β-glucosidases of dicots are localized to the apoplast, while those of monocots are localized to the cytoplasmic plastids (Esen, 1993). To study the localization of β-glucosidases, the simultaneous azo-coupling technique (Ashford, 1970) and one-step fluorescence optical procedure (Gierse and Barz, 1976) were used for histochemical localization in early years. Some drawbacks of these two methods limit the applications in localization studies. For instance, the azo-coupling technique could not be applied to polyphenol containing plants, and the non-specific substrates, 6-bromo-2-naphthyl-β-glucoside and 4-umbelliferyl β-glucoside, respectively, were used in these methods. By
using the first method, β-glucosidase activity aiming at DIMBOA-glucoside was found to be restricted to the phloem of the small vascular bundles in maize at particulate sites in the cytoplasm (Mace, 1973). Using the second method, Gierse and Barz (1976) investigated the distribution of β-glucosidase activity in chickpea, and found the enzyme activity predominantly in the cortex cells, but also in several other tissues. Because of the non-specificity of these methods, it is not possible to examine the specific β-glucosidases in plant tissue except using immunohistochemical approaches in which the immunoglobulin is specific against the protein of interest. In recent years, the techniques have improved from immunofluorescence to immunogold labeling, and from polyclonal antibody to monoclonal antibody (Ibrahim, 1992). By using immunofluorescent techniques, the coniferin specific β-glucosidase from spruce was localized at the inner layer of the secondary cell wall in 9-day-old hypocotyl (Marcinowski et al., 1979), in accordance with the enzyme extraction data (Marcinowski and Grisebach, 1978). By the same means, coniferin specific and isoflavone 7-glucoside specific β-glucosidases were also inspected in stem and root sections of chickpea (Burmeister and Hösel, 1981). The coniferin specific β-glucosidase was found in the cell walls of the tracheary elements and of the endodermis, epidermis and exodermis; while the isoflavone 7-glucoside specific β-glucosidase was predominantly located in the parenchymatic cortex cell cytoplasm (Burmeister and Hösel, 1981). This is an exception to the general rule that β-glucosidases in dicotyledonous plants are localized in cytoplasm. To extract β-glucosidases from various plant tissue/organelle preparations is an alternative way to understand the localization of the enzymes. By extracting from the developing xylem of mature Pinus banksiana wood, with buffer containing sodium chloride and Triton X-100, two acidic glycoproteins were confirmed to have coniferin-hydrolyzing β-glucosidase activity. Due to the solubility in buffer containing low salt and mild detergent, these enzymes might be cell wall-associated, but not cell wall-bound proteins, unlike the coniferin β-glucosidase in spruce (Leinhos et al., 1994). A β-glucosidase specific to the glucoside of 2-hydroxy-cis-cinnamic acid (coumarinyl glucoside), but not the trans isoform (o-coumaryl glucoside) was demonstrated to be located in the extracytoplasmic space of sweet clover (Oba et al., 1981). A dihydroflavonol glucoside β-glucosidase in Petunia
hybrida was isolated from the cell wall, whereas several β-glucosidases with unknown function such as the β-glucosidases from Lactuca sativa pith (Giordani and Lafon, 1993), barley and oat (Li et al., 1989), maize (Nagahashi et al., 1992), and soybean (Nari et al., 1982/83), were also isolated or detected from cell wall.

The Importance of Isoflavone-Conjugate-Specific β-Glucosidase in Disease Resistance

Constitutive Conjugates Serve as Precursors for Inducible Pterocarpan Phytoalexins

The phytoalexins will not be synthesized until the plant is challenged biotically or abiotically, as mentioned in the "General Introduction". A prevailing view held in the literature for a number of years is that phytoalexin is quantitatively synthesized de novo from the early precursors of primary metabolism. Although researchers found isoflavone conjugates occasionally in plants, they did not think the conjugates played any roles in the induced phytoalexin biosynthesis. However, in soybean and chickpea, the isoflavones and their conjugates, the metabolic intermediates of glyceollin biosynthesis in the phenylpropanoid pathway, are constitutively present in plant organs and serve as precursors for inducible pterocarpan phytoalexins (Graham et al., 1990; Barz and Mackenbrock, 1994). In soybean, the isoflavones daidzein and genistein and their conjugates are differentially distributed in organs (Graham, 1991b). For instance, daidzein-7-O-glucoside (syn. daidzin, for abbreviation, DZ1), 6″-O-malonyl-7-O-β-glucosyl daidzein (DZ2), and 6″-O-malonyl-7-O-β-glucosyl genistein (GT2) are major isoflavones in 7-day-old cotyledons. In roots and hypocotyls, DZ2 is the predominant conjugate, while in the primary leaf, GT2 and flavonoids and their glycosides are the majority (Graham, 1991b). Furthermore, in root tips of light grown soybean, DZ2 reaches the highest amount at about 6000 nmoles/g fresh tissue, followed by free daidzein at 1200 nmoles (Graham, 1991b). Upon infection by Phytophthora sojae race 1, the amount of daidzein in the proximal cell population of soybean cv. Williams 79 decreases rapidly, concomitant with the quick and large accumulation of glyceollins within 48 hr. On the other hand, total daidzein conjugates increased at the first day and then held level for another 2 days. However, in the compatible reaction (P. sojae race 1 vs. Williams), the decrease of daidzein and increase of glyceollins
were delayed, and only low levels of glyceollin were formed within 48 hr (Graham et al., 1990). Another significant difference between compatible and incompatible responses was the large accumulation of daidzein conjugates in the former reaction. A similar study in soybean leaves and hypocotyls revealed the accumulation of isoflavones and their conjugates in response to *Phytophthora sojae* (Morris et al., 1991). On the basis of the preformation of isoflavone conjugates and the turnover between these conjugates and glyceollin, Graham et al (1990) suggested that glyceollin accumulation might not be solely from *de novo* synthesis upon challenge by pathogens. Daidzein released through enzymatic reactions could be used to synthesize the phytoalexin (Graham et al., 1990). Likewise, isoflavone conjugates, including medicarpin-3-O-glucoside-6"-malonate, afromosin-7-O-glucoside, and afromosin-7-O-glucoside-6"-malonate, were major constitutive secondary metabolites in alfalfa cell cultures (Kessmann et al., 1990). Among these isoflavone conjugates, only medicarpin conjugates responded to elicitation, while the amount of afromosin conjugates did not change. In addition, the application of a competitive phenylalanine lyase inhibitor, L-a-aminooxy-β-phenylpropionic acid (L-AOPP), showed that only a small amount of \(^{14}\)C labeled phenylalanine was incorporated in medicarpin, suggesting that phytoalexin synthesis might be from the preformed conjugates (Kessmann et al., 1990). A study in chickpea backed this suggestion up (Barz and Welle, 1992). In chickpea, the AOPP was used to block the *de novo* synthesis of the phenylpropanoid pathway, but the researchers still found a considerable amount of phytoalexins in chickpea cell culture. They also observed that the pre-existing formononetin 7-O-glucoside-6"-malonate was hydrolyzed to release the aglycone and the formation of pterocarpan phytoalexin occurred consequently (Mackenbrock and Barz, 1991). Besides isoflavone conjugates, two phytoalexins, medicarpin and maackiain, were also formed constitutively as 3-O-glucoside-6"-malonates conjugates in chickpea cell culture (Mackenbrock et al., 1993). The ratio of free aglycone and conjugate was highly dependent on the concentration of elicitor. When a low to moderate amount of elicitor was applied, more conjugates were found in cell culture, whereas high doses of elicitor favored the release of aglycones (Mackenbrock et al., 1993). This finding could apply to the real world where the host
encountering less density of pathogens or other non-pathogens will synthesize either isoflavone conjugates or pterocarpan phytoalexin conjugates constitutively, and then hydrolysis takes place when the host faces bigger challenges.

**The Hydrolysis and Formation of Conjugates Are Through Enzymatic Reactions**

Of particular importance is the fact that hydrolysis and formation of isoflavone conjugates are mediated by enzymes. In chickpea, the enzymes catalyzing the formation of conjugates, UDP-glucose:isoflavone-7-O-glucosyltransferase and isoflavone-7-O-glucoside-6'-malonate malonyltransferase were purified separately (Köster and Barz, 1981; Köster et al., 1984). The hydrolytic enzymes, isoflavone-7-O-glucoside-6’-malonate malonylsterase and isoflavone-7-O-glucoside β-glucosidase were also isolated to homogeneity (Hösel and Barz, 1975; Hinderer et al., 1986). These four enzymes had been suggested to catalyze the reactions to form or break down the pterocarpan phytoalexin conjugates, and they were highly specific to their natural substrates (Mackenbrock et al., 1993). Interestingly, these four enzymes were regulated by the concentration of elicitor, which was consistent with the phenomenon that the ratio of conjugate and aglycone depended upon the elicitor concentration. At low elicitor levels, isoflavone glucosyltransferase and malonyltransferase were highly induced, while they were suppressed by higher amounts of elicitor. An inverse situation was observed for malonylsterase and β-glucosidase (Mackenbrock et al., 1993). This findings suggest that enzymatic regulation is important for conjugate metabolism upon elicitor challenge.

In soybean, such enzymes regulating the conjugate pools (Figure 1.1) have not yet been reported, except for relatively non-specific β-glucosidases hydrolyzing isoflavone glucosides (Matsuura and Obata, 1993). It is a reasonable assumption that soybean also contains these regulating enzymes because of the similarity in the structure of isoflavones and phytoalexins between soybean and chickpea (Graham and Graham, 1991b). Among these four enzymes, β-glucosidase is of particular interest because the released isoflavones, genistein and daidzein, both play defensive roles. Genistein was found to be toxic to the soybean pathogen *Phytophthora sojae* at 60-120 mM and fungicidal at 240 mM (Rivera-
Vargas et al., 1993). In addition, genistein exhibits antifungal activity against *Rhizoctonia solani, Sclerotium rolfsii, Cercospora beticola,* and *Monilinia fructicola* (Johnson et al., 1976; Weidenbomer et al., 1990). Daidzein, on the other hand, not only serves as the precursor of glyceollin (Ebel, 1986), but also inhibits the growth of *Fusarium culmorum* (Kramer et al., 1984). Because of the toxicity of the isoflavones *per se* and their involvement in the synthesis of the more toxic substance, glyceollin, isoflavone-conjugate-specific β-glucosidases have been proposed to play a role in broad spectrum resistance or tolerance in soybean. For a better understanding of the isoflavone-conjugate-specific β-glucosidase in soybean defense, we purified this protein and characterized its unique properties. The objectives of this study reported in this chapter were to 1) evaluate the distribution of specific and non-specific β-glucosidases in soybean organs, particularly in roots, 2) determine the best source of enzyme for purification, 3) investigate whether the isoflavone-conjugate-specific β-glucosidase could be regulated by elicitor, and 4) determine if this enzyme is inducible, and if transcription and translation inhibitors could block its induction.
Figure 1.1: The enzyme regulation of isoflavone conjugate pools. Isoflavone 7-O-glucoside-6'-O-malonates (DZ2 and GT2) are hydrolyzed by a malonyltransferase to release isoflavone 7-O-glucosides (DZ1 and GT1), which are then cleaved by a specific β-glucosidase to liberate their free aglycones.
MATERIALS AND METHODS

**Chemicals**

Acetonitrile (HPLC grade), p-nitrophenyl β-glucopyranoside, actinomycin D, and cycloheximide were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A). Chloroform, methanol, ethyl acetate, benzene, and diethyl ether were obtained from Mallinckrodt AR. Acetic acid and HCl were purchased from EM Science, NJ. Phytophthora sojae wall glucan was prepared according to Ayers et al. (1976), and as noted previously (Graham and Graham, 1991b).

**Plant Material**

Seeds of soybean (*Glycine max* L.) cultivars Williams, Williams 79, Williams 82 and Harosoy seeds were obtained from Dr. A. F. Schmitthenner (Department of Plant Pathology, The Ohio State University, Columbus, OH). Seedlings were grown at 26°C with 500 mE/m²/s of light on a 14-h photoperiod for 7 to 8 days (Graham, 1991b). The seedlings were watered every other day during growth without fertilization.

**The Preparation of Substrates for the Isoflavone-Conjugate-Specific β-Glucosidase**

Soybean cv. Williams seeds (50 grams) were ground to a fine powder using a coffee grinder and suspended in 100 ml chloroform for 30 min with stirring to remove soybean seed lipid. The solids were filtered through Whatman #1 filter paper or recovered by low speed centrifugation (3/4 speed for 10 min, International Equipment Co., Needham, HTS., Mass., USA). This step was repeated with chloroform and then with hexane for 30 minutes each. The defatted soybean flour was extracted twice with distilled water for another 30 min. The pooled extract is a crude substrate mixture containing isoflavone conjugates and their free aglycones. To eliminate the free aglycones, which interfered the evaluation of enzyme products, several methods were evaluated. First, the dilute crude extract, ca. 10 ml, was passed slowly through a PrepSep C18 column (Fisher Scientific, Springfield, NJ) previously washed with 100% methanol and distilled water. After washing with distilled
water, the bound chemicals were eluted in one step by at least 2 ml 100% acetonitrile. The eluent was evaporated to dryness, taken up in distilled water and partitioned with ethyl acetate, chloroform, benzene, or diethylether. In a second method, 1 ml various percentages of acetonitrile was used to fractionate the bound chemicals from the C18 column. In a third method, the concentrated eluent from the first method was subjected to preparative TLC (Silica Gel G, 20 × 20 cm, 1000 m, Analtech, DE), developed by chloroform:methanol:20% acetic acid (7/3/1). The materials were monitored as dark spots by a UV lamp (Spectroline® model ENF-280C, Spectronics Corps., NY) at short wavelength (254nm). The dark bands were scraped off the plate, and then eluted with absolute methanol. The efficiency of these three methods in preparing suitable substrates was evaluated by analytical high performance liquid chromatography (HPLC) as described below.

**Enzyme Assay**

The standard assay for the isoflavone-conjugate-specific β-glucosidase was carried out as follows. Five microliters of partially purified substrate mixture (≥ 1mM final concentration) was dissolved in 0.1M phosphate-0.05M citrate buffer, pH 5.0, and the same volume of enzyme preparation were incubated at 40°C for 30 min. The reaction was stopped by adding 90 μl of methanol, and activity was measured by HPLC as described by Graham (1991a) except that a 20 minute gradient was used. With the use of appropriate standards, the integrated area of released aglycones was converted into concentrations. For chromogenic substrates such as p-nitrophenyl-β-glucoside (pNPG), the assay solution containing 90 μl of 2 mM substrate in 0.1M phosphate-0.05M citrate buffer, pH5.0, and 10 μl of enzyme solution was incubated at 40°C for 30 min. The reaction was then stopped by adding 900 μl of 1 M sodium carbonate. The p-nitrophenol liberated from pNPG was measured at 400 nm, and the concentration calculated by Beer’s law in which the ε value (mM⁻¹cm⁻¹) is equal to 19.30. One unit of β-glucosidase activity was defined as the amount of enzyme that produced 1 mmol of product per minute. Specific activity was expressed as enzyme units per mg protein.
Distribution of β-Glucosidases in Soybean Organs

The distribution of isoflavone-conjugate-specific β-glucosidase and other non-specific β-glucosidases in soybean organs, using the isoflavone conjugate mixture and pNPG as substrates, respectively, was investigated in the soybean cultivars Williams, Williams 79, Williams 82, and Harosoy. Different organs of each cultivar were examined. The organs were dissected into shoot apical meristem (SAM), stem, leaf, cotyledon, hypocotyl, and root. The plant materials were prepared according to the method mentioned earlier.

Enzyme Extraction

Every step was conducted at 0 to 4°C, unless mentioned otherwise. Plant materials were ground with a pre-cooled mortar and pestle with 200 mM phosphate buffer, pH 6.0 at 1:1 (W/V) ratio. The crude extract was filtered through four-layers of cheesecloth, and centrifugated at 10,000 x g for 15 min. This low speed pellet (cell wall fraction) was washed twice with 200 mM phosphate buffer, pH 6, and then extracted twice with the same buffer containing 0.5 M sodium chloride. The precipitate was removed by centrifugation (10,000 x g for 10 min) and the supernatant was the cell wall extract. The enzyme activities of crude extract and cell wall extract were determined by spectrophotometric and HPLC methods.

Distribution and Developmental Regulation of Isoflavone-Conjugate-Specific β-Glucosidase in Soybean Roots

The isoflavone-conjugate-specific β-glucosidase was further monitored in distinct sections of 7-day-old etiolated Williams seedlings, including root tip (RT), elongation zone (EZ), lateral root zone (LRZ), lower hypocotyl (LH), hypocotyl hook (HH), primary leaf (PL), cotyledon (COT), and seed coat (SC). For this purpose, soybean cv. Williams seeds were surface sterilized briefly with 80% ethanol and wrapped in wet germination papers (Anchor Paper, Packaging Converters, Hudson, WI) for germination and growth as described elsewhere (Graham, 1991b). Each section was collected from six individual
etiolated soybean seedlings. These subsamples were pooled and the enzyme was extracted as previously described. The developmental distribution of this enzyme was inspected in the same sections at various times after germination, except day 2 in which EZ and LRZ were not differentiated.

**Enzyme Source for Purification**

**Soybean seeds as source**

Soybean seeds (100 gm) were soaked in tap water at room temperature overnight, then drained and rinsed twice with cool water. The soaked seeds were homogenized in a Waring blender with 400 ml 100 mM phosphate buffer, pH 6.6. The debris were removed by filtering through cheesecloth, and the extract was centrifuged at 3,000 × g for 10 min. The supernatant was acidified with 0.1N HCl to pH 5.0, and centrifuged again at 8,000 × g for 20 min (Matsuura and Obata, 1993). The resulting supernatant served as the source of crude enzyme.

**Soybean roots as source**

Seven-day-old soybean roots were washed with tap water to remove planting media and harvested by cutting them from the base of the crown with a razor blade. Roots with scars or rotten lesions were discarded. The roots were frozen and then ground into powder with an ice cold mortar and pestle. The crude extract and cell wall wash were obtained as before.

The intercellular fluid of roots was collected by using a design described below. About 30 g roots were cut into relatively small pieces and immersed in a beaker containing 100 ml phosphate buffer, 100 mM, pH 6.0, containing 0.5 M sodium chloride. The roots were placed in a desiccator and infiltrated under vacuum for 30 min, tapping the desiccator to facilitate release of bubbles. The infiltrated roots were blotted with paper towels and put in a polystyrene centrifuge tube (30 ml in size) with three holes on the bottom, which was placed in a bigger centrifuge bottle (250 ml in size). The whole set was centrifuged at 200 × g for 10 min at 4°C. Care was taken not to spin too fast in order to avoid cytoplasmic
protein contamination. The intercellular fluid which had been spun down in the bigger bottle was pooled and served as the infiltration fluid.

*Induction of Isoflavone-Conjugate-Specific β-Glucosidase by Phytophthora sojae Wall Glucan*

To understand whether the isoflavone-conjugate-specific β-glucosidase was inducible by *Phytophthora sojae* elicitor, the cut cotyledon assay (Graham and Graham, 1991b) was performed. The concentration of elicitor used in this experiment was 100 mg/ml, the saturated concentration for reaching maximal phytoalexin induction. Ten cotyledon section 1 tissues (as defined by Graham and Graham, 1991) were harvested and weighed at various times after elicitation. The SI (section 1) tissues were extracted by grinding the tissues in 400 µl of 100 mM phosphate-50 mM citrate buffer, pH 5, with a polypropylene pestle (Kontes Glass Co., Vineland, NJ). The crude extract was centrifuged for 10 min at 18,000 × g, and the supernatant was assayed for enzyme activity as described. The precipitate was resuspended and centrifuged in the same buffer for two cycles, and the pellet was blotted by Kimwipes and weighed. The pellet then was incubated with 10 µl natural substrate mixture at 40°C for 30 min, and the reaction stopped with 90 µl methanol. After centrifugation, the supernatant was taken directly for HPLC.

*Suppression of Isoflavone-Conjugate-Specific β-Glucosidase After Elicitation by Actinomycin D and Cycloheximide*

The cut cotyledon assay was conducted as usual, except the application of elicitor (100 mg/ml) was made along with actinomycin D or cycloheximide. The final concentrations of actinomycin D and cycloheximide applied on cut cotyledons were 1000, 200, 50, and 20 mM, and 200, 50, and 10 mM, respectively. The enzyme activity of the crude extract and cell wall fraction were measured at 8 h post-elicitation.
RESULTS

Preparation of Substrates for Isoflavone-Conjugate-Specific \( \beta \)-Glucosidase

To measure the isoflavone-conjugate-specific \( \beta \)-glucosidase activity, the use of isoflavone conjugates as substrate was of particular importance, since the use of artificial substrates such as \( p \)NPG or 4-methylumbelliferyl-\( \beta \)-glucoside during the purification could lead to other non-specific \( \beta \)-glucosidases which have low affinity to isoflavone conjugates (Hösel and Conn, 1982). The easiest and most efficient way to prepare the isoflavone conjugates as substrates was 20% acetonitrile fractionation from a PrepSep C18 column loaded with crude isoflavone extract. The preparation consisted of malonyl glucosyl daidzein (DZ2), malonyl glucosyl genistein (GT2), daidzein glucoside (DZ1), and genistein-glucoside (GT1), as shown in Figure 1.2A. Solvent partitioning against water did not separate free aglycones from their conjugates, except with ether. Alternatively, preparative TLC isolated these isoflavones into three groups monitored by a UV lamp at 254 nm bands, i.e. malonyl glucosyl conjugates, glucosyl conjugates, and free aglycones. These occurred at Rf values 0.28, 0.55, and 0.86, respectively. Preparative TLC could not separate daidzein and its conjugates from genistein and its conjugates when chloroform: methanol: 20% acetic acid (7/3/1) was used as development solvent. Due to the lower yield and hazardous process, which might be harmful to enzyme activity in the latter two methods, 20% acetonitrile fractionation from PrepSep C18 column was selected to be the sole means to prepare the substrate mixture for routine enzyme assay. The HPLC chromatogram of substrate mixture was used to monitor the absence of free isoflavones (Figure 1.2A).

Enzyme Assay

For isoflavone-conjugate-specific \( \beta \)-glucosidase activity, the same amount of enzyme solution and substrate were incubated at 40°C for 30 min, and the products, free daidzein (peak 5) and genistein (peak 6) were shown clearly in the HPLC chromatogram (Figure 1.2B). Subsequently, the integrated area of peak daidzein and genistein were
Figure 1.2 HPLC chromatograms before and after enzymatic digestion. Enzyme solution was mixed with partially purified substrates and the reaction stopped at time 0 (A), and time 30 min (B). The products, daidzein (peak 5) and genistein (peak 6) are shown. Peak 1, DZ1; peak 2, DZ2; peak 3, GT1; peak 4, GT2.
converted to concentration (nmole/ml) on the basis of the response factors which had been established by previous work (Graham, 1991a). Using these values, the enzyme activity was deduced (mmol/min).

Distribution of β-Glucosidases in Soybean Organs
The specific activity of isoflavone-conjugate-specific β-glucosidase was found mainly in the soybean root cell wall fraction (Figure 1.3A), regardless of cutivar, when isoflavone conjugates were used as enzyme substrates, indicating this particular enzyme was relatively abundant in the root cell wall fraction. However, the utilization of pNPG for monitoring non-specific β-glucosidases revealed that non-specific enzyme activity is distributed quite evenly in all soybean organs with the greatest amount in shoot apical meristems (SAM) in both the crude extract supernatant and cell wall fraction (Figure 1.3B).

Distribution and Developmental Regulation of Isoflavone-Conjugate-Specific β-Glucosidase in Soybean Roots
The distribution of isoflavone-conjugate-specific β-glucosidase was further examined in 7-day-old etiolated cv. Williams soybean seedlings. This specific enzyme was present at greatest concentration in the root tip, where the malonylated conjugate of daidzein as well as free daidzein have previously been shown to be plentiful (Graham, 1991b). Somewhat lower levels of enzyme were found in the elongation zone and root hair zone (Figure 1.4). Again, the cell wall fraction contained more enzyme than the crude soluble extract. This specific enzyme in the root tip did not reach its highest concentration until 6 to 7 days (Figure 1.5). Intriguingly, the amount of isoflavone-conjugate-specific β-glucosidase in the root hair zone was fairly high at day 2, then dropped dramatically one day later, and climbed again at day 7 (Figure 1.5). From previous studies (Graham, 1991b), it is suggested that this later rise might be the contribution of lateral root tips.
Figure 1.3: The distribution of isoflavone-conjugate-specific β-glucosidase (A) and nonspecific β-glucosidase (B) in soybean. CR, crude extract; CW, cell wall wash.
Figure 1.4: Distribution of the isoflavone-conjugate-specific \( \beta \)-glucosidase in soybean cv. Williams root and other organs. The crude enzyme was extracted with 0.1 M phosphate buffer, pH 6.0, and the cell wall fraction was washed by 0.5 M NaCl in the same buffer. The enzyme activity unit (U) was defined as 1 \( \mu \)mole/min. RT, root tip; EZ, elongation zone; RHZ, root hair zone; LH, lower hypocotyl; HH, higher hypocotyl; COT, cotyledon; PL, primary leaf; SC, seed coat. ■ cell wall extract; □ crude supernatant.
Figure 1.5: Developmental distribution of the isoflavone-conjugate-specific β-glucosidase in soybean root. A: crude supernatant; B: cell wall extract. Abbreviations are the same as in Figure 1.4.
**Enzyme Source for Purification**

Imbibed soybean seeds were not selected to be the source of enzyme because of the low specific activity (Table 1.1). On the other hand, the high specific activity of root intercellular fluid obtained from vacuum infiltration suggested that it could be a good candidate, but the low amount of proteins made the purification very difficult. Thus, the final source of isoflavone-conjugate-specific β-glucosidase for purification was from the cell wall wash, in which the concentration of protein was moderate, and the specific activity was the highest (Table 1.1).

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Quantity</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imbibed seeds</td>
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<td>6093</td>
<td>501</td>
<td>0.08</td>
</tr>
<tr>
<td>Infiltrated roots</td>
<td>66</td>
<td>18</td>
<td>114</td>
<td>6</td>
</tr>
<tr>
<td>Cell wall wash</td>
<td>780</td>
<td>124</td>
<td>8775</td>
<td>71</td>
</tr>
</tbody>
</table>

Table 1.1: The determinant factors of enzyme sources for starting enzyme purification.

**Induction of Isoflavone-Conjugate-Specific β-Glucosidase by Phytophthora sojae Wall Glucan**

The isoflavone-conjugate-specific β-glucosidase in soybean cultivars was induced slightly by *Phytophthora sojae* wall glucan at 8 to 12 h post-elicitation (Figure 1.6). In Williams, the maximal induction only reached 0.03 and 0.3 mU/mg tissue in the crude extract and cell wall fractions, respectively. In contrast, the induction of this enzyme in the cell wall fraction in the soybean cultivars Williams 79 and Williams 82, which carry *Rps*...
genes, was 3-fold and 2-fold higher, respectively, than the enzyme activity in cv. Williams, a universal susceptible cultivar without known Rps gene (Figure 1.6). However, the low overall induction of enzyme activity suggested that the isoflavone-conjugate-specific β-glucosidase might be mainly constitutive in soybean cultivars.

**Suppression of Isoflavone-Conjugate-Specific β-Glucosidase After Elicitation by Actinomycin D and Cycloheximide**

Actinomycin D and cycloheximide are non-specific transcriptional and translational inhibitors, respectively. As shown in Figure 1.7, the enzymes in cv. Williams crude extract supernatant were less sensitive to both inhibitors than the those in the cell wall fraction, but the induction by *P. sojae* wall glucan was all suppressed, suggesting *de novo* enzyme synthesis might be involved in this induction.
Figure 1.6: Induction of the isoflavone-conjugate-specific β-glucosidase in soybean cultivars by *Phytophthora sojae* wall glucan. Enzyme activity was measured in glucan-treated cotyledon tissue (S1) from (A) crude extract supernatant and (B) cell wall fraction.
Fig. 1.7: The suppression of glucan-induced isoflavone-conjugate-specific β-glucosidase activity by actinomycin D and cycloheximide. The enzyme activity of the cotyledon tissues treated with elicitor or elicitor/inhibitor was examined in (A) the crude extract and (B) cell wall fraction. ■ Actinomycin D; □ Cycloheximide.
DISCUSSION

For purifying an enzyme with a specific function, a specific substrate for measuring the enzyme activity is requisite (Conn and Hösel, 1981). Unfortunately, the isoflavone conjugate specific β-glucosidase substrates such as daidzein 7-O-glucoside (daidzin, DZ1 for abbreviation) are not commercially available, or very expensive. The isolation of substrates was the first step in this study. Matsura and Obata (1993) reported the purification of β-glucosidases from soybean cotyledons by using DZ1 and GT1 as substrates. The authors claimed to have isolated and separated them from each other by means of a gel filtration on a Sephadex G-25 column. However, it seems unlikely that it would be possible to differentiate DZ1 from GT1 by means of size exclusion column chromatography, since the difference in the two molecular weights is only an oxygen atom. Moreover, in the beginning, it is not known whether or not the isoflavone-conjugate-specific β-glucosidase is able to hydrolyze the β-glucosidic bond when the glucose is malonylated. It is a reasonable assumption that the hydrolysis of malonylated isoflavone glucosides to release free isoflavones is conducted by two enzyme activities, i.e. an esterase for removing the malonyl group first and then a β-glucosidase, since such enzymes have been identified in chickpea (Hösel and Barz, 1975; Köster et al., 1984). A substrate mixture containing all four isoflavone conjugates could allow us to measure both enzyme activities at the same time by an analytical HPLC method. The increased amount of free isoflavones is used for β-glucosidase activity, and the decreased amount of DZ2 or GT2 is used for esterase activity. The presence of free DZ and GT in the substrate mixture would probably interfere with the enzyme activity calculation, and thus the aglycones should not exist in this mixture. In a previous study, the isoflavone species were obtained from a PrepSep C18 column that was saturated with a crude soybean flour extract and subsequently eluted with 100% acetonitrile or methanol (Graham, 1990b). It is known that isoflavone glucosides are slightly more hydrophilic than the isoflavone aglycones. Therefore, several hydrophobic solvents were used to partition the crude isoflavones prepared from PrepSep C18 column against water. Only ether could remove the free isoflavones successfully. Preparative TLC
is another common means to get a large quantity of substances. The developing solvent system we used, chloroform:methanol: 20% acetic acid (7/3/1 lower layer) (Kudou et al., 1991), separated isoflavone-7-O-glucoside-6'-O-malonates, isoflavone-7-O-glucosides, and free isoflavones with increasing Rf values. The disadvantages of these later two methods were the longer time and smaller scales for the preparations. The use of various percentages of acetonitrile stepwise to elute the isoflavone conjugates was the fastest and most efficient method to get a large quantity of substrates. This substrate mixture needed to be stored at -20°C to prevent simultaneous or microbial hydrolysis. A control was always run to eliminate this potential error. In the substrate mixture, DZ2 and GT2 are usually the major conjugates, whereas DZ1 is intermediate and GT1 is the least prevalent. Substrate concentration was always adjusted so that the concentration of DZ1 was always over ten fold the Km of the known chickpea isoflavone glucoside specific β-glucosidase for its natural substrate (Hösel and Barz, 1975).

The soybean isoflavone-conjugate-specific β-glucosidase was shown to be predominant in the root cell wall fraction, regardless of the examined cultivars (Figure 1.3). The similarity of distribution of the enzyme in various soybean cultivars, including some with Rps genes (Williams 79, Williams 82 and Harosoy) and one lacking the genes (Williams), suggests that constitutive levels of this enzyme are not involved in race-specific resistance. It is interesting that this enzyme is mainly in the root, particularly in the root tip, while the isoflavone glucoside specific β-glucosidase in chickpea was isolated from root, leaf, and stem as isoforms (Hösel and Barz, 1975). However, this does not mean that the total enzyme activity in other organs is low, but just relatively less in comparison. In contrast, the non-specific β-glucosidases monitored by p-nitrophenyl-β-glucoside were spread over the all soybean organs, although a relatively higher non-specific β-glucosidase activity was found in the shoot apical meristem (SAM).

The criteria for compartmentalization of proteins in plant tissues have been defined (Li et al., 1989). The soluble cytoplasmic proteins can be obtained from the supernatant by disrupting cells and removing the organelles and broken cell walls. The soluble apoplastic proteins are washed from tissues by buffer without damaging the plasmalemma. There are
two kinds of cell-wall-associated proteins including the ionically bound proteins and covalently bound or entrapped proteins. The ionically bound proteins can be washed off by salt with high ionic strength, but the entrapped proteins cannot. According to these criteria, the isoflavone-conjugate-specific \( \beta \)-glucosidase may be ionically bound to the cell wall. It is not excluded that this protein could also be a peripheral membrane protein in the intercellular space. In fact, after extraction twice by 0.2 M phosphate buffer, pH 6, containing 0.5 M NaCl, there still was a considerable amount of \( \beta \)-glucosidase activity remaining in the cell wall when \( p \)-nitrophenyl \( \beta \)-glucoside was used as substrate (data not shown). The enzyme activity in the supernatant of crude extracts may come from at least two \( \beta \)-glucosidases, one of them being the isoflavone-conjugate-specific enzyme and the other a non-specific and relative heat stable protein.

The accumulation of DZ2 to 6000 n mole/g tissue in root tips (Graham, 1990b) is consistent with the idea that the isoflavone conjugate specific \( \beta \)-glucosidase is present close to its natural substrates. Isoflavones and their conjugates were recovered in seed and root exudates (Graham, 1990b). These isoflavones conjugates exhibit not only the chemotactic activities, but also are positive nod gene regulators of \textit{Bradyrhizobium japonicum} (Smit et al., 1992). The chemotatic ability of GT2 and DZ2 to \textit{B. japonicum} is at 1 n mole and 100 n mole ranges, respectively (Khan and Bauer, 1988). GT2 is enriched in seed, and therefore it is possible that \textit{B. japonicum} is attracted by GT2 toward soybean imbibed seed and when the concentration of GT2 gets higher, then the bacterium shifted toward the root tip where the DZ2 concentration is higher. Consequently, the nod gene turns on, and nodulation takes place. On the other hand, free daidzein and genistein are involved in chemotaxis of \textit{Phytophthora sojae} zoospore at 10 nM as well (Morris and Ward, 1992). The amount of free genistein seems not likely to reach 240 mM, a fungicidal dose (Rivera-Vargas et al., 1993), in the rhizosphere. Moreover, the attracted zoospore encysts immediately when it reaches the soybean root and germinates and penetrates subsequently (Deacon and Donaldson, 1993). Therefore, the equipped sensor for DZ and GT is considered as a virulence factor of \textit{P. sojae}. Intriguingly, the isoflavone conjugate specific \( \beta \)-glucosidase is
secreted into root tip exudates, where free daidzein is dominant. However, treatment of this root tip exudate with heat (100°C, 5min), causes DZ2 to be predominant (Graham, 1991b).

The level of the isoflavone-conjugate-specific β-glucosidase varies a great deal in developmental stages. At 6 days, this protein reached its highest amount in the root tip, and by the seventh day, lateral root tips contributed much enzyme activity too (Figure 1.4). In contrast, enzyme activity in other parts of the root did not show significant change with age. Although it is obvious that the root tip is the best enzyme source, to collect a reasonable amount of root tips is not practically possible. It is not known which types of cells produce more isoflavone conjugate specific β-glucosidase, since there are several types of cell populations in the root tip.

The only study of a β-glucosidase hydrolyzing isoflavone conjugates from soybean was reported by a Japanese group studying the objectionable aftertaste of soybean products (Matsuura and Obata, 1993; Matsuura et al., 1995). Three isozymes were separated by ion exchange on CM-Sephadex C-50 from imbibed seed extract. Due to this previous work, although the distribution study showed that the root cell wall fraction was a good candidate for starting the protein purification, we also tried to use the imbibed seed preparation as a start. Unfortunately, there were tremendous loads of proteins in this seed extract, and the isoflavone conjugate hydrolyzing activity was relatively low (0.08 mU/mg proteins, Table 1.1). Due to the appearance of the isoflavone conjugate specific β-glucosidase in the cell wall fraction, it might be possible that the specific enzyme extracted from the intercellular space would be relatively more pure. In spite of a 75-fold purer protein than in the seed extract, only 18 mg proteins were in the infiltrated fluid from 66 g root. This low amount of protein was too little to do further purification. Washing at least 7 days old root cell wall fraction with a high salt solution ultimately allowed the purest quality and largest quantity of starting enzyme preparation.

The isoflavone-conjugate-specific β-glucosidase was not induced substantially by P. sojae wall glucan in soybean cultivars (Figure 1.6). The selection of cotyledon instead of root for determining the induction was based on the fact that the amount of enzyme was not high in cotyledons so that the induction would not be overshadowed by high background.
The slight induction of the enzyme activity by elicitor is similar to other enzymes in the phenylpropanoid pathway in which a strong induction occurs at about 8-10 h post-elicitation (Ebel, 1986). It is not clear if this enzyme is inducible in root, where it is present abundantly. Although the induced enzyme activity was higher in soybean cultivars with Rps genes, this enzyme was not truly highly inducible in these resistant cultivars. The fact that much more abundant DZ2 accumulated in elicitor-treated proximal cells in cv. Williams, but not in Williams 79, both infected by P. sojae race 1, suggests that the activity of β-glucosidase in Williams is constitutive and is not turned on by some kind of signal(s) which may be encoded by Rps genes. The slight induction of the isoflavone-conjugate-specific β-glucosidase differs from that of β-glucosidase from chickpea. In chickpea suspension culture, the activity of isoflavone conjugate hydrolyzing enzymes, esterase and β-glucosidase, was induced by higher concentrations of elicitor, from 20 to 35 and from 300 to 480 mkat/kg protein, respectively, consistent with the increase of free aglycones (Barz and Mackenbrock, 1994). Interestingly, low or moderate levels of elicitor increase the activity of conjugate forming enzymes, glucosyltransferase and malonyltransferase, and therefore, the concentration of conjugates are raised, implying a downstream regulatory mechanism controlled by elicitor (Barz and Mackenbrock, 1994). The non-specific transcriptional and translational inhibitors, actinomycin D and cycloheximide, suppressed the induction of β-glucosidase by elicitor (Figure 1.7), suggesting de novo protein synthesis.

The negative values of relative enzyme activity infer that wounding (water control of cut cotyledon assay) also contributed a minor induction of β-glucosidase and that this induction was inhibited by these non-specific inhibitors as well. Indeed, in a recent study, wounding helped phenolic polymer deposition and wound exudate was comprised of cell competency factors that facilitate phenolic polymer and glyceollin responses to elicitor (Graham and Graham, 1996a). The β-glucosidase may be involved in both reactions indirectly by releasing the free isoflavones. However, the true relationship of isoflavone-conjugate-specific β-glucosidase and wound competency factors needs further study.
CHAPTER 2

PURIFICATION OF ISOFLAVONE-CONJUGATE-SPECIFIC β-GLUCOSIDASE FROM SOYBEAN

INTRODUCTION

A great number of β-glucosidases have been purified from plants, animals and microorganisms. This kind of hydrolytic enzyme was one of the classical proteins described over a century ago. For instance, a β-glucosidase cleaving a cyanogenic glucoside was demonstrated in 1837 by Liebig and Wohler (cited in Hösel, 1981). The possible roles of released aglycones were discussed in the first chapter. The β-glucosidases purified from plants with known or unknown physiological functions will be reviewed here. The principles of each purification step used in this study will be briefly covered as well.

Purification of β-Glucosidases from Plants

Several classic cyanogenic glucoside specific β-glucosidases have been purified from Rosaceous plants. The cyanogenic diglucoside, amygdalin, was hydrolyzed by amygdalin hydrolase (AH), and the product, prunasin was cleaved subsequently by another β-glucosidase, prunasin hydrolase (PH). These two distinct β-glucosidases were separated from black cherry seeds by DEAE-cellulose chromatography (Kuroke et al., 1984). The AH was further purified over 200 fold to yield two isoforms following concanavalin A-Sepharose 4B, CM-cellulose, and Ultrogel-HA (hydroxyapatite) chromatography, and Polybuffer Exchanger 94 chromatofocusing column to over 200 fold (Kuroki and Poulton,
1986). The final step, a chromatofocusing column, differentiated the AH1 and AH2 by pH values of 6.44 and 6.37, respectively. The isoforms of amylodalin hydrolase were not due to proteolysis since the presence of protease inhibitors did not alter the elution profiles (Kuroke and Poulton, 1986). Dhurrin specific β-glucosidase, on the other hand, was purified from *Sorghum bicolor*. The sorghum seedlings were extracted with 0.2 M Tris-HCl buffer, pH 7.6, containing 0.4 mM EDTA, 10 mM sodium diethyldithiocarbamate, and 2 mM DTT. The supernatant was incubated with calcium phosphate, and then precipitated by ammonium sulfate. The pellets between 30-65% were collected and desalted by Sephadex G-25. The protein solution was loaded on a Whatman DE-52 column and eluted with a linear sodium chloride gradient from 0 to 0.6 M in 0.2 M Tris-HCl, pH 7.6, containing 2 mM DTT. The active fractions were concentrated by ultrafiltration, and applied to a column of Matrix Red A gel equilibrated with the same buffer. The enzyme was in the unbound fractions, and passed through a concanavalin A-Sepharose 4B column. Again the β-glucosidase found in the pass-through was concentrated and subjected to an Ultrogel ACA 34 column. Two β-glucosidases, dhurrinase 1A and 1B, were eluted on the basis of different molecular weights, and these two formed a tetramer-dimer relationship, since they showed a single monomeric subunit upon SDS-PAGE (Hösel et al., 1987). Interestingly, the *Hevea* linamarase, the β-glucosidase hydrolyzing the cyanogenic glucoside linamarin, was found to be a non-specific β-glycosidase, which also cleaved other glycosides with low velocity. The linamarase was extracted from leaves with 50 mM Tris-HCl buffer, pH 7.2, and the supernatant was fractionated by ammonium sulfate precipitation. The precipitation from 30-80% ammonium sulfate saturation was collected, resuspended, and dialyzed against 5 mM Tris-HCl buffer, pH 7.2. The protein solution was applied to a DEAE column (Biogel A) and developed with a NaCl gradient (0-300 mM). The active fractions were concentrated by ultrafiltration and the buffer changed to phosphate-citrate buffer, pH 4.5, simultaneously. The solution was loaded on a CM-Biogel column, and elution was performed using a gradient of phosphate-citrate buffer, pH4.5, from 1.1 to 250 mM citrate. After ultrafiltration, the concentrated protein was subjected to Sephadex G-150 using the same buffer. This enzyme is the only one to cleave all β-glucosides and β-galactosides in *Hevea* (Selmar et
al., 1987). On the other hand, another linamarase was isolated using a simplified extraction procedure from cassava. The enzyme was extracted from leaves with a low pH phosphate buffer (0.1 M, pH 3.5). The supernatant was brought to 40% ammonium sulfate saturation and the pellet was solubilized and dialyzed against 0.05 M phosphate buffer, pH 4.5. The protein solution was treated with active charcoal twice and concentrated by ammonium sulfate precipitation at 40%. After dialysis, the concentrated protein was loaded onto a Sephadex G-200 column, and the linamarase activity was eluted near the void volume (Mkppong et al., 1990).

A number of coniferin specific β-glucosidases were purified from mainly gymnosperms, and exemplified as follows. A coniferin-specific β-glucosidase was extracted from spruce hypocotyl particulate fraction by treatment with 0.6 M sodium chloride and the fractionated with ammonium sulfate. The major activity was recovered in the range of 45-70% saturation. After dialyzing, the protein preparation was applied to a CM-cellulose column, and eluted with a linear gradient of 0-1 M sodium chloride. Two β-glucosidases were separated by applying the active fractions from CM-cellulose to a Sepharose 6 B column. Glucosidase II was obtained by eluting the column with buffer containing 0.5 M NaCl, and glucosidase I was further purified by repeated gel filtration on Sepharose 6 B (Marcinowski and Grisebach, 1978). Similarly, the chickpea coniferin specific β-glucosidase was isolated from cell suspension culture. The chickpea cell wall fraction was also extracted by a phosphate-citrate buffer, pH 5, with 0.5 M NaCl, and the solubilized proteins were treated with ammonium sulfate to 65% saturation. After desalting by Sephadex G-25, the crude protein solution was subjected to Sephadex G-200. Two β-glucosidase peaks were found using p-nitrophenyl β-glucoside as substrate. Only one coniferin specific β-glucosidase was collected and purified to near homogeneity by CM-Sephadex chromatography (Hösel et al., 1978). In contrast, the soybean coniferin and syringin specific β-glucosidase was not absorbed by CM-Sephadex C-50 column at pH 5. Therefore, the non-binding fractions were pooled and chromatographed on a DEAE 'Cellex' column, Sephadex G-200 column, and by isoelectric focusing. The final step separated the specific enzyme from other β-glucosidases which had no affinity to coniferin.
and syringin (Hösel and Todenhagen, 1980). An acidic coniferin-hydrolyzing β-glucosidase was purified from the developing xylem of *Pinus banksiana*. The frozen xylem tissue was extracted by sodium acetate buffer, pH 5.8, with 0.05 M sodium chloride and 0.1% Triton-X 100, and the crude extract was fractionated by ammonium sulfate at 40-80% saturation. After dialysis, two coniferin-hydrolyzing β-glucosidases were separated by preparative isoelectric focusing gel electrophoresis in the range of pH 3 and 4. The proteins in this zone were subjected to a high performance liquid chromatographic cation-exchange column (CEC) and the specific β-glucosidase was recovered with 5% yield and 30-fold purity (Leinhos et al., 1994). Another β-glucosidase from the xylem of lodgepole pine (*Pinus contorta*) was purified by anion exchange, hydrophobic interaction, and size exclusion chromatography. In the beginning, the extract was brought to 70% saturation with ammonium sulfate, and then desalted by Bio-Gel P6DG column. The desalted protein solution was applied to a Q-Sepharose column and developed with a linear gradient of 0.05 to 0.4 M NaCl. The active fractions were pooled and solid ammonium sulfate was added to 2 M. Then a hydrophobic interaction column, tert-butyl Macroprep, was used and elution was carried out with a decreasing salt gradient of ammonium sulfate (2-0 M). The active fractions were desalted again and applied to another anion exchange column, QMA MemSep 1010. The β-glucosidase active fractions were obtained by elution with a 0.02 to 0.12 M NaCl gradient. The homogeneous coniferin specific β-glucosidase was finally purified by using a size exclusion column, Superose 12 HR 10/30 (Dharmawardhana et al., 1995).

Two specific β-glucosidases involved in indole alkaloid biosynthesis in *Catharanthus roseus* were partially purified by one column chromatography. The crude extract from cell culture was brought to 0-45% ammonium sulfate saturation. After dialysis, the protein solution was layered onto a DEAE-cellulose column (Whatman DE-52), which was developed with a non-linear gradient of 0-200 mM KCl in 20mM phosphate buffer, pH 7.8. The two specific β-glucosidases hydrolyzing strictosidine, a alkaloid glucoside, were obtained in the latter gradient fractions, and three non-specific specific β-glucosidases were found in the non-binding and early gradient fractions (Hemscheidt and Zenk, 1980).
The DIMBOA-glucoside specific $\beta$-glucosidase was partially purified from maize leaves which were extracted by 100 mM phosphate buffer, pH 7.5, containing PVPP and 16 mM $\beta$-mercaptoethanol. Proteins were salted out by 30-60% ammonium sulfate fractionation, and after desalting, applied to a Superose 12 HR 10/30 size exclusion column or a Mono P HR 5/5 isoelectric focusing column. The latter chromatography separated the specific enzyme and a non-specific one better (Cuevas et al., 1992).

An isoflavone-glucoside-specific $\beta$-glucosidase was purified as early as in 1975 (Hösel and Barz) from chickpea. The extract supernatant was brought to 35-65% ammonium sulfate saturation. The fraction was desalted by Sephadex G-25 and subsequently applied to a column of DEAE-cellulose, which was eluted with a linear gradient of Tris-HCl buffer from 0.02 to 0.5 M. The active fractions were further resolved by CM-Sephadex chromatography into minor and major peaks when the isoflavone glucoside was used as substrate (Hösel and Barz, 1975). A $\beta$-glucosidase that hydrolyzed isoflavone glucosides (daidzin and genistin) was also purified from imbibed soybean seeds (Matsuura and Obata, 1993). The imbibed soybean seeds were extracted by phosphate buffer, pH 6.6, and the supernatant was acidified to pH 5 by 0.1 N HCl. Ammonium sulfate was added in the protein solution to give 75% saturation. Followed by dialysis, the protein preparation was subjected to a CM-Sephadex C-50 column, and the column was eluted by a 0-1M linear gradient of NaCl. Three $\beta$-glucosidases were revealed in CM-Sephadex chromatogram using natural substrates (Matsuura and Obata, 1993). The strongest peak, $\beta$-glucosidase-C was further applied to a Butyl-Toyoperal 650C hydrophobic interaction column, equilibrated with phosphate-citrate buffer containing 1.2 M ammonium sulfate. A reverse linear gradient from 1.2 to 0 M ammonium sulfate in the same buffer was used to elute the active protein. The active fractions were concentrated and subjected to Sephadex G-150 size exclusion chromatography, and a homogeneous $\beta$-glucosidase was purified 456-fold (Matsuura et al., 1995).

A flavone 7-0-glucoside specific $\beta$-glucosidase was isolated from Chamonmilla recutita flower heads. The crude extract from ligulate florets of chamomile was adjusted to 10% PVPP, and the PVPP removed by centrifugation. Solid ammonium sulfate was added
to this crude supernatant to 35% saturation. After desalting, the protein solution was loaded on a Mono Q column and eluted with a linear NaCl gradient from 0 to 1 M NaCl. The active fractions were combined and solid ammonium sulfate was added to a final concentration of 0.75 M. The solution was chromatographed on a Bio-Gel TSK Penyl-5-PW hydrophobic interaction column, and bound proteins were released by a decreasing linear gradient of ammonium sulfate from 0.75 to 0 M. The active fractions were concentrated and applied to a Superose 12 column. The flavone glucoside specific β-glucosidase was purified 29-fold (Maier et al., 1993).

A furostanol glycoside 26-O-β-glucosidase from *Costus speciosus* rhizomes was obtained. The extraction buffer, phosphate buffer, pH 7.0, containing Polyclar AT and sodium isoascorbate was used to extract the rhizomes. The crude supernatant was brought to 30-60% ammonium sulfate saturation, and desalted by passing through the Pharmacia PD-10 column. The protein solution was loaded onto DEAE-cellulose (Whatman DE52) and eluted with phosphate buffer, pH 7.0, containing 0.5 M KCl. The active fractions were concentrated by ammonium sulfate precipitation and desalted as before. Ammonium sulfate was added to this protein preparation to 1 M. After removing the insoluble materials, the supernatant was subjected to HPLC using a TSKgel Ether-5PW column. Bound proteins were eluted by linearly decreasing the ammonium sulfate concentration to 0 M. The active fractions were desalted and concentrated by Centricon 30, and then applied to another HPLC TSKgel DEAE-5PW column. The pure specific β-glucosidase was eluted at room temperature with a linear gradient of 0.1-0.5 M KCl (Inoue and Ebizuka, 1996).

An auxin-binding protein (p60) from maize was identified with a molecular weight of 60 kDa by photolabeling with azido-IAA. The purified protein turned out to be a β-glucosidase. Etiolated maize coleoptiles were extracted with 100 mM Tris-Citric acid buffer, pH 8.0, containing sucrose, EDTA, ascorbic acid, MgCl₂, DTT, and protease inhibitors. Particulate material was isolated by calcium chloride precipitation and extracted with n-butanol. The water phase containing the microsomal extract was dialyzed and applied to an S-Sepharose column coupled to a Q-Sepharose column. After loading and washing, the S-Sepharose column was removed and the Q-Sepharose column was washed.
with 50 mM NaCl in 10 mM Tris-Mes buffer, pH 6.5, containing 5 mM MgCl₂. Bound proteins were eluted stepwise with 120 mM NaCl in the same buffer. The fractions were applied to an affinity chromatography column (1-NAA-Sepharose), which was washed with 0.4 M NaCl, and then eluted with 5 mM 1-NAA and 5 mM IAA in buffer containing 0.4 M NaCl. The eluate was further subjected to hydroxylapatite column chromatography and eluted twice with a linear gradient from 15 to 500 mM sodium phosphate. The p60 protein showed high substrate specificity to indoxyl-\(O\)-glucoside, but not cellobiose, IAA-inositol or IAA amino acid conjugates (Campos et al., 1992).

Hydrolysis of conjugated gibberellins by \(\beta\)-glucosidases was demonstrated in dwarf rice. The enzyme was partially purified from rice seeds or shoots using \(p\)-nitrophenyl \(\beta\)-glucoside as a substrate. The seed extract was brought to 90% ammonium sulfate saturation. The precipitate was dissolved and dialyzed. The protein preparation was subjected to chromatography on a CM-Sephadex C-50 column developed with a linear gradient of NaCl from 0 to 1 M of NaCl in phosphate-citrate buffer, pH 5.0. Four \(\beta\)-glucosidases were separated by CM-Sephadex C-50. CM1 and CM4 from seeds and shoots, respectively, favored GA₄-O-2-glucoside and GA₃-O-2-glucoside, and to a lesser extent GA₅-O-2-glucose ester and GA₆-O-13-glucoside, as substrates (Schliemann, 1984).

By using the artificial substrate, \(p\)-nitrophenyl \(\beta\)-glucoside, a few \(\beta\)-glucosidases with non-identified or proposed functions were also purified. A cell wall associated \(\beta\)-glucosidase from soybean was isolated to homogeneity by size exclusion and hydroxyapatite chromatography. The cell wall fragments were partially purified from cell suspension culture, and extracted with 0.05 M succinate buffer, pH 5, containing 1 M NaCl. The supernatant was saturated to 30-80% by solid ammonium sulfate, and the resuspended protein solution was subjected to Sephadex G-100 chromatography. The active fractions were collected and subjected to hydroxyapatite chromatography (HA Ultrogel). The elution was carried out by a linear gradient of phosphate buffer of 0.25-0.4 M. This \(\beta\)-glucosidase hydrolyzed gentiobiose, cellobiose, and sophorose (Nari et al., 1982/83). A \(\beta\)-glucosidase was isolated from \textit{Carica papaya} fruit pulp and purified 1000-fold to homogeneity. The crude extract from papaya fruit was brought to 2.8 M ammonium sulfate, the pellet was
dissolved in 50 mM phosphate buffer, pH 7.6, and the sulutive was dialyzed against 1 M ammonium sulfate. The protein solution was applied to a Phenyl-Sepharose CL-4B column, and the elution was carried out by stepwise washing including 1 M NaCl, a linear gradient of NaCl from 1 M to 100 mM, 50 mM phosphate buffer alone, pH 7.6, and the same buffer containing 50% ethylene glycol. The active fractions were pooled and concentrated by ultrafiltration, and then loaded onto a Sephacryl S-200 column. The fractions from gel filtration with β-glucosidase activity also had β-galactosidase activity. Final separation of these two enzymes was achieved by preparative isoelectric focusing. The β-glucosidase had a $K_m$ of $1 \times 10^{-4}$ M for p-nitrophenyl β-glucoside (Hartmann-Schreier and Schreier, 1986).

Two other β-glucosidases were also partially purified from the acetone powder of grapefruit, showing no different properties but different molecular weights. The extraction buffer was 100 mM phosphate-citrate buffer, pH 5, containing Tween 80 and PVP. The crude extract was concentrated by ultrafiltration and then loaded onto an Ultrogel AcA 44 column. Two enzyme peaks were revealed in the chromatogram, and each of them was subjected to DEAE-Sepharose CL-6B chromatography and eluted with a linear gradient of sodium chloride from 0 to 0.5 M (Lecas et al., 1991).

An improved chromatography procedure was conducted to isolate peroxidase and β-glucosidase from barley (Hordeum vulgare) seedlings. The plant seedlings were extracted by de-ionized water and brought to 50-90% ammonium sulfate saturation. After dialysis, the supernatant was loaded onto a DEAE cellulose column connected to a hydroxylapatite column. The peroxidase was not bound by either column, but β-glucosidase was bound by the second column. The hydroxylapatite column was separated from the DEAE-cellulose column and eluted with 0.3 M phosphate buffer, pH 7.0. The β-glucosidase was demonstrated to be homogeneous by native PAGE, SDS PAGE, and analytical gel isoelectric focusing (Rescigno et al., 1993). A β-glucosidase and a β-galactosidase were also found in barley meal to hydrolyze lactose. The barley meal was suspended in 0.1 M ammonium acetate buffer, pH 6.0, and the supernatant was acidified by dilute HCl to pH 5.0. The resulting pellet was removed by centrifugation. The crude extract was fractionated by solid ammonium sulfate. The pellet between 40% to 60% was collected, suspended, and
dialyzed. The preparation was passed through DEAE-cellulose equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The β-glucosidase activity was found in the pass-through and after changing the buffer system to ammonium acetate buffer, pH 5, the solution was loaded onto a Blue Sepharose column. The elution was achieved by an increasing linear gradient of KCl. This β-glucosidase favored cellulbiose and o-nitrophenyl β-glucoside with $K_m$ values of 6 and 2.5 mM, respectively (Simos and Georgatsos, 1988). A β-glucosidase proposed to be involved in the seed germination of cereal was purified from barley flour using $p$-nitrophenyl β-glucoside as a substrate. The flour was extracted in 50 mM phosphate buffer, pH 6.0 and ammonium sulfate was added to the supernatant to 65% saturation. The pellet was collected, re-dissolved, dialyzed and freeze-dried. The freeze-dried extract was solubilized in a minimal volume and applied to a Sephadex G-75 column. Active fractions were pooled, dialyzed, and freeze-dried. The powder was resuspended in 50 mM sodium acetate buffer, pH 5.0, and chromatographed on a cation exchange column, Mono S HR 5/5. β-Glucosidase activity eluted at 75 mM NaCl, showing a single band of 60 kDa in SDS-PAGE. The β-glucosidase had high activity towards sophorose, laminaribiose, cellulbiose, cellopentaose, but not gentiobiose (Leah et al., 1995).

A maize β-glucosidase was extracted from coleoptiles with 50 mM sodium acetate buffer, pH 5.0. The pH of the crude extract was adjusted to pH 4.6, and most of the contaminating proteins were cryoprecipitated at 0°C for 24 hours. After centrifugation, the supernatant was chromatographed by an Accell CM column using a 4.8 to 6.8 pH gradient of the same buffer. Two peaks were resolved, but only one SDS-PAGE band was shown at 60 kDa (Esen, 1992). Similarly, two membrane-associated β-glucosidases, pm60 and pm58, from maize coleoptiles were solubilized by Triton X-114 from purified microsomal vesicles. The membrane vesicles were obtained from two-phase partitioning and extracted with Triton X-100, followed by another extraction of Triton X-114. The protein pm60 had β-glucosidase activity using $p$-nitrophenyl β-glucoside as a substrate (Feldwisch et al., 1994).
Principles of Purification Procedures for Purifying the Isoflavone-Conjugate-Specific β-Glucosidase from Soybean

A great number of β-glucosidases have been purified from plants by means of many combinations of column chromatography including ion exchange, size exclusion, hydrophobic interaction, affinity and hydroxyapatite columns and other techniques such as ammonium sulfate fractionation and preparative gel electrophoresis. To attempt the purification of the isoflavone-conjugate-specific β-glucosidase from soybean root, ammonium sulfate precipitation, and chromatography including ion exchange, hydrophobic interaction, size exclusion, lectin affinity, and a specially designed substrate affinity procedure were used. The principles and applications of these techniques are briefly reviewed here.

Ammonium Sulfate Fractionation

Salting out of proteins is one of the most popular techniques in enzyme purification. The hydrophobicity of a protein is the most important determinant for how much salt is needed to precipitate it. A typical protein molecule has hydrophobic patches contributed by hydrophobic amino acid side chains, and these patches are surrounded by water molecules in an aqueous solution to retain the solubility. If high salt concentration is present in this solution, the salt ions become solvated and the free water molecules are scarce. Therefore, the surrounding water molecules are pulled away from the hydrophobic side chains so that the aliphatic surfaces are exposed and interact with each other to form aggregates. Although several ions are effective to salt out proteins according to the Hofmeister series, ammonium sulfate is the most common and effective salt with a number of advantages such as low cost, less hydrated heat, lower density, and stabilization of proteins (Scopes, 1987).

Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography is a purification technique based on the principle described above, but the hydrophobic binding occurs between the protein molecule and the nonpolar stationary phase on the column matrix in the presence of salt.
The affinity between protein and matrix is highly dependent on the length of the hydrocarbon chains on the matrix and the number, size, shape and hydrophobicity of the non-polar patches on the protein's surface. Therefore, the strength of hydrophobic interaction does not rely on the pI of protein, where the net change of protein is zero and the solubility in water is minimal, but on the local hydrophobic patches. Usually, the bound proteins are released by reducing the concentration of salt in the mobile phase. Some other chaotropic reagents such as alcohols, ethylene glycol, urea, guanidine HCl, and surfactants may promote the desorption as well. In addition, changing the pH of the mobile phase also results in elution. However, some chemicals and changes in pH may cause protein conformation changes and concomitant activity loss (Pennings et al., 1991).

**Lectin Affinity Chromatography**

After extensive study of lectin-carbohydrate interactions, it is now known that different lectins are able to distinguish various carbohydrate mixtures. Therefore, lectin was introduced to purify and analyze the heterogeneity of glycoproteins (Merkle and Cummings, 1987). A great number of plant cell wall proteins were demonstrated to be glycoproteins (Fry, 1991), and plant glycoproteins have both the typical high mannose glycans and complex Asn-linked glycans (Faye et al., 1989). The lectin Concanavalin A (ConA) from *Cancavalia ensiformis* specifically recognizes terminal α-D-glucose and α-D-mannose; the lectin GNA from *Galanthus nivalis* recognizes terminal mannose α(1-3), α(1-6), and α(1-2) linked to mannose; TPA from *Tetragonolobus purpureas* recognizes terminal α-L-glucose; and SBA (soybean agglutinin) from *Glycine max* recognizes terminal D-galactose and N-acetyl-D-galactosamine (Teissere et al., 1994). A β-glucosidase partially purified from soybean cell walls was recognized by SBA (Teissere et al., 1994). Therefore, soybean agglutinin was chosen in this study.

**Substrate Affinity Chromatography**

It is known that the heterocyclic isoflavone backbone is somewhat hydrophobic. Genistein, for instance, is soluble in usual organic solvents but practically not soluble in
water. Although the glucose or malonyl glucose moiety helps a little in solubility, the hydrophobic isoflavone backbone is associated strongly with long chain aliphatic group in the reverse phase columns through the hydrophobic interaction. If a C18 reverse phase column is saturated with the isoflavone conjugates, either isoflavone-7-O-glucoside or isoflavone-7-O-glucoside-6"-O-malonate, the isoflavone-conjugate-specific β-glucosidase might be able to bind the substrates at the protein's active center. A small amount of excess isoflavone conjugates can then be used to compete with the C18 binding sites, and consequently the protein is eluted. This novel design was the first time to be used for protein purification.

**Ion Exchange Chromatography**

Ion exchange chromatography is a kind of adsorption chromatography where the solid matrix has charged groups chemically linked to an inert support. This is one of the most commonly practiced chromatographic methods for protein purification because of the ease of use, large capacity, and low cost. Ion exchange chromatography is the separation of proteins on the basis of their charge. Proteins possess both positively and negatively charged groups, termed amphoteric on their surface. The net charge of a certain protein is dependent on its pI and the pH of its environment. If the pH of the environment is above the pI, the protein have a net negative charge, while below it, the overall charge is positive. Therefore, the stationary phase of ion exchangers are derivatized with positively-charged groups such as diethylaminoethyl (DEAE) to bind negative-charged proteins or negatively-charged groups such as carboxymethyl (CM) to bind positive-charged proteins. These are termed anion and cation exchangers, respectively. There are three ways to elute the bound proteins from the matrices including a change of pH (up for cation exchangers and down for anion exchangers), an increase of ionic strength, and the use of affinity elution (Scopes, 1987). Continuous pH gradients are rarely used because of the low reproducibility and the difficulty to produce these at constant ionic strength (Roe, 1989). Ionic strength gradients are more commonly used, and the salt concentration needed to elute proteins is dependent on the isoelectric point (Scopes, 1987)
Size Exclusion Column Chromatography

As the name of this method implies, size exclusion or gel filtration chromatography is a method to perform a fractionation on the basis of the relative size of protein molecules. Basically, the separation is achieved by using a stationary phase gel matrix with pores of a specific diameter. The smaller protein molecules in a protein mixture of the mobile phase can enter the matrix pores and thus delay the movement in the column, while the larger proteins are excluded from the matrix and pass through the column more rapidly. Those intermediate sized proteins can enter the pores but spend less time within it than smaller proteins do. Therefore, proteins appear in order of their increasing size (Preneta, 1989).

In this chapter, we attempted the purification of isoflavone-conjugate-specific β-glucosidase by using chromatographic methods mentioned above, and evaluated the successfulness of the chromatography. Due to the instability of this enzyme, protein stabilization by adding different chemicals was inspected as well. The final purification procedures was described and the protein yield and purity were also determined.
MATERIALS AND METHODS

Chemicals

Ammonium sulfate, acrylamide, methylene-bis-acrylamide, DEAE Sephadex A-50, CM Sephadex C-50, Sephadex G-25, soybean agglutinin, Coomassie brilliant blue and silver stain chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA. Econo-Pac methyl HIC cartridge was purchased from BIO-RAD Laboratories, Hercules, CA, USA. Dialysis membrane, Spetra/Por® CE, MWCO 10,000 was obtained from PGC Scientifics, Gaithersburg, MD, USA. Butyl Sepharose 4 FAST FLOW HIC resin was purchased from Pharmacia Biotech AB, Uppsala, Sweden.

Plant Material

Soybean cv. Williams was planted and grown as described previously (Chapter 1). At least 6-day-old seedlings were harvested and the roots were detached to be the enzyme source.

Heat Treatment

The soybean root cell wall wash was obtained as described in Chapter 1. The enzyme preparation was heated in a water bath at various temperatures and for different times. If the isoflavone-conjugate-specific β-glucosidase is relatively heat stable, other heat labile proteins can be removed with the precipitate. The enzyme activity after treatment was measured as noted in Chapter 1.

Precipitation Technique

The protein preparation was fractionated by sequential ammonium sulfate precipitation. Three fractions from 0-40%, 40-60%, and 60-80% were carried out by slowly adding solid ammonium sulfate to the protein solution on ice with stirring. Each fraction was allowed to stand for at least 2 hours on the ice, and then the protein precipitate was
collected by centrifugation at 10,000 × g for 10 min. The precipitate was dissolved in 20 mM phosphate buffer, pH 6.0, and then either dialyzed against the same buffer (MWCO 10,000) or desalted by a gel filtration column, Sephadex G-25.

**Column Chromatography on DEAE Sephadex A-50**

About 10 g of DEAE Sephadex A-50 resin (dry beads) was swollen in 20 mM phosphate buffer, pH 6.0, in a boiling water bath for 2 hours or at room temperature for 24 hours. The unheated resin was degassed under vacuum. The complete swollen resin was cooled down to 4°C and then packed in a low pressure open column (Bio-Rad). For packing, the column was tilted to avoid bubbles when pouring the resin slurry with the help of a reservoir, and then set vertically immediately (Strobel, 1990). The desalted protein solution from the 40-60% ammonium sulfate fraction was applied to a DEAE Sephadex A-50 column (2.8 × 35 cm) equilibrated with 20 mM phosphate buffer, pH 6.0. The column was washed to baseline absorbance (A280) with the same buffer, and then eluted with an 800-ml linear gradient of sodium chloride in the same buffer from 0 to 1 M with a starting flow rate of about 15 ml/h.

**Column Chromatography on CM Sephadex C-50**

The CM Sephadex C-50 resin was prepared in the same way as the DEAE-Sephadex A-50 gel. The active pool from DEAE Sephadex A-50 was subjected to CM Sephadex C-50 column (2.8 × 19 cm) equilibrated with the same buffer used in anion exchange, and then developed with a linear gradient of sodium chloride from 0 to 1 M (400 ml) with the same flow rate as the DEAE Sephadex column.

**Hydrophobic Interaction Chromatography**

The commercial Econo-Pac methyl HIC cartridge (5 ml bed volume) and Butyl Sepharose (25 ml) was used for hydrophobic interaction chromatography at different purification stages. The enzyme solution from the root intercellular fluid obtained by infiltrating fresh roots with 100 mM phosphate buffer, pH 6.8, containing 250 mM sodium
chloride was brought to 2.4 M ammonium sulfate and then applied to an Econo-Pac methyl HIC cartridge equilibrated with the same buffer having 250 mM NaCl and 2.4 M ammonium sulfate. The column was washed to baseline absorbance followed by a 100 ml-negative linear gradient of ammonium sulfate from 2.4 to 0 M in the same buffer system with 250 mM NaCl. Different batches of enzyme solutions were initially tested by adding various concentrations of ammonium sulfate or sodium chloride to determine the best binding conditions. Apart from the DEAE and CM Sephadex ion exchanges, another HIC column, Butyl Sepharose column (1.7 x 11 cm), was used to extend the purification steps. The application of the Butyl Sepharose column will be described later.

Lectin Affinity Chromatography

Soybean roots were infiltrated by 100 mM phosphate-50 mM citrate buffer, pH 5.0, containing 250 mM sodium chloride, and the intercellular fluid was obtained by a gentle centrifugation as described in Chapter I. The intercellular fluid was incubated with 1 ml soybean agglutinin (SBA) for 6 hours at 4°C, and then the mixture was poured into an empty low pressure column (5 ml). The filtrate was collected, and the soybean agglutinin was washed extensively with the same buffer. After washing, the column was eluted with 1 ml of 10 mM N-acetyl-galactosamine (GalNac) twice (Exp I). Another crude enzyme solution prepared from root cell wall wash (the cell wall fraction washed by 100 mM Tris-HCl buffer, pH 7.2 containing 250 mM sodium chloride) was mixed with soybean agglutinin overnight (> 12 hrs). After incubation, the enzyme solution was filtered through an empty column and the lectin was washed with buffer. The bound protein was eluted by 1 ml of 200 mM galactose and another 1 ml of 500 mM galactose (Exp II).

Substrate Affinity Chromatography

A PrepSep C-18 column was saturated with substrate mixture obtained from the method described in Chapter I. The saturation of the column was determined by measuring the amount of substrates before and after application using HPLC. A concentrated protein solution, 0.8 ml, obtained from the active pool of DEAE Sephadex A-50 column which was
previously precipitated by 80% ammonium sulfate saturation and dialyzed, was applied to
the saturated column and then washed with 200 mM phosphate buffer, pH 6.0. The column
subsequently was eluted with about 0.3 ml very concentrated substrate mixture twice. The
protein samples were collected for assaying enzyme activity and analyzing purity by SDS
polyacrylamide gel electrophoresis.

Size Exclusion Chromatography

A Dextran-based gel, Sephadex G-150, was used in this study. The gel powder was
mixed with excess buffer and then the gel slurry was heated in a water bath to 100°C. This
method allows the gel to swell within a few hours and removes any dissolved air. Fine
particles were decanted off before packing into a column. The packing was performed as
described above for preparing the DEAE-Sephadex column. A concentrated protein sample,
0.5 ml, either from the crude cell wall wash or a partialy purified preparation, was loaded
onto the column (1.7 × 19 cm) previously equilibrated with at least two bed volumes of
buffer. The column was then developed after the protein sample was absorbed into the gel.
The void volume was determined by applying 0.75mg/ml Blue Dextran onto the column.

SDS Poly-Acrylamide Gel Electrophoresis

Denaturing gel electrophoresis was conducted according to the standard Laemmli
(1970) procedure in 7.5% polyacrylamide gel by using a modular Mini-Protein II
electrophoresis system (Bio-Rad). In sample buffer, β-mercaptoethanol might or might not
be included, and the protein sample was incubated with sample buffer with or without
heating as noted specifically. The molecular markers were used from 14 to 70 KD (Sigma
Chemical Co.). The protein gel was visualized by staining with Coomassie Brilliant Blue or
silver stain prepared from individual chemicals.
Purification of Isoflavone-conjugate-specific β-Glucosidase from Imbibed Soybean Seeds

The crude extraction from imbibed soybean seeds was carried out by the method described in Chapter 1. The acid treated supernatant was brought to 30-80% ammonium sulfate fractionation, and the precipitate was collected by centrifugation and then dissolved in 100 mM phosphate-50 mM citrate buffer, pH 5.0. The protein preparation was desalted by dialysis in a dialysis bag (MWCO 10,000) against the same buffer for overnight, and then applied to a CM-Sephadex C-50 column (2.8 × 19 cm). After removing the unbound proteins by excess buffer, the enzyme activity was eluted with a sodium chloride linear gradient from 0 to 1 M (800 ml) at flow rate 20 ml/h. The enzyme activity was assayed by p-nitrophenyl β-glucoside and natural substrate mixture.

The Chemicals for Stabilization of Isoflavone-conjugate-specific β-Glucosidase

The isoflavone-conjugate-specific β-glucosidase was found to be very unstable during the purification steps. The following chemicals were added into the extraction buffer to stabilize the crude enzyme. Enzyme activity was examined subsequently at various time courses and temperatures.

1. 100 mM phosphate buffer, pH 6.
2. 100 mM phosphate buffer, pH 6 + 5 mM DTT
3. 100 mM phosphate buffer, pH 6 + 5 mM β-mercaptoethanol
4. 100 mM phosphate buffer, pH 6 + 5 mM DTT + 1 mM PMSF
5. 100 mM phosphate buffer, pH 6 + 1 mM PMSF
6. 100 mM phosphate buffer, pH 6 + 10 mM EDTA
7. 100 mM phosphate buffer, pH 6 + 10 mM EDTA + 5 mM DTT
8. 100 mM phosphate buffer, pH 6 + 10 mM EDTA + 1 mM PMSF
9. 100 mM phosphate buffer, pH 6 + 10 mM EDTA + 5 mM DTT + 1 mM PMSF
Final Purification Procedures of the Isoflavone-conjugate-specific β-glucosidase from Soybean Roots

All procedures were carried out at 4°C. Seven to ten day old Soybean cultivar Williams roots were washed by tap water to remove planting media and botted with paper towels. The roots were frozen at -20°C until used. The frozen roots were ground by prechilled mortar and pestle to almost a powder, and then extracted three times (1 g tissue/1 ml buffer) by incubation at least an hour with cold 0.2 M sodium phosphate buffer, pH 6.0. The cell wall fraction was then extracted with the same volume of 0.2 M phosphate buffer, pH 6.0, containing 0.5 M NaCl overnight. The root residue was further extracted one more time with the same buffer for 3 hours. The two crude cell wall washes were pooled, 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) was added and the extract incubated at 40°C overnight. The crude cell wall wash was filtered through four layers of cheesecloth, and clarified by centrifugation at 10,000 × g for 20 minutes. The cell wall wash was subsequently brought to 40% saturation with solid ammonium sulfate with gentle stirring for 1 h, and the protein precipitate was spun down by centrifugation at 10,000 × g for 20 minutes. Solid ammonium sulfate was added to the 40% saturated protein supernatant to achieve 60% saturation. The pellet was collected by centrifugation at 10,000 × g for 20 minutes and resuspended in 20 mM phosphate buffer, pH 6.0. The protein preparation was clarified by centrifugation and desalted using a Sephadex G-25 column (1.7 × 20 cm) equilibrated with 20 mM phosphate buffer, pH 6.0, containing 3 mM DTT and 10% glycerol. The desalted crude cell wall wash was applied to a DEAE-Sephadex A-50 column (2.8 × 35 cm) equilibrated with the same buffer system. After washing with the same buffer to remove unbound protein, the column was eluted with an 800 ml linear gradient of 0-1 M sodium chloride in the same buffer. Enzyme activity found in the unbound fractions was pooled. The protein solution was loaded onto a column of CM-Sephadex C-50 (2.8 × 19 cm), equilibrated with 20 mM phosphate buffer, pH 6.0, containing 3 mM DTT, 5 mM ascorbate and 10% glycerol. The elution was carried out with a 400 ml linear gradient of sodium chloride from 0 to 1 M in the same buffer system. Chromatographic fractions having isoﬂavone-conjugate-specific β-glucosidase activity were pooled and divided into
two parts with the same volume. One was lyophilized and stored at -20°C for further characteristic studies. Alternatively, solid ammonium sulfate was added to a final concentration of 2.4 M into the CM-Sephadex active fractions, and then loaded onto the Butyl Sepharose 4 Fast Flow column (1.7 x 15 cm) using the same buffer with 3 mM DTT and 10% glycerol. Elution was achieved with a decreasing salt gradient of ammonium sulfate (2-0 M). The fractions were dialyzed against 20 mM phosphate buffer, pH 6.0, with 3 mM DTT and 10% glycerol, and analyzed by SDS-PAGE. The fractions were pooled into four groups (I- IV): Group I (fraction #104-110), Group II (fraction #111-115), Group III (fraction #116-120).
RESULTS

Heat Treatment

The crude enzyme preparation from the root cell wall wash was sensitive to heat above 50°C. At 50°C for 45 min, 87% enzyme activity remained, but at 60°C for 5 min, only 23.5% enzyme activity was recovered. Enzyme treated at 70°C for 10 min lost activity completely (Table 2.1).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Enzyme Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>45</td>
<td>87</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>60</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>60</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>70</td>
<td>1</td>
<td>45</td>
</tr>
<tr>
<td>70</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>70</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.1: The heat sensitivity of the isoflavone-conjugate-specific β-glucosidase from soybean root cell wall wash.

Precipitation Technique

Protein salting out by ammonium sulfate is one of the most widely used techniques in enzyme purification. As shown by 7.5% SDS-PAGE (Figure 2.1), the ammonium sulfate 0-40% fraction did not precipitate many proteins from the root cell wall wash (lane A,
Most enzyme activity was found in the range of 40-60% ammonium sulfate saturation in both the cell wall wash and crude extract (lane B and D, Figure 2.1). Some enzyme activity was recovered from the 60-80% fraction, but more small proteins or peptides were in this preparation (lane C, Figure 2.1).

Figure 2.1: SDS-PAGE of protein patterns from various ammonium sulfate fractionations. Every fraction was treated with SDS alone without β-mercaptoethanol. Resolving gel contained 7.5% acrylamide, and was stained by Coomassie Blue. The isoflavone-conjugate-specific β-glucosidase is showed as arrow indicated.

Lane A: crude cell wall wash, 0-40% ammonium sulfate fractionation
Lane B: crude cell wall wash, 40-60% ammonium sulfate fractionation
Lane C: crude cell wall wash, 60-80% ammonium sulfate fractionation
Lane D: crude supernatant, 40-60% ammonium sulfate fractionation
Column Chromatography on DEAE Sephadex A-50

At pH 6.0, almost all of the isoflavone-conjugate-specific β-glucosidase activity from the root cell wall wash was found in the non-binding fractions (Figure 2.2) from the DEAE column, whereas a small peak of specific activity was bound and eluted by a NaCl gradient (Figure 2.2). A considerable amount of protein was bound by the DEAE Sephadex A-50 resin, which showed a relatively low activity to isoflavone conjugates (Figure 2.2). The enzyme activity assay was conducted by HPLC using isoflavone conjugates as substrates.

Column Chromatography on CM Sephadex C-50

The active fractions from the DEAE-Sephadex A-50 column were pooled and applied to a CM Sephadex C-50 column directly under the same condition, pH 6.0. The isoflavone-conjugate-specific β-glucosidase did not pass through the column, but was bound by this cation exchanger at pH 6.0. The bound enzyme was eluted by a sodium chloride gradient right after the major protein peak, indicating a good separation (Figure 2.3). Only one peak was found using the isoflavone conjugate mixture as substrate. The purification scheme is summarized in Table 2.2. In another experiment, the crude extract supernatant from imbibed soybean seeds was brought to 30-80% ammonium sulfate saturation and dialyzed against 100 mM phosphate-50 mM citrate buffer, pH 5.0. The desalted enzyme solution was applied to a CM-Sephadex C-50 column and developed with a sodium chloride linear gradient from 0 to 1 M in the same buffer. The chromatogram is shown in Figure 2.4 using p-nitrophenyl β-glucoside as a substrate. The enzyme activity peak was eluted by 0.6 M sodium chloride, similar to the activity peak in Figure 2.3, which was eluted by 0.56 M sodium chloride. The pooled fractions were measured for β-glucosidase activity by both artificial and natural substrates, and the calculations are recorded in Table 2.3 and 2.4.
Figure 2.2: The chromatogram of the DEAE-Sephadex A-50 column. Soybean root cell wall wash was brought to 40-60% ammonium sulfate saturation and dialysed against 20 mM phosphate buffer, pH 6.0. The protein preparation was applied to the DEAE-Sephadex A-50 column and the elution was carried out by a linear gradient of 0-1 M sodium chloride. ■ enzyme activity ♦ A280.
Figure 2.3: The chromatogram of the CM-Sephadex C-50 column. Crude cell wall wash was obtained from soybean roots as described in Materials and Methods. The crude protein solution was brought to 40-60% ammonium sulfate saturation. The precipitate was collected and resuspended in phosphate buffer, pH 6.0. After dialysis, the protein solution was applied to a DEAE-Sephadex A-50 column. Active fractions obtained from the DEAE-Sephadex A-50 column were pooled and loaded onto a CM-Sephadex C-50 column equilibrated with 20 mM phosphate buffer, pH 6.0. The bound proteins were eluted out by a linear gradient of 0-1 M sodium chloride. ■ --- enzyme activity ♦ ---♦ A280.
Figure 2.4: The chromatogram of the CM-Sephadex C-50 column. The protein solution was obtained from soybean cv. Williams imbibed seeds extracted by 0.1 M phosphate buffer, pH 6.6. The crude extract was acidified to pH 5.0 and the supernatant was brought to 30-80% ammonium sulfate saturation. After dialysis against 100 mM phosphate-50 mM citrate buffer, pH 5.0, the protein sample was subjected to CM-Sephadex C-50 column equilibrated previously with the same buffer. The elution was achieved by a linear gradient of 0-1 M NaCl. The β-glucosidase activity was measured using p-nitrophenyl β-glucoside as a substrate. ■ enzyme activity, ♦ A280
<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity* (mU)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (mU/mg protein)</th>
<th>Yield (%)</th>
<th>Purity (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>root cell wall wash</td>
<td>24663</td>
<td>536</td>
<td>46</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 40-60%</td>
<td>13986</td>
<td>137</td>
<td>102</td>
<td>57</td>
<td>2</td>
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<tr>
<td>Dialysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>4758</td>
<td>10</td>
<td>459</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>A-50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM Sephadex C-50</td>
<td>51</td>
<td>0.05</td>
<td>1057</td>
<td>0.2</td>
<td>23</td>
</tr>
</tbody>
</table>

* The enzyme activity was measured by HPLC using a natural isoflavone conjugate mixture as substrate. The activity was calculated from the concentration of released free daidzein.

Table 2.2: The purification scheme of the isoflavone-conjugate-specific β-glucosidase from soybean root.

**Hydrophobic Interaction Chromatography**

The use of hydrophobic interaction columns revealed a good resolution of the enzyme (Figure 2.5), although different resins showed slightly different patterns. The infiltrated fluid from soybean roots containing 250 mM sodium chloride was brought to 2.4 M ammonium sulfate and applied to an Econo-Pac HIC cartridge (5 ml bed volume) with methyl functional groups. A negative linear gradient of 2.4-0 M ammonium sulfate in 100 mM phosphate, pH 6.8 was used to elute the bound proteins. The isoflavone-conjugate-specific β-glucosidase activity was found at the end of the gradient, suggesting that the enzyme has hydrophobic regions and is therefore highly associated with the hydrophobic resin (Figure 2.5).
<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity (mU)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (mU/mg protein)</th>
<th>Yield (%)</th>
<th>Purity (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude supernatant, pH 6.6</td>
<td>2804</td>
<td>6093</td>
<td>0.5</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Acid treatment, pH 5.0</td>
<td>2786</td>
<td>2399</td>
<td>1.2</td>
<td>99</td>
<td>3</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 30-80%</td>
<td>1684</td>
<td>1011</td>
<td>1.7</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td>Dialysis</td>
<td>926</td>
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<td>7</td>
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<td>Sephadex G-25</td>
<td>2875</td>
<td>232</td>
<td>12</td>
<td>102</td>
<td>27</td>
</tr>
<tr>
<td>CM Sephadex C-50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction #83-88</td>
<td>1987</td>
<td>16</td>
<td>126</td>
<td>71</td>
<td>274</td>
</tr>
<tr>
<td>Fraction #89-97</td>
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<td>9</td>
<td>46</td>
<td>14</td>
<td>101</td>
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</table>

Table 2.3: The purification procedure of the isoflavone-conjugate-specific β-glucosidase from imbibed soybean seeds. The activity was measured by the spectrophotometric method using p-nitrophenyl β-glucoside as a substrate.
<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity* (mU)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (mU/mg protein)</th>
<th>Yield (%)</th>
<th>Purity (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude supernatant, pH 6.6</td>
<td>501</td>
<td>6093</td>
<td>0.08</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Acid treatment, pH 5.0</td>
<td>552</td>
<td>2399</td>
<td>0.2</td>
<td>110</td>
<td>2</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 30-80%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialysis</td>
<td>147</td>
<td>285</td>
<td>0.5</td>
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<td>6</td>
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<tr>
<td>Sephadex G-25</td>
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<td>1.1</td>
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<tr>
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<td>Fraction #89-97</td>
<td>99</td>
<td>9</td>
<td>11</td>
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<td>139</td>
</tr>
</tbody>
</table>

* The enzyme activity was measured by HPLC using a natural isoflavone conjugate mixture as substrate. The activity was calculated from the concentration of released free daidzein.

Table 2.4: The purification procedure of the isoflavone-conjugate-specific β-glucosidase from imbibed soybean seeds. The enzyme activity was measured by the HPLC method using isoflavone conjugates mixture as substrates.
Figure 2.5: Chromatogram of the hydrophobic interaction chromatography. Solid ammonium sulfate was added to the soybean root intercellular fluid to bring it to a final concentration of 2.4 M, and then applied to Econo-Pac methyl HIC cartridge (Bio-Rad) equilibrated with 100 mM phosphate buffer, pH 6.8. After washing, the bound proteins were eluted with a decreasing linear salt gradient from 2.4 to 0 M in 100 mM phosphate buffer, pH 6.8. ■ —■ enzyme activity monitored by isoflavone conjugates ◆ —◆ A280.
**Lectin Affinity Chromatography**

The isoflavone-conjugate-specific β-glucosidase from either intercellular fluid (Exp. I) or cell wall wash (Exp. II) was not successfully recognized by soybean agglutinin (SBA). No significant amounts of enzyme were bound and only about 0.6% enzyme activity was eluted by sugars (Table 2.5).

<table>
<thead>
<tr>
<th></th>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total Activity (mU)*</td>
<td>Recovery Rate (%)</td>
</tr>
<tr>
<td>Before SBA binding</td>
<td>256</td>
<td>100</td>
</tr>
<tr>
<td>After SBA binding</td>
<td>277</td>
<td>108</td>
</tr>
<tr>
<td>10 mM GalNac 1°</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>10 mM GalNac 2°</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>200 mM Galactose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>500 mM Galactose</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* The same as in Table 2.2.

Table 2.5: The lack of binding of the β-glucosidase to soybean agglutinin.

**Substrate Affinity Chromatography**

A PrepSep C18 column saturated with isoflavone conjugates was used as an affinity column to selectively bind the proteins associated with isoflavone conjugates. About 16% of enzyme was bound by this affinity column, but only 6% activity was recovered (Table 2.6). The binding and elution were confirmed by a SDS-PAGE gel shown in Figure 2.6.
The SDS-PAGE gel also revealed that other than the isoflavone-conjugate-specific β-glucosidase, other proteins were also associated with this putative affinity column (Figure 2.6).

<table>
<thead>
<tr>
<th></th>
<th>Total Activity (ml)*</th>
<th>Recovery Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before application</td>
<td>52.4</td>
<td>100</td>
</tr>
<tr>
<td>After application</td>
<td>44.2</td>
<td>84</td>
</tr>
<tr>
<td>Elution</td>
<td>3.2</td>
<td>6</td>
</tr>
</tbody>
</table>

*The same as Table 2.2.

Table 2.6: The application of a substrate affinity column.

Figure 2.6: Evaluation of substrate affinity column chromatography by SDS-PAGE on 7.5% gel. Samples obtained from DEAE-Sephadex column and concentrated were treated with SDS only without β-mercaptoethanol. The protein of interest, the isoflavone-conjugate-specific β-glucosidase is indicated. Lane A: protein sample before application; Lane B: Protein sample after application; C: The first eluent by excess isoflavone conjugates.
Size Exclusion Chromatography

The void volume of a Sephadex G-150 column (1.7 x 19 cm) was about 18 ml. The desalted protein solution from 40-60% ammonium sulfate precipitation was applied to this size exclusion column, and eluted with 20 mM phosphate buffer, pH 6.0, at 4°C. The isoflavone-conjugate-specific β-glucosidase activity was found immediately after the void volume and spanned a few fractions resulting in a broad peak (Figure 2.7A). A similar chromatogram was obtained using partially purified enzyme solution (DEAE and CM-Sephadex column chromatography) (Figure 2.7B). The broad peak of gel filtration suggests heterogeneity in molecular weight. This could be the result of aggregation or of different degrees of glycosylation.

The Chemicals for Stabilization of Isoflavone-conjugate-specific β-Glucosidase

Due to the extensive loss of enzyme activity during these various purification procedures, several chemicals were tested in order to stabilize the protein. The enzyme activity was relatively stable at room temperature for up to 4 days in all treatments except the #9 chemical combination consisting of 10 mM EDTA, 5 mM DTT, and 1 mM PMSF (Figure 2.8A). Under cold conditions (4°C), the sulfhydryl-containing reagents, DTT and β-mercaptoethanol, facilitated enzyme activity losses (Figure 2.8B), consistent with the experience that enzyme activity was lost when developing buffer used in chromatography had 5 mM β-mercaptoethanol in it. Freezing and thawing were not good to maintain the enzyme activity, especially in the phosphate buffer containing EDTA, PMSF, or DTT (Figure 2.8C). Importantly, none of these reagents stabilized the enzyme significantly above the use of buffer alone.
Figure 2.7: The chromatograms of size exclusion chromatography on Sephadex G-150. The crude cell wall wash was fractionated by 40-60% ammonium sulfate, and dialyzed against 20 mM phosphate buffer, pH 6.0 (A). Alternatively, the partially purified enzyme solution from DEAE- and CM-Sephadex ion change was lyophilized and dissolved in 20 mM phosphate buffer, pH 6.0 (B). The concentrated protein solutions (A) and (B) were loaded onto Sephadex G-150 and eluted with the same buffer. Void volume was about 18 ml as indicated by the arrow.
Figure 2.8: The effect of several chemicals on the stability of the isoflavone-conjugate-specific β-glucosidase at various temperatures for different time periods. The enzyme was extracted with 100 mM phosphate buffer, pH 6.0 (1) or the same buffer containing 5 mM DTT (2), 5 mM β-mercaptoethanol (3), 5 mM DTT and 1 mM PMSF (4), 1 mM PMSF (5), 10 mM EDTA (6), 10 mM EDTA and 5 mM DTT (7), 10 mM EDTA and 1 mM PMSF (8), or 10 mM EDTA, 5 mM DTT, and 1 mM PMSF (9). The crude extracts were left at room temperature (A), 4°C (B), and -20°C (C), and activity was measured at time 0, day 1, day 2 and day 4, respectively. Activities are expressed as percent of activity at time 0.
Development of the Final Purification Procedures for the Isoflavone-conjugate-specific β-glucosidase from Soybean Roots

Because HIC chromatography suggested that the protein was relatively hydrophobic, 10% glycerol was also examined for stabilization of the enzyme. In combination with DTT, glycerol led to very significant stabilization. Thus, 10% glycerol and 3 mM DTT, were subsequently used in all purification procedures. The final purification protocol resulted in an enzyme purification of about 19-fold in two chromatographic steps (Table 2.7), or 17-fold in three chromatographic step (Table 2.8). The resultant preparation after DEAE- and CM-Sephadex column chromatography (Figure 2.9A and B) was near homogeneous as observed on a silver-stained SDS-PAGE gel (Figure 2.10A). In addition, the active fractions from DEAE and CM Sephadex ion exchanges were pooled and brought to 2.4 M ammonium sulfate. This enzyme preparation was subjected to hydrophobic interaction chromatography, eluted with a linear 2.4-0 M gradient. The activity peak was eluted by 0.62 M ammonium sulfate (Figure 2.9C). Four groups were defined (see Materials and Methods) and fractions within a group were pooled, desalted, and subjected to a SDS-PAGE to examine the purity. The enzyme after hydrophobic interaction chromatography lost about three fourths of its activity (Table 2.8) and the SDS-PAGE gel revealed that Group I had more of the enzyme than Group III (Figure 2.10B), although the latter had more activity. In addition, the purification was not improved by hydrophobic interaction column in which 17-fold purity was similar to the two chromatographic purification schemes (compare Tables 2.7 and 2.8).
<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity* (mU)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (mU/mg protein)</th>
<th>Yield (%)</th>
<th>Purity (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW crude</td>
<td>8775</td>
<td>124</td>
<td>71</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 40-60%</td>
<td>10541</td>
<td>49</td>
<td>216</td>
<td>120</td>
<td>3</td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>2961</td>
<td>12</td>
<td>238</td>
<td>34</td>
<td>3.4</td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>1736</td>
<td>1.3</td>
<td>1343</td>
<td>20</td>
<td>19</td>
</tr>
</tbody>
</table>

*The same as in Table 2.2.

Table 2.7: The purification procedures for the isoflavone-conjugate-specific β-glucosidase from soybean roots by two chromatographic steps.
<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity* (mU)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (mU/mg protein)</th>
<th>Yield (%)</th>
<th>Purity fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW crude</td>
<td>8775</td>
<td>124</td>
<td>71</td>
<td>100%</td>
<td>1 fold</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 40-60%</td>
<td>10541</td>
<td>49</td>
<td>216</td>
<td>120%</td>
<td>3 fold</td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>2961</td>
<td>12</td>
<td>238</td>
<td>34%</td>
<td>3.4 fold</td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>1576</td>
<td>1.6</td>
<td>989</td>
<td>18%</td>
<td>14 fold</td>
</tr>
<tr>
<td>Butyl Sapharose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I (104-110)</td>
<td>51</td>
<td>1</td>
<td>52</td>
<td>0.6%</td>
<td>0.7 fold</td>
</tr>
<tr>
<td>Group II (111-115)</td>
<td>166</td>
<td>0.5</td>
<td>337</td>
<td>1.9%</td>
<td>4.8 fold</td>
</tr>
<tr>
<td>Group III (116-120)</td>
<td>392</td>
<td>0.3</td>
<td>1209</td>
<td>4.5%</td>
<td>17.0 fold</td>
</tr>
</tbody>
</table>

*The same as in Table 2.2.

Table 2.8: The purification procedures for the isoflavone-conjugate-specific β-glucosidase from soybean roots by three chromatographic steps.
Figure 2.9: The final purification of isoflavone-conjugate-specific β-glucosidase from soybean roots (see Materials and Methods). A. DEAE-Sephadex A-50 column chromatography, B. CM-Sephadex C-50 column chromatography. C. Butyl Sepharose 4 Fast Flow hydrophobic interaction chromatography. ■—■ The isoflavone-conjugate-specific β-glucosidase •—• A280.

(to be continued)
Figure 2.9: continued
Figure 2.10: Documentation of the final purification of the isoflavone-conjugate-specific β-glucosidase from soybean roots. Each purification stage including crude cell wall wash, DEAE- and CM-Sephadex column chromatography (A) and final hydrophobic interaction chromatography (B) was subjected to SDS-PAGE on 7.5% gels and visualized by silver staining. A) Lane 1: protein marker (66, 45, and 29 kDa); Lane 2: crude cell wall wash; Lane 3: 40-60% ammonium sulfate saturation and desalted by Sephadex G-25; Lane 4: DEAE-Sephadex A-50 column chromatography active-fraction pool; Lane 5: CM-Sephadex C-50 column chromatography active-fraction pool; B) Lane 6: HIC, Group I; Lane 7: HIC, Group II; and Lane 8: HIC, Group III.
DISCUSSION

Many β-glucosidases have been purified since the early 1970s. Along with the improvement of techniques, the amino acid sequences of β-glucosidases from a variety of sources are now known. From the N-terminal sequence data, there is a consensus region present. It seems a good idea to purify β-glucosidases by means of affinity chromatography in which the specific antibody against the consensus region is linked with the stationary phase. However, this principle did not apply to β-glucosidase purification in several recent reports (Dharmawardhana et al., 1995; Leah et al., 1995; Inoue and Ebizuka, 1996) because the wide distribution of this kind of enzyme makes the separation of a specific enzyme from a β-glucosidase mixture more difficult. Most of these β-glucosidases have activity toward the artificial substrate and other natural glucosides with various $K_m$ and $V_{max}$. In contrast, traditional column chromatography plays an important role in the purification of β-glucosidases, including ion exchange, gel filtration, hydroxylapatite, hydrophobic interaction, and chromatofocusing.

Heat treatment could not be used as a purification step for the isoflavone-conjugate-specific β-glucosidase from soybean roots, since 76% enzyme activity was lost at 60°C for 5 min. Although the isoflavone-conjugate-specific β-glucosidase only lost 13% activity within 45 min at 50°C, there was no apparent precipitates in this treatment. Comparing with other β-glucosidases, many of them are relatively unstable even at 50°C. For instance, one from maize lost its activity completely at 50°C for 30 min (Esen, 1992), and another flavone 7-O-glucoside-specific β-glucosidase denatured rapidly above 45°C (Maier et al., 1993).

Protein precipitation by high salt is highly dependent on the nature of the protein, especially the number and size of hydrophobic patches on the protein surface. A membrane-associated protein or a trans-membrane protein will not necessarily be salted out at lower percentages of ammonium sulfate fractionation. Trial fractionation with ammonium sulfate has to be done to reach a compromise between purification and recovery (Scopes, 1987). As
shown in Figure 2.1, no significant amount of protein was precipitated in 0-40% fraction; the protein was mainly found in the 40-60% fraction. Although the isoflavone-conjugate-specific b-glucosidase activity was measured in the 60-80% fraction as well, the SDS-PAGE data suggested that more smaller proteins or peptides were precipitated in this fraction and therefore a compromise was made. Ammonium sulfate has a slight acidifying action, so at least 50 mM buffer should be used and a trace amount of heavy metals will have a considerable effect on enzyme activity in higher percentage cuts (Scopes, 1987).

Frequently it is necessary to remove salts for the next step in purification. There are usually two ways to remove ammonium sulfate after the protein pellet is dissolved. These are dialysis or column chromatography. Dialysis can be carried out simply with dialysis membrane against a mass of buffer, or for large quantities, diafiltration with a stirrer cell device. Gel filtration column chromatography such as Sephadex G-25 (Pharmacia) and Bio-Gel P 6 (Bio-Rad), with exclusion limits of 5,000 and 6,000 Da, respectively (Robyt and White, 1987) can be used to remove the salt, since most proteins have molecular weights over 5,000 or 6,000 Da. Another pre-packed column for desalting is the PD-10 column from Pharmacia which works similarly (Harris, 1989). For the isoflavone-conjugate-specific b-glucosidase, desalting by means of gel filtration seemed better than the use of dialysis membrane in terms of total activity (Table 2.3 and 2.4). About 2-3 fold higher total activity was recovered using a gel filtration column. In fact, more protein precipitation was observed when the protein solution was dialyzed overnight at 4°C. It is possible that this protein had more opportunities to aggregate with each other in a dialysis bag over a longer period of time than in the mobile phase of gel filtration for a relatively short time. Alternatively, dilution of a protein may lead to dissociation of subunits which may be inactive, and this enzyme does have subunits showed in SDS-PAGE gels when it treated with SDS alone and SDS plus b-mercaptoethanol (See Chapter 3).

Ion exchange is often used following the crude extraction, ammonium sulfate fractionation, and dialysis because of its high capacity. In an early previous experiment, higher levels of b-glucosidases were extracted by alkaline buffers such as borate buffer, pH 8.5, and phosphate buffer, pH 8.0, concomitant with more proteins, than by neutral or
slightly acidic buffers from imbibed soybean seeds when \( p \)-nitrophenyl \( \beta \)-glucoside was used as a substrate. This was consistent with the finding that higher levels of \( \beta \)-glucosidase activity were found in particulate fractions isolated from corn roots under alkaline compared to acid conditions (Nagahashi et al., 1985). However, the \( \beta \)-glucosidase activity was unstable in the alkaline condition in which three fourths to two thirds of its activity was lost over one night. In addition, the isoflavone-conjugate-specific \( \beta \)-glucosidase was not active in under alkaline conditions. Therefore, slightly acidic or neutral buffer systems were used in ion exchange column chromatography. In a trial for determining the binding ability of DEAE-Sephadex A-50 resin at various pH values, the isoflavone-conjugate-specific \( \beta \)-glucosidase was only partially bound at pH 7.0, and about 90% of \( \beta \)-glucosidase activity was found in the unbound fractions at pH 6.0 (Figure 2.2). However, there was a considerable amount of other proteins associated with this anion resin and eluted by a linear gradient of sodium chloride at pH 6.0. In fact, to remove other proteins by means of DEAE-Sephadex improved the purification about 5 fold (Table 2.2). Because this study focused on the isoflavone-conjugate-specific \( \beta \)-glucosidase, the enzyme activity was always monitored by specific substrates. There was a small peak eluted by 0.38 M NaCl (Figure 2.2), and it is likely to be another \( \beta \)-glucosidase with lower affinity to isoflavone conjugates.

Only two isoflavone glucoside specific \( \beta \)-glucosidases had been purified from chickpea and soybean to date (Hösel and Barz, 1975; Matsuura and Obata, 1993; Matsuura et al., 1995). It was of particular importance that we tried to repeat the purification work from soybean done by the Japanese group. The \( \beta \)-glucosidases were isolated from imbibed soybean seeds by acid precipitation, CM-Sephadex C-50, Butyl-Toyopearl 650C and Sephadex G-150 column chromatography by Matsuura et al (1995). As shown in Table 2.3, after three steps, a non-specific \( \beta \)-glucosidase was purified 274-fold with high specific activity (126 mU/mg protein) toward \( p \)-nitrophenyl \( \beta \)-glucoside. In contrast, the same fraction showed very low specific activity (14 mU/mg protein) toward an isoflavone conjugate mixture. These data suggested that these purification procedures favored the isolation of a non-specific \( \beta \)-glucosidase instead of the isoflavone-conjugate-specific \( \beta \)-glucosidase. Indeed, the purified \( \beta \)-glucosidase C from imbibed soybean seeds revealed
higher hydrolysis rates with p-nitrophenyl β-glucoside (100%), and very low rates with daidzein glucoside (38%) and genistein glucoside (12%) (Matsuura and Obata, 1993; Matsuura et al., 1995), indicating that this particular enzyme was not an isoflavone-conjugate-specific β-glucosidase. Moreover, in Table 2.3, the isoflavone-conjugate-specific β-glucosidase from soybean roots had been purified only 23-fold, because in root this enzyme was more abundant and the starting crude cell wall wash was about 575-fold more pure than the starting extract from imbibed seeds.

In initial experiments, a considerable loss of enzyme activity was observed after two chromatographic steps with only 0.2% recovery after DEAE-Sephadex and CM-Sephadex columns (Table 2.2). The tremendous loss of activity was not acceptable for further studies such as the determination of enzyme characteristics. Several chemicals were used to stabilize the enzyme including, sulfhydryl protectants, protease inhibitors, and heavy metal chelating agents. An inhibition study (see Chapter 3) disclosed that silver and mercury ions could inhibit the isoflavone-conjugate-specific β-glucosidase activity at 1 mM and the presence of excessive β-mercaptoethanol restored the enzyme activity from this suppression. It is also likely that there are protease and heavy metals from soybean root and ammonium sulfate contamination respectively, so a common protease inhibitor, PMSF, and a chelator, EDTA, were included in the extraction buffer, 200 mM phosphate buffer, pH 6.0. With different combinations of these chemicals, the enzyme activity did not decrease rapidly at room temperature except in the cocktail of PMSF, DTT, and EDTA (Figure 2.8A). At 4°C, the isoflavone-conjugate-specific β-glucosidase activity declined gradually with exceptions of DTT and β-mercaptoethanol (Figure 2.8B), probably because the free sulfhydryl groups were oxidized more quickly under this condition. Additionally, freezing and thawing also deteriorated the enzyme activity. Therefore, the presence of at least 10% glycerol to reduce the physical damage of freeze and thaw was necessary. This may also stabilize the enzyme in solution since HIC chromatography suggested it is relatively hydrophobic. The purification procedures had been accomplished using the usual buffer with 5 mM β-mercaptoethanol, but the enzyme activity was lost dramatically overnight. Due to the quick oxidization of β-mercaptoethanol within 24 hours in aerobic conditions,
the oxidized β-mercaptoethanol can even accelerate the inactivation processes, since it activates the formation of disulfide bond between two sulfhydryl groups in the active center (Scopes, 1987). This prompted us to choose DTT over β-mercaptoethanol because DTT is more stable, and has a lower redox potential and order. In addition, DTT does not form mixed disulfides with proteins, but β-mercaptoethanol does (Deutscher, 1990). Polyphenolic compounds such as flavonoids and tannins may inhibit enzyme activity by forming hydrogen bonds with peptide bond oxygens or by covalently modifying amino acid residues including those which possess hydroxyls, thiols, and primary amines (Gegenheimer, 1990). In addition, although it is not known if the enzyme products, daidzein and genistein, could inhibit enzyme activity, the product inhibition was observed in several β-glucosidases (Kuroki and Poulton, 1986; Inoue and Ebizuka, 1996). Insoluble polyvinylpyrrolidone, PVPP, thus, was added into the crude cell wall wash. As described in Materials and Methods, the final purification procedures, with the use of 1% PVPP in crude cell wall wash, and 3 mM DTT and 10% glycerol in all chromatographic steps, did help to stabilize the enzyme and resulted in 20% recovery after CM-Sephadex column chromatography (Table 2.8).

Hydrophobic interaction chromatography has drawn attention recently since this technique is dependent on the different hydrophobicity on the surface of the constituent proteins and is hence complementary to those chromatographic techniques based on the charge or the size of the protein (Pennings et al., 1991). It is convenient to use HIC when the protein solution contains salts without changing buffer or desalting, and usually the capacity is as high as ion exchange. By using the HIC methyl cartridge from Bio-Rad, proteins in the intercellular fluid did not bind to the alkyl group of the resin until the concentration of ammonium sulfate was 2.4 M, about 50% saturation, which is consistent with the principle that a salt concentration just below that used for salting out the protein is normally used (Kennedy, 1990). The isoflavone-conjugate-specific β-glucosidase is not like those proteins, such as globulins, membrane-associated proteins, and others that precipitate in a low range of ammonium sulfate saturation (20-40%), suggesting that this enzyme is not highly hydrophobic. However, it is hydrophobic enough to bind the methyl group tightly.
and hence delay its elution to the end of the gradient (Figure 2.5). Due to the good resolution from this chromatogram, the protein solution obtained from DEAE and CM-Sephadex ion exchanges was also subjected to a larger column, Butyl Sepharose 4 Fast Flow, using the same condition as mentioned above. A sharp activity peak was eluted at 0.62 M ammonium sulfate, right after a broad protein peak (Figure 2.9C). However, the calculation of total enzyme activity uncovered that only one fourth of the enzyme activity was recovered, and this step also did not improve the purity (Table 2.9). An analytic SDS-PAGE gel showed that Group I and II (#104-110 and #111-115) from this solution contained higher levels of β-glucosidase than Group III did (Figure 2.10B). However, the activity increased from Group I to III, indicating that the isoflavone-conjugate-specific β-glucosidase was denatured during the chromatographic process. Hydrophobic interaction is one of the forces to maintain protein tertiary structure (Rees et al., 1994), and this hydrophobic interaction is similar to the interaction between HIC resin and proteins. Therefore, partial or complete unfolding of the protein with concomitant loss of biological activity is very possible, particularly when the protein binds too tightly to the resin (Pennings et al., 1991). This may explain why the enzyme activity was lost dramatically in the process of Butyl Sepharose chromatography. Clearly, the butyl group is more hydrophobic than the methyl group, and the same condition used in the HIC methyl cartridge was not suitable for use in Butyl Sepharose, because 2.4 M of ammonium sulfate made the protein bind more tightly. During the elution, therefore, irreversible conformational change may have occurred and promoted protein aggregation. Trials and errors should have been done previously to determine the best binding condition for Butyl Sepharose column. Interestingly, the presence of 2.4 M ammonium sulfate did not interfere with the enzyme activity. Some studies showed that several plant and fungal β-glucosidases retained their activity under denaturing conditions (Esen and Gungor, 1993). The high salt was precipitated when methanol was added to stop the reaction and removed by centrifugation.

Lectin affinity chromatography is an attractive technique for separating oligosaccharides and glycoproteins in complex mixtures, because the separation is not
dependent on size and charge of the macromolecules, but on specific interactions of oligosaccharides with immobilized lectins which recognize certain structures of oligosaccharides. Lectin affinity also allows purification of extremely small amounts of material and provides knowledge on the structural characteristics of oligosaccharides (Merkle and Cummings, 1987). It was not known whether the isoflavone-conjugate-specific \( \beta \)-glucosidase was a glycoprotein or not, although it was possible because most cell wall associated proteins are glycoproteins. Although soybean agglutinin did not bind the isoflavone-conjugate-specific \( \beta \)-glucosidase (Table 2.5), it does not rule out that this enzyme still could be a glycoprotein. Further application of other lectins is necessary to make a final conclusion.

The substrate affinity chromatography was designed exclusively for this study. Theoretically, the isoflavone-conjugate-specific \( \beta \)-glucosidase would bind to its substrates and be eluted by adding excessive substrates. Indeed, the enzyme was bound and eluted out as expected (Figure 2.6). However, the recovery rate was only 6\%, which was too low for scaling up, and some other proteins were eluted out as well. It is likely that the enzyme cleaved the substrates and was released from the binding sites before the elution took place, although the whole process was conducted at 4\(^\circ\)C where only 18\% enzyme activity remained compared to its optimum temperature at 30\(^\circ\)C (see Chapter 3). Apart from the isoflavone-conjugate-specific \( \beta \)-glucosidase bound by this substrate affinity column, some other proteins also have affinity to isoflavone conjugates. It is conceivable that enzymes involved in isoflavone metabolism may have affinity to these isoflavone conjugates too. Alternatively, some bare C18 functional groups in the PrepSep column may provide nonspecific binding sites to proteins. This potential affinity chromatography might be improved by using smaller C18 column such as HPLC guard column (Alltech Co.) and applying crude enzyme solution accompanied by a \( \beta \)-glucosidase competitive inhibitor such as glucono-\( \delta \)-lacton which suppresses the enzyme activity to avoid cleavage occurring before elution.

Both crude and partially purified enzyme applied on size exclusion chromatography resulted in a broad peak, and the first fraction having enzyme activity was near the void
volume (Figure 2.8). The exclusion limit of Sephadex G-150 is about 300 kDa, which means proteins more than 300 kDa will be found in the void volume. It is likely that the isoflavone-conjugate-specific β-glucosidase formed various degrees of aggregates with itself or some other proteins. The more aggregated proteins form, the less activity remains, and this is consistent with the result from Butyl Sepharose HIC column in which large aggregation was found in the Group I, but almost all of activity was lost.
CHAPTER 3

THE CHARACTERIZATION OF THE ISOFLAVONE-CONJUGATE SPECIFIC
β-GLUCOSIDASE PURIFIED FROM SOYBEAN

INTRODUCTION

Due to the wide distribution of β-glucosidases in the living world (see Chapter 1), it is reasonable that some β-glucosidases share similar physical and catalytic properties. In fact, the subunit molecular weights of 55 to 65 kDa and acidic pH optima (pH 5-6) are very common in β-glucosidases (Esen, 1993), and the substrates include not only β-glucosides but also some others such as fucosides and galactosides (Esen, 1993). There are a very few examples that β-glucosidases can also use α-linked glucosides as substrates with an extremely low relative activity. In this chapter, the characteristics of β-glucosidases with or without known physiological functions are reviewed individually. It is worth noting that some so-called “specific” β-glucosidases do not necessarily have a narrow range of substrates, and sometimes the $K_m$ of a β-glucosidase for its physiological substrate is not lower than that for synthetic substrates.
Characteristics of Purified or Partially Purified β-Glucosidases from Plants

I. Gymnosperms

The spruce coniferin β-glucosidase from a hypocotyl cell wall fraction showed high activity with a pH optimum between 4.5 and 5.5, and a temperature optimum between 40 and 45°C. Its \( K_m \), \( V_{max} \), and the ratio of \( V_{max}/K_m \) for coniferin were 1.3 mM, 144 mkat/kg, and 111 mkat/kg x mM, respectively, which were lower than those for o-nitrophenyl β-glucoside. The coniferin β-glucosidase is composed of only one polypeptide chain with Mr 58.6 kDa (Marcinowski and Grisebach, 1978).

Two cytosolic glycoproteins from lignifying xylem of *Pinus banksiana* with coniferin-hydrolyzing β-glucosidase activity were isolated. They both were glycoprotein with molecular weights of 110 and 90 kDa. The pI was 3.8 for both and they mainly hydrolyzed coniferin only (Leinhos et al., 1994).

A β-glucosidase specific for coniferin was also purified from the xylem of lodgepole pine (*Pinus contorta* var latifolia Engelm). The molecular weight of the native enzyme was 60 kDa determined by gel filtration chromatography, and there were two subunits of 28 kDa (and/or 24 kDa) revealed by SDS-PAGE. The optimum pH for coniferin hydrolytic activity at 30°C was between 5.4 and 5.9, and the pI was 4.5. This protein was demonstrated to be a glycoprotein as well. The coniferin β-glucosidase was highly specific to its native substrate, coniferin, and a related one, syringin, with a \( K_m \) of 0.18 mM and 0.29 mM, respectively. Although it also hydrolyzed synthetic substrates such as o-nitrophenyl β-glucoside, p-nitrophenyl β-glucoside (\( K_m =1.9 \) mM), and 4-methylumbelliferyl β-glucoside (\( K_m =2.3 \) mM), the \( K_m \) was higher and relative activity was lower. Among three inhibitors used, bromoconduritol, conduritol β-epoxide, and glucono-δ-lactone, only the latter one was effective in inhibiting coniferin β-glucosidase (Dharmawardhana et al., 1995).
II. Angiosperms

Dicotyledonous Plants

Coniferin-Specific β-Glucosidases

The chickpea coniferin-specific β-glucosidase from a crude cell wall preparation of cell cultures had several isoenzymes from PI 8.5 to 10 with identical catalytic activity. The $K_m$ and $V_{\text{max}}$ for coniferin were 0.8 mM and 6 mmol/min/mg protein. The molecular weight was 110 kDa, with two non-identical subunits of Mr 63 and 43. Maximum hydrolytic activity was at pH 5 (Hösel et al., 1978). This enzyme was also purified from chickpea stem and root and showed similar kinetics. Among the known β-glucosidase inhibitors, 50 μM of bromoconduritol inhibited enzyme activity up to 89%, and 2 mM of glucono-δ-lactone suppressed 61% of its activity ($K_i = 1.4$ mM). The coniferin-specific β-glucosidase had a low temperature optimum at 28-30°C, and had carbohydrate content, indicating a glycoprotein nature (Surholt and Hösel, 1980).

Similarly, a β-glucosidase from soybean specifically hydrolyzed coniferin and syringin with a $K_m$ of 0.6 and 0.3 mM, and $V_{\text{max}}$ of 0.127 and 0.115, respectively. The activity was maximal at pH 4.6-6, and the isoelectric pH was 4.2-4.4. Molecular weight was 45 kDa. Glucono-δ-lactone was a good inhibitor with $K_i$ value of 10 mM (Hösel and Todenhagen, 1980).

Cyanogenic Glucoside-Specific β-Glucosidases

Flax seeds have large amounts of cyanogenic compounds, linustain and linamarin. Two cyanogenic β-glucosidases, linustatinase and linamarase, which hydrolyze linustain and linamarin sequentially, were isolated and purified. Linustatinase had five different isozymic forms with pIs between 7 and 8, while linamarase had one major form with a pI between 4 and 5. Linustatinase was found to be a dimer with molecular weights of 39 and 19 kDa, respectively, whereas linamarase appeared to be a $\alpha_5\beta_5$ complex with
molecular weights of and of 62.5 and 65 kDa, respectively. Both enzymes were
glycoproteins containing mannose and glucose. $K_m$ values of linustatinase and linamarase
for their natural substrates were 2.6 mM and 8.2 mM and the pH optima were 4-6 and 5.5-6,
respectively. Both enzymes were sensitive to glucono-δ-lactone with $K_i$ values of 0.11 mM
and 0.27 mM. However, only linustatinase was inhibited by high concentrations of
linustatin (10 mM) and another substrate, neolinustatin (5 mM), while linamarase was not.
With respect to substrate specificity, linustatinase had high relative activity toward β-bis-
glucosides with β-1,6 (such as linustatin, neolinustatin, and amygdalin) and β-1,3 (such as
neocycasin A) linkages, while linamarase had low activities for them. On the other hand,
linamarase favored β-monoglucosides such as linamarin and prunasin. Interestingly,
linustatinase had high relative activity for the β-monoglucoside, dhurrin as well. As for
artificial substrates, both enzymes had highest relative activity toward p-nitrophenyl β-
glucoside, but also had minor activity for p-nitrophenyl β-galactoside (Fan and Conn,
1985).

Two isoenzymes of amygdalin hydrolase (AH I and AH II) from black cherry
(Prunus serotina Ehrh.) seeds were monomeric with Mr 60 kDa and had isoelectric points
of 6.6 and 6.5, respectively. Both were glycoproteins with positive periodic acid-Schiff
staining. Maximum pH for hydrolytic activity was shown in the range of 4.5 and 5.0. AH I
and AH II had $K_m$ values for amygdalin of 2.5 and 2.1 mM, respectively. The

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efficiently inhibited AH I and II with $K_i$ values of 1.7 and 1.3 mM (Kuroki and Poulton, 1986).

The product of amygdalin hydrolases, prunasin, was further hydrolyzed by prunasin hydrolase (PH) which was also purified from black cherry seeds. There were three forms of prunasin hydrolase, PH I, PH IIa, and PH IIb. Both PH I and PH IIb were monomeric with molecular weights of 68 kDa, while PH IIa was a dimer with a native molecular weight of 140 kDa, with 69.5 kDa for two identical subunits. These three isozymes were all glycoproteins and had a pH optimum at pH 5.0. Michaelis constants of PHI, IIa, and IIb for prunasin, were 1.73, 2.3, and 1.35 mM, respectively. The three forms of prunasin hydrolase also used $\alpha$-nitrophenyl $\beta$-glucoside, $p$-nitrophenyl $\beta$-glucoside, and $p$-nitrophenyl $\beta$-galactoside with decreasing activity rates. Castanospermine competitively inhibited PH I and IIb with $K_i$ values of 0.19 and 0.09 mM, respectively. Metal ions including $\text{Cu}^{2+}$, $\text{Mg}^{2+}$, $\text{Zn}^{2+}$, $\text{Pb}^{2+}$, $\text{Fe}^{2+}$, $\text{Fe}^{3+}$, and $\text{Ag}^+$, had little or no effect at 0.1 mM final concentration. Organic substances such as $\beta$-mercaptoethanol (5 mM), DTT (5 mM), EDTA (5 mM), $p$-chloromercuriphenylsulfonate (1 mM), iodoacetamide (1 mM), and iodoacetate (1 mM) did not reduce the enzyme activity significantly (Kuroki and Poulton, 1987).

In the rubber tree, *Hevea brasiliensis*, there was only one $\beta$-glucosidase detectable in leaves, and this $\beta$-glucosidase was also a linamarase hydrolyzing the abundant substrate, linamarin. Although the linamarase had $K_m$ and $V_{\text{max}}$ for linamarin of 7.6 mM and 191 mmol/min/mg protein, this enzyme turned out to be a nonspecific $\beta$-glycosidase with a broad substrate spectrum, particularly for $\alpha$-nitrophenyl $\beta$-glucoside, the $K_m$ and $V_{\text{max}}$ of which were 0.5 mM and 189 mmol/min/mg protein, respectively. Using $p$-nitrophenyl $\beta$-glucoside as substrate, the enzyme exhibited a pH optimum at pH 5.6, and a temperature optimum at 62°C. An interesting characteristic of this enzyme was that it formed many
oligomers which were interconvertible by dissociation processes. The monomer is a single protein of 64 kDa (Selmar et al., 1987).

The linamarase in cassava consisted of a single polypeptide with a molecular mass of 65 kDa. The enzyme had different isozyme forms on isoelectric focusing with pIs of 4.4, 3.4, and 3.0. The $K_m$ and $V_{max}$ for linamarin were 1.9 mM and 490 μmol/min/mg protein, respectively. The optimum temperature was 55°C, and the pH optimum for hydrolyzing p-nitrophenyl β-glucoside was pH 7.0. This β-glucosidase also cleaved prunasin with a rate similar to p-nitrophenyl β-glucoside, but hydrolyzed o-nitropheyl β-glucoside with a lower rate (Mkpong et al., 1990).

**β-Glucosidases Involved in Metabolism of Secondary Metabolites**

Two β-glucosidases involved in indole alkaloid biosynthesis in *Catharanthus roseus* were separated. The pH optimum for these two enzymes was between 6.0 and 6.4, and the temperature optimum for both enzymes was 30°C. $K_m$ values for β-glucosidase I and II were 0.2 mM and 0.1 mM, and the respective $V_{max}$ values were 0.23 and 0.12 nmol/min when the native substrate, strictosidine, was used. The molecular masses of enzyme I and II were very large at 230 kDa and > 450 kDa, respectively. The strictosidine-specific β-glucosidase I and II could be differentiated by using the inhibitor glucono-δ-lactone. Enzyme I was less sensitive to this inhibitor with an IC$_{50}$ at about 10 mM, while enzyme II needed around 100 mM to inhibit 50% activity (Hemscheidt and Zenk, 1980).

In *Costus speciosus*, a furostanol glycoside 26-O-β-glucosidase hydrolyzed furostanol glycosides to yield spirostanol glycosides (steroid saponins) during postharvest treatment and storage. The enzyme was highly specific for cleavage of the natural substrates with a $K_m$ for protogracillin of 50 μM. This enzyme showed a molecular weight of 110 kDa, with two subunits, 54 and 58 kDa each. The optimum pH of the purified enzyme was determined to be between 5.0 and 5.5 when protogracillin was the substrate. Glucono-δ-
lactone was effective in inhibiting this β-glucosidase and showed 50% inhibition at 0.25 mM, while condurito β-epoxide was less effective with an IC_{50} of 2.4 mM. Diosgenin (a sterol), an aglycone of spirostanol glycosides suppressed 50% of the enzyme activity at 79 μM (Inoue and Ebizuka, 1996).

**Miscellaneous β-Glucosidases**

A β-glucosidase with no known physiological function was isolated from papaya fruit. The pI of this β-glucosidase was 5.2, and the molecular weight was 54 kDa with two identical subunits (27 kDa each). The optimum pH and temperature were of 5.0 and 50°C. The enzyme followed typical Michaelis kinetics with a K_m and V_{max} of 0.11 mM and 0.03 mmol/min (Hartmann-Schreier and Schreier, 1986).

A glycoprotein with β-glucosidase activity was purified from soybean cell walls. The protein was a single polypeptide with Mr of 60 kDa (Nari et al., 1982/83).

Two β-glucosidases of unknown function were purified from grapefruit with different molecular weights of 98 and 50 kDa. For both enzymes, the optimum pH of activity was 5.0, and the optimum temperature was 45°C. The enzymes had a K_m and V_{max} for p-nitrophenyl β-glucoside of 1.72 mM and 0.205 nkat/ml, respectively. These enzyme also hydrolyzed some naturally occurring aromatic glucosides such as benzyl- and phenyl-ethyl β-glucosides with lower hydrolyzing rate than the synthetic substrates. In addition, some primary alcohol glucosides, including geranyl-, neryl-, and citronellyl- β-glucosides were also good substrates, but tertiary alcohol glucosides, e.g. linalyl β-glucoside and terpinyl β-glucoside, were not. Several cations such as Cu^{2+} and Ca^{2+} (10 mM final concentration) could inhibit both enzyme activities, and PCMB (p-chloromercuribenzoate) was also very effective at 10 mM. The product, glucose, and the classical β-glucosidase inhibitor, glucono-δ-lactone were competitive inhibitors. The K_i was 170 mM for glucose, and 0.215 mM for glucono-δ-lactone (Lecas et al., 1991).
Monocotyledonous Plants

Cyanogenic Glucoside-Specific β-Glucosidases

Two different dhurrin specific β-glucosidases were purified from Sorghum bicolor. The optimum activity of both enzymes was at pH 6-6.2. Dhurrinase 2 required DTT to maintain its activity, while dhurrinase 1 did not. SDS-PAGE revealed that dhurrinase 1 had a monomeric subunit with Mr of 57 kDa, while the native form in the seedlings had a molecular weight of 200-240 kDa (a tetramer, termed dhurrinase 1A), which dissociated during the process of purification without losing activity to a dimeric form with Mr 100-110 kDa (termed dhurrinase 1B). Dhurrinase 2 appeared to be a tetrameric protein with molecular weight of 250-300 kDa, with a monomer of 61 kDa. Both enzymes preferred dhurrin as their substrate with the same K_m of 0.15 mM, but dhurrinase 1 hydrolyzed another structure-similar cyanogenic β-glucoside, sambunigrin, faster that dhurrinase 2 did, while the latter also cleaved 4-methylumbelliferyl β-glucoside, but not the former. Three β-glucosidase inhibitors, nojirimycin, glucono-δ-lactone, and 1-amino-β-D-glucoside were effective in inhibiting the dhurrinase activity, with a K_i of 0.15, 0.6, and 0.8 mM, respectively for dhurrinase 1, and 0.13, 0.12, and 1 mM for dhurrinase 2. Furthermore, these two β-glucosidases were not glycoproteins (Hösel et al., 1987)

β-Glucosidases Hydrolyzing Pre-formed Toxin Glucosides

The hydroxamic acid glucoside specific β-glucosidase, and a non-specific β-glucosidase from maize were partially purified. The p-nitrophenyl β-glucoside β-glucosidase had a Mr of 60 kDa and an isoelectric point of 6.4, while the hydroxamic acid glucoside β-glucosidase had a Mr of 158 kDa and a pI of 4.8. The specific β-glucosidase had an optimum pH of 6.0, and the K_m for DIMBOA-glucoside and p-nitrophenyl β-glucoside were 0.11 and 0.46 mM, respectively. A complete inhibition of DIMBOA-
glucoside β-glucosidase was obtained at 100 mM castanospermine, and 50% inhibition at a concentration about 20 mM (Cuevas et al., 1992).

Avenacosidase, a β-glucosidase hydrolyzing a pre-formed toxin (avenacoside B) to yield 26-desgluco-avenacoside B was purified from oats. This enzyme, having a single subunit with Mr of 60 kDa (therefore termed As-P60), aggregated to form various degrees of aggregation from molecular weights of 300-350 kDa to larger than $10^6$ kDa. Detergents or high salts concentration such as 0.1% SDS, 2% Triton X-100, 50-500 mM NaCl or KCl, and 3 M urea, could not change the multiple banding patterns. However, freezing and thawing completely dissociated the aggregates to dimers with total loss of enzyme activity. As-P60 had a higher affinity towards avenacosides A and B, with a $K_m$ of 12 μM than towards p-nitrophenyl β-glucoside, with a $K_m$ of 2.2 mM. This enzyme did not hydrolyze disaccharides, but cleaved p-nitrophenyl β-fucoside, p-nitrophenyl β-galatoside, o-nitrophenyl β-glucoside, and o-nitrophenyl β-galatoside in decreasing order (Gus-Mayer et al., 1994).

**Putative Hormone Conjugate β-Glucosidases**

Using photoaffinity labeling with the auxin analog, 5-azido-[7-$^3$H]indole-3-acetic acid, a protein with molecular weight 60 kDa (p60) was identified and subsequently purified. This protein turned out to be a β-glucosidase. Protein p60 was a dimer with a molecular weight of 130 kDa, and had different isoforms. The enzyme activity showed maximal activity between pH 5.3 and 5.9, using p-nitrophenyl β-glucoside as a substrate. The p60 β-glucosidase hydrolyzed p-nitrophenyl β-glucoside, p-nitrophenyl β-arabinoside, salicin, and laminariaribiose with relative activities of 100%, 27%, 14%, and 17.5%, respectively. On the other hand, this enzyme accepted intoxyl-O-glucoside as a substrate, but not other auxin conjugates such as indole-3-myo-inositol and indole-3-aspartate, and this enzyme was inhibited by IAA, 1-NAA, and the auxin transport inhibitor NPA,
suggesting that the p60-associated β-glucosidase may be involved in phytohormone regulation. For instance, 1 mM to 2 mM of 1-NAA inhibited enzyme activity up to 40%, while other plant hormones including abscisic acid, gibberellins, or cytokinins did not affect the enzyme activity (Campos et al., 1992).

Another protein was identified by photoaffinity labeling as well from membrane vesicles of maize with the same molecular weight, 60 kDa, (pm60) as the one described in the last paragraph. The pm60 also had β-glucosidase activity, hydrolyzing p-nitrophenyl β-glucoside with a pH optimum of 5.0, with a $K_m$ and $V_{max}$ of 0.652 mM and 6.24 mmol/min/mg protein. Although p-nitrophenol could be detected after incubating the enzyme with p-nitrophenyl β-galactoside, p-nitrophenyl β-xyloside, p-nitrophenyl α-gluco- side, and p-nitrophenyl α-arabinofuranoside at 30°C for 15 min, the specific activity was very low. Again, this enzyme was also influenced by auxins. The enzyme activity was reduced to 20% and 45% by 1 mM of 1-NAA and IAA with a $K_i$ of 0.43 and 1.14 mM, respectively. The well known inhibitor of β-glucosidase, glucono-δ-lactone, and the product, glucose, suppressed the enzyme activity with a $K_i$ of 0.8 and 148 mM, respectively. Both p60 and pm60 shared an identical N-terminal sequence over a stretch of 20 amino acid. The only marked difference was solubility of these two proteins; p60 was solubilized by acetone, n-butanol, and Triton X-100, while pm60 was only solubilized by Triton X-114 (Deldwisch et al., 1995).

**β-Glucosidase Involved in Carbohydrate Catabolism**

A 60-kDa β-glucosidase (BGQ60) was purified from barley seeds. This β-glucosidase was able to hydrolyze β-linked, synthetic oligosaccharides composed of glucose and mannose. The major activity for hydrolyzing natural substrates was found to be the cleavage of disaccharides with (1-2)-, (1-3)-, and (1-4)-β-linkages, but not (1-6)-β-linkages. The $K_m$ values for sophorose, cellobiose, and laminaribiose were 6.3 mM, 4.5
mM, and 3.0 mM, respectively. It was able to use short-chain cello-oligosaccharides such as cellopentaose as substrates. In addition, this enzyme also hydrolyzed indoxyl β-D-glucoside and salicylic acid β-D-glucoside at a low rate, but not gibberellin glucosides. The pH optimum and temperature optimum for activity of BGQ60 using p-nitrophenyl β-glucoside as a substrate was pH 5.0 and 60°C (Leah et al., 1995).

**Miscellaneous β-Glucosidases from Monocots**

Using a DEAE cellulose coupled with a hydroxylapatite column, a β-glucosidase was distinguished from a peroxidase in barley seeds. The molecular weight of this β-glucosidase was 53.7 kDa, determined by SDS-PAGE, and was a single protein. The isoelectric point was 7.65, and the enzyme had 19% carbohydrate, indicating a glycoprotein. The pH optimum was 5.25. The β-glucosidase hydrolyzed p-nitrophenyl β-glucoside most rapidly, and 4-methylumbelliferyl β-glucoside and p-nitrophenyl β-galactoside were hydrolyzed with decreasing rates (Rescigno et al., 1993).

A lactose-hydrolyzing β-glucosidase from barley meal was purified to homogeneity. This enzyme showed a band on SDS-PAGE with molecular weight of 55 kDa, and it did not stain for glycoprotein. There were four catalytically active bands of pI 8.5, 8.8, 9.0 and 9.2 separated by isoelectric focusing. The enzyme acted optimally at pH 4.5-5.0, showed high affinity toward o-nitrophenyl β-glucoside and cellobiose with a $K_m$ of 2.5 and 6 mM, respectively, and much weaker affinity towards o-nitrophenyl β-galactoside and lactose with a $K_m$ of 27 and 100 mM, respectively. This enzyme was insensitive to concentrations of monovalent salts up to 400 mM, except AgNO₃, which totally inhibited enzyme activity at 1 mM or higher. Among organic substances, only glucono-δ-lactone was an effective inhibitor to suppress the hydrolysis of cellobiose by 73% at 1 mM (Simos and Geogatsos, 1988).

A maize β-glucosidase was purified to a monomer with a Mr of 60 kDa. The pI of this enzyme was about 5.2, and the pH optimum was around 5.8. The optimum temperature
for the activity of maize β-glucosidase was 50 C. Two sulfhydryl blockers, Hg²⁺ and Ag⁺, were potent inhibitors of the enzyme activity with 100% inhibition at 32 μM for Ag⁺ and 256 μM for Hg²⁺. Alkylation did not reduce enzyme activity using 1.56 to 50 mM of iodoacetamide and iodoacetate respectively (Esen, 1992).

The Properties and Characteristics of Flavone and Isoflavone Glucoside Specific β-Glucosidases

Due to the focus of this study on the isoflavone conjugate specific β-glucosidase, the characteristics of highly related isoflavone- and flavone-7-O-glucoside β-glucosidases are highlighted in this section. Only four reports for two isoflavone glucoside specific β-glucosidase and one flavone glucoside specific β-glucosidase have been made.

Hösel and Barz purified the first isoflavone-7-O-glucoside specific β-glucosidase in 1975. Several isozymes were purified from leaves, roots and hypocotyls of chickpea seedlings. All β-glucosidases had native molecular weights ranging from 125 to 135 kDa, consisting of two identical subunits with molecular weight of 68 kDa. The pH optimum for enzyme activity using p-nitrophenyl β-glucoside as a substrate was between 7.0 and 7.5, with an additional minor optimum at 4.5-5.0. The temperature optimum was found to be 45°C. For substrate specificity, the enzyme had a pronounced specificity for the 7-position of the aglycone, including formononetin 7-O-β-glucoside, biochanin A 7-O-β-glucoside, and biochanin A 7-O-β-glucoapioside with $K_m$ values of 30, 20, and 150 mM, respectively. They also show high affinity to other non-isoflavone natural substrates or synthetic isoflavones such as apigenin 7-O-β-glucoside and 2-methyl-4'-nitro-isoflavone 7-O-β-glucoside with $K_m$ values of 50 and 70 mM, respectively. The $K_m$ values for synthetic substrates were much higher. For example, for p-nitrophenyl β-glucoside, p-nitrophenyl β-galactoside, and 4-methylumbelliferyl β-glucoside, the $K_m$ values were 1.5, 2, and 0.17 mM, respectively. The well-known β-glucosidase inhibitor, glucono-δ-lactone showed a
50% inhibition at 1 mM. Mercapto groups were not thought to be involved in the enzyme mechanism, since \( p \)-chloromercuribenzoate (0.1 mM) was not an inhibitor. On the other hand, this enzyme was highly sensitive to \( \text{Hg}^{2+} \) (1 mM), while \( \text{Ag}^+ \) exerted moderate inhibition, and \( \text{Cu}^{2+} \) had no effect. No preincubation of enzyme with \( \text{Hg}^{2+} \) rendered the \( K_i \) of 9 mM, whereas preincubation reduced the \( K_i \) to 3 mM (Hösel and Barz, 1975).

The second putative isoflavone-glucoside-specific \( \beta \)-glucosidase was purified from soybean seeds. Owing to the bad and strong aftertaste of isoflavones, i.e. daidzein and genistein, but not isoflavone glucosides in soybean products, the \( \beta \)-glucosidase has to be controlled in food processing of soybean. Matsuura and Obata (1993) isolated three \( \beta \)-glucosidases from soybean flour, and among them \( \beta \)-glucosidase B and C had nearly all of the hydrolyzing action on daidzin and genistin (glucosides of daidzein and genistein). The molecular weight of \( \beta \)-glucosidase B was estimated to be 52 kDa, while the \( \beta \)-glucosidase C had a native molecular mass of 81 kDa, with monomer of 36 kDa. The pl of \( \beta \)-glucosidase C was 6.68, and the pH optimum was at 4.5, while the pH optimum of \( \beta \)-glucosidase B was at 5.5. Both enzymes showed maximum activity at 45°C. These enzymes all hydrolyzed daidzin and genistin at lower rates when compared to \( p \)-nitrophenyl \( \beta \)-glucoside. \( \beta \)-Glucosidase C also cleaved amygdalin, esculin and gentiobiose, and the relative rate of hydrolysis of the latter two substrates was even higher than the rate for daidzin and genistin. The \( K_m \) values for \( p \)-nitrophenyl \( \beta \)-glucoside, daidzin and genistin were 0.2 mM, 0.13 mM and 0.27 mM, respectively (Matsuura et al., 1995). Similar to the chickpea isoflavone glucoside-specific \( \beta \)-glucosidase, the two \( \beta \)-glucosidases were sensitive to glucono-\( \delta \)-lactone and \( \text{Hg}^{2+} \), and moderately sensitive to \( \text{Ag}^+ \). The \( K_i \) of glucono-\( \delta \)-lactone for \( \beta \)-glucosidase C was 0.5 mM. PCMB, iodoacetate, and other metal ions had no effect (Matsuura and Obata, 1993). Taken together, the \( \beta \)-glucosidases purified from imbibed soybean seeds seem not likely to be the isoflavone conjugate-specific enzymes.
A flavone-glucoside-cleaving β-glucosidase was purified to electrophoretic homogeneity from the ligulate florets of chamomile. The molecular weight of the native enzyme ranged between 500 kDa (gel filtration) and 334 kDa (native PAGE). Only one protein with Mr of 60 kDa was found on SDS-PAGE, suggesting multimeric forms of this enzyme in the native condition. The isoelectric point was determined by chromatofocussing at 4.6. This enzyme was not a glycoprotein. The optimal conditions for enzyme activity were found to be at 37°C and at pH 5.6. Kinetic studies uncovered that the flavone-glucoside β-glucosidase showed a significant specificity for flavone 7-O-glucosides including apigenin-, luteolin-, and naringenin-7-O-glucoside with a $K_m$ of 0.35, 0.56, and 0.64 mM, respectively. The glucose moiety on the other position, e.g. luteolin-5-O-glucoside, reduced the enzyme activity to one sixth of its original level. No flavonol-3-O-glucosides were used as the substrate by this enzyme. In addition, the specific enzyme did not hydrolyze apigenin-7-O-apiosylglucoside or apigenin-7-O-rhamnosylglucoside, but cleaved apigenin-7-O-acetylglucoside with low activity. The enzyme accepted $p$-nitrophenyl β-fucoside, $p$-nitrophenyl β-glucoside, $p$-nitrophenyl β-galactoside, and $o$-nitrophenyl β-glucoside as substrates with decreasing rates; however, the $K_m$ values for these substrates were 2.1 mM, 9.42 mM, 24.8 mM, and 0.23 mM, respectively.

In this study, the properties of the isoflavone conjugate specific β-glucosidase from soybean were characterized. The characteristics of this enzyme are also compared to those of other β-glucosidases, in particular with the isoflavone- and flavone-glucoside-hydrolyzing enzymes. The isoflavone-conjugate-specific β-glucosidase seems to form different degrees of aggregation in the native condition and hydrolyzes the isoflavone-7-O-glucoside-6"-O-malonate directly with the same or even greater speed as cleavage of the isoflavone-7-O-glucoside. Importantly, this enzyme was truly isoflavone specific, unlike the previously reported enzyme from soybean described above. The defensive role of this enzyme in soybean is discussed as well.
MATERIALS AND METHODS

Chemicals

All inorganic and organic chemicals used in inhibition studies were obtained from Sigma Chemical Co., except calcium chloride which was from Fisher Scientific. All chemicals tested for substrate specificity were purchased from Sigma Chemical Co., except rutin and isoquercitrin which were from Roth.

pH and Temperature Optima

The enzyme preparation used for determining the characteristics of the isoflavone-conjugate-specific β-glucosidase were obtained using DEAE- and CM-Sephadex column chromatography as described in the Chapter 2, or as otherwise noted.

To determine the pH optimum of the isoflavone-conjugate-specific β-glucosidase activity, the substrate, 4-methylumbelliferyl-β-glucoside, was prepared in various buffers varying in pH from 2.6 to 10.6. A 50 mM of stock solution of 4-methylumbelliferyl-β-glucoside was diluted with buffers to a final concentration of 2 mM. Buffer systems used in this study were 200 mM phosphate buffer, ranging from pH 5.8 to 8.0, 200 mM phosphate-100 mM citrate buffer, ranging from pH 2.6 to 7.0, and glycine-NaOH buffer, ranging from 8.6 to 10.6 (Stoll and Blanchard, 1990). Sixty µl of 2 mM substrate in different buffers was mixed with 5 µl of enzyme solution and incubated at 30°C for 30 min. The reaction was stopped by adding 65 µl of 1 M sodium carbonate and then diluted with 870 µl distilled water to a final volume of 1 ml. The solution was read using a spectrophotometer (Shimadzu, UV-160) at 360 nm, and product concentration was calculated according to Beer’s law where ε = 18.25 mM⁻¹ cm⁻¹. The temperature optimum of enzyme activity was resolved by incubating the enzyme solution with isoflavone
conjugates prepared as mentioned in the Chapter 1 over a temperature range of 0 to 86°C for 30 min. The enzyme activity was monitored by the HPLC method described earlier.

Effects of Metal Ions and Organic Chemicals on Enzyme Activity

The enzyme solution was prepared as follows. The dialyzed 40-60% ammonium sulfate fractionated protein solution was subjected to a native polyacrylamide gel electrophoresis with 7.5% resolving gel. After electrophoresis, the gel with isoflavone-conjugate-specific β-glucosidase activity was sliced and put in a dialysis bag filled with the running buffer. The enzyme was electroeluted by putting the dialysis bag in a horizontal gel electrophoresis system (BPL, Inc.) and electroelution conducted with low voltage for two hours at room temperature. Then the eluted protein was dialyzed against 100 mM phosphate-50 mM citrate buffer, pH 5.0, and concentrated by lyophilization (Dura-Dry™, FTS Systems). Glycerol was added to the protein solution to 20% final concentration. One mM final concentration of the following metal ions were tested for inhibition effects: AgNO₃, CaCl₂, CoCl₂, CuSO₄, HgCl₂, MgCl₂, MnCl₂, and ZnCl₂. In addition, organic chemicals (1 mM) and solvents (5%) were also examined including iodoacetate, glucono-δ-lactone, dimethyl sulfoxide (DMSO), phenylmethylsulfonyl fluoride (PMSF), p-chrolomercuribenzoate (PCNB), eserine, ethyleneglycol monomethylether (EGMME), and ethyleneglycol dimethylether (EGDME). The standard reaction mixture consisted of 5 μl of isoflavone conjugates, 5 μl of appropriate amount of enzyme and 5 μl of each chemical. Each reaction was incubated at 40°C for 30 min and stopped by adding 85 μl methanol. The relative enzyme activity was calculated from two replicates.

Effect of β-Mercaptoethanol on Enzyme Activity and Silver and Mercury Ion Inhibition

The reducing agent β-mercaptoethanol (2-ME) was added to the enzyme solution obtained from electroelution at 0 to 2000 mM final concentration. Five μl natural
substrate and 5 μl of various concentrations of 2-ME were mixed prior to adding another
5 μl enzyme solution. The incubation was at 40°C for 30 min, and stopped by adding 85
μl methanol. The data were plotted to yield the tolerance of the isoflavone-conjugate-
specific β-glucosidase to 2-ME. For restoration of activity after inhibition with silver
and mercury ions, 250 mM 2-ME was present in a sample solution consisting of 5 μl of
substrate and 5 μl of different concentrations of inhibitors before the enzyme was
introduced. The incubation condition was the same as mentioned above and methanol
was added to 100 μl final volume to stop the reaction.

**Enzyme Kinetics and Substrate Specificity**

The near homogeneous isoflavone-conjugate-specific β-glucosidase obtained
from column chromatography was used in kinetic studies. The kinetic studies employed
the Michaelis-Menten Equation to determine the $K_m$ and $V_{\text{max}}$ by the Lineweaver-Burk
reciprocal plot. The initial velocity of the reaction was determined from the linear
portions of the curves for each substrate. The substrates were used at 2 mM or as
otherwise mentioned. The standard assay by spectrophotometer used the appropriate
concentration of isoflavone-conjugate-specific β-glucosidase (10 μl) and substrate (90
μl) in 200 mM phosphate-100 mM citrate buffer, pH 5.0, and was incubated at 40°C for
$T_1$ and $T_2$ (where $T_2$>$T_1$, and both were in the linear range of the initial velocity). The
reaction was stopped by alkalinization of the assay mixture with an equal volume of 1 M
sodium carbonate, and the mix was then diluted to 1 ml with distilled water. The activity
was measured by determining the absorbance of the released aglycone. The velocity ($v$)
was calculated by the following formula, $v=[P]_2-[P]_1/T_2-T_1$, and the product
concentration was determined by Beer's law. The wavelengths and $e$ values (mM$^{-1}$cm$^{-1}$)
of each product are listed in Table 11. The poorly soluble substrates such as 4-
methyllumbellifery β-glucoside and p-nitrophenyl α-mannoside were dissolved in
ethyleneglycol monomethylether to make a 20 to 50 mM stock solution, and then diluted

137
to 2 mM with 200 mM phosphate-100 mM citrate buffer, pH 5.0, before use. The substrates tested by this spectrophotometric method were as follows, \( p \)-nitrophenyl \( \beta \)-glucoside, \( p \)-nitrophenyl \( \beta \)-galactoside, \( p \)-nitrophenyl \( \beta \)-mannoside, \( p \)-nitrophenyl \( \beta \)-arabinoside, \( p \)-nitrophenyl \( \alpha \)-glucoside, \( p \)-nitrophenyl \( \alpha \)-galactoside, \( p \)-nitrophenyl \( \alpha \)-mannoside, 4-methylumbellifery \( \beta \)-glucoside, and salicin.

<table>
<thead>
<tr>
<th>Products</th>
<th>Wavelengths (nm)</th>
<th>( \varepsilon ) values (mM(^{-1}) cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p )-nitrophenol</td>
<td>400</td>
<td>19.3</td>
</tr>
<tr>
<td>( o )-nitrophenol</td>
<td>420</td>
<td>4.55</td>
</tr>
<tr>
<td>4-methylumbelliferone</td>
<td>360</td>
<td>18.25</td>
</tr>
<tr>
<td>salicyl alcohol</td>
<td>295</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table 3.1: The wavelengths and \( \varepsilon \) values of the aglycones used in the study of substrate specificity.

The substrates esculin and arbutin were also assayed by the spectrophotometric method with modifications. Due to the lack of known \( \varepsilon \) values for these two glucosides, the following method was developed. The UV spectrum of 100 \( \mu \)l of esculin in 900 \( \mu \)l of phosphate citrate buffer showed a peak at 330 nm, while 100 \( \mu \)l of esculin in 900 \( \mu \)l of 100 mM sodium carbonate, the peak shifted from 330 to 374 nm. The activity assay was conducted as described above, and two different incubation times resulted in two different readings at 374 nm, but with a similar spectrum (Figure 3.1A). Here, the
Figure 3.1: The spectrum of esculin in alkaline condition shows a peak at 374 nm (A) and the standard curve of concentration of esculin versus corresponding absorbance at 374 nm (B). In enzyme assay solution, 90 µl of esculin incubated with 10 µl of enzyme solution for 15 (T1) and 30 (T2) minutes at 40°C, and then reaction was stopped by adding 100 µl of 1 M Na2CO3. The solution was diluted with 800 µl of distilled water and measured the absorbance at 374 nm.
longer incubation time had a lower reading than the shorter one, indicating that more
esculin was consumed by the enzyme. The difference of the two readings was converted
to concentration by a linear standard curve made from various concentration of esculin
(100 μl) in 100 μl Na₂CO₃ and 800 μl H₂O (Figure 3.1B). The enzyme activity against
arbutin was determined similarly at 267 nm. Activity against several natural substrates
were assayed by the HPLC method. Two to five mM of substrates were used except DZ1,
DZ2, GT1 and GT2 which were assayed at 0.2 mM, 0.18 mM, 0.79 mM, and 1.15 mM,
respectively. The isoflavone conjugates (DZ1, DZ2, GT1, and GT2) used in these kinetic
studies were purified by preparative HPLC (Spectroflow, Kratos) with non-acidic water
and acetonitrile as eluting solvents. One ml of partially purified isoflavone conjugates
(same preparation as used in the assay used in purification) was injected manually into
the HPLC, and the column washed with non-acidic water to base line absorbance. After
washing, the usual analytical linear gradient (described above) was started and every peak
was collected and analyzed by analytical HPLC described in Chapter 1. The standard
assay by HPLC was performed in a reaction mixture containing 10 μl of substrate and 5
μl of appropriate enzyme solution at 40°C or 30°C (for isoflavone conjugates only). The
reaction was stopped by adding 85 μl of methanol and the products subjected to HPLC.
The substrates assayed by the HPLC method included four isoflavone conjugates, two
flavonol glycosides (rutin and isoquercitrin), phloridzin (a chalcone glucoside), and
amygdalin (a cyanogenic diglucoside). Commercial almond emulsin β-glucosidase, a
classic non-specific β-glucosidase, was also used to determine the Kₘ and Vₘₐₓ for
some substrates for comparison.

**Hydrolysis of GT2 by the Isoflavone-Conjugate-Specific β-Glucosidase**

During the enzyme kinetic study, GT2 seemed to be hydrolyzed by isoflavone-
conjugate-specific β-glucosidase directly without observing any intermediate, GT1, in the
HPLC chromatogram, even at very short time incubation times (e.g. 1 min). If the
purified enzyme had both esterase and β-glucosidase activities, it is still possible that the enzyme could hydrolyze GT2 to GT with little or no accumulation of GT1. To rule out this possibility, a competitive β-glucosidase inhibitor, glucono-δ-lactone, was used to completely suppress the isoflavone-conjugate-specific β-glucosidase activity. If the enzyme had esterase activity, the reactant, GT2, would be cleaved and the product, GT1, would accumulate and be seen in the HPLC chromatogram. The inhibitory activity of glucono-δ-lactone was determined by incubating a variety of concentrations of glucono-δ-lactone at a range of 1 mM to 1 M, along with isoflavone conjugates and the enzyme solution. Methanol was added to stop the reaction and activity was measured by HPLC. The data showed that 100 mM of the inhibitor would stop about 99% enzyme activity. Therefore, 100 mM of glucono-δ-lactone was used for these studies.

**Electrophoresis Under Nondenaturing Conditions and Activity Staining**

Crude cell wall wash and chromatographic preparations were subjected to a 7.5% gel discontinuous PAGE under nondenaturing conditions according to the Laemmli procedure, but without SDS and β-mercaptoethanol in the sample buffer. Samples were electrophoresed in a ice bath to maintain the stability of the enzyme. For β-glucosidase activity staining, the gel was equilibrated in two changes of 100 mM phosphate-50 mM citrate buffer, pH 5.0, for 10 min each at 4°C, followed by incubation in 0.2 mM 4-methyl umbelliferyl β-glucoside (Hösel and Barz, 1975), or 1.3 mM 6-bromo-2-naphthyl β-glucoside-1.93 mM coupling dye (fast blue BB salt) (Esen, 1992), or 0.1% (wt/vol) esculin and 0.03% (w/v) ferric chloride (Kwon et al., 1994) dissolved in phosphate-citrate buffer, pH 5.0, for an hour. After washing the gel three times with distilled water, the gel incubated with 4-methyl umbelliferyl β-glucoside was examined under UV light for blue fluorescing bands of aglycone; the gel incubated with 6-bromo-2-naphthyl β-glucoside showed red bands and was stored in fixative (1 methanol: 1 acetic acid: 5 water) for 16-24 hours, and then air dried; and the gel incubated with esculin and ferric chloride showed black band with a transparent background. In additional studies, native PAGE
was performed as described, and after electrophoresis, the gel was sliced into 2 mm pieces, each of which was then put in a microfuge tube. Then 200 μl of 200 mM phosphate-100 mM citrate buffer, pH 5.0 was added to each tube for equilibration for about 30 min. The gel then was pulverized in the presence of 200μl of isoflavone conjugates and incubated for 2 hours at 40 C. The reaction was stopped by putting the tube in a boiling water bath for 1 min, and the activity was assayed by HPLC. The slice containing isoflavone-conjugate-specific β-glucosidase activity was further analyzed by SDS-PAGE. The protein in the active slice was either electroeluted and then subjected to SDS-PAGE or the entire slice was applied directly to SDS-PAGE with or without the presence of reducing agent. The predominant band treated from gels in which the sample was treated with SDS alone was in some experiments excised again and incubated with SDS and β-mercaptoethanol. This sample was re-subjected to SDS-PAGE. After electrophoresis, all gels were silver stained.
Results

**pH and Temperature Optima**

The optimum pH of the isoflavone-conjugate-specific \( \beta \)-glucosidase at 30\(^\circ\)C was approximately pH 5.8 in citrate-phosphate buffer and pH 6.0 in phosphate buffer (Figure 3.2A). The optimum temperature for isoflavone conjugate hydrolytic activity at pH 5.0 was found to be 30\(^\circ\)C (Figure 3.2B) with a shoulder at 35 and 40\(^\circ\)C.

**Effects of Metal Ions and Organic Chemicals on Enzyme Activity**

Inhibition experiments were carried out with the enzyme electroeluted from non-denaturing PAGE, using isoflavone conjugates as substrates. Only silver and mercury ions were found to be harmful to the enzyme activity at a 1 mM level. Other chemicals, including \( \text{Cu}^{2+} \), \( \text{Mg}^{2+} \), \( \text{Co}^{2+} \), \( \text{Mn}^{2+} \), \( \text{Ca}^{2+} \), and \( \text{Zn}^{2+} \) had no effect, while \( \text{Mn}^{2+} \) and \( \text{Zn}^{2+} \) had a moderate effect on enzyme activity at 10 mM level (Table 3.2). The organic chemicals did not inhibit the isoflavone-conjugate-specific \( \beta \)-glucosidase activity at 1 mM. Even a common \( \beta \)-glucosidase inhibitor, glucono-\( \delta \)-lactone was not inhibitory at 10 mM (Table 3.2). Iodoacetate showed moderate inhibition at 10 mM. However, PMSF was not inhibitory. Control studies showed that the inhibition activity of PMSF at 10 mM came from the solvent ethanol. Other common organic solvents such as DMSO, ethyleneglycol monomethylether (EGMME), and ethyleneglycol dimethylether (EGDME) were also not effective.
Figure 3.2: The optimal conditions for the isoflavone conjugate specific β-glucosidase from soybean. A. pH optimum, using three different buffer systems, including phosphate-citrate buffer ◆—◆, phosphate buffer ■—■, and glycine-NaOH buffer ▲—▲. B. temperature optimum.
<table>
<thead>
<tr>
<th>Inorganic Chemicals</th>
<th>Relative Activity* (%)</th>
<th>Organic Chemicals</th>
<th>Relative Activity* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNO₃ 1 mM</td>
<td>35</td>
<td>Iodoacetate 1 mM</td>
<td>97</td>
</tr>
<tr>
<td>10 mM</td>
<td>0</td>
<td>10 mM</td>
<td>74</td>
</tr>
<tr>
<td>HgCl₂ 1 mM</td>
<td>62</td>
<td>PCMB 0.1 mM</td>
<td>102</td>
</tr>
<tr>
<td>10 mM</td>
<td>23</td>
<td>1 mM</td>
<td>103</td>
</tr>
<tr>
<td>CaCl₂ 1 mM</td>
<td>99</td>
<td>Glucono-δ-lactone 10 mM</td>
<td>99</td>
</tr>
<tr>
<td>10 mM</td>
<td>94</td>
<td>control</td>
<td>97</td>
</tr>
<tr>
<td>MgCl₂ 1 mM</td>
<td>94</td>
<td>PMSF in EtOH 1 mM</td>
<td>89</td>
</tr>
<tr>
<td>10 mM</td>
<td>82</td>
<td>control</td>
<td>97</td>
</tr>
<tr>
<td>CoCl₂ 1 mM</td>
<td>97</td>
<td>10 mM</td>
<td>41</td>
</tr>
<tr>
<td>10 mM</td>
<td>102</td>
<td>control</td>
<td>37</td>
</tr>
<tr>
<td>MnCl₂ 1 mM</td>
<td>99</td>
<td>Eserine 1 mM</td>
<td>87</td>
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<tr>
<td>10 mM</td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuSO₄ 1 mM</td>
<td>99</td>
<td>DMSO 5%</td>
<td>117</td>
</tr>
<tr>
<td>10 mM</td>
<td>115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnSO₄ 1 mM</td>
<td>104</td>
<td>EGMME 5%</td>
<td>109</td>
</tr>
<tr>
<td>10 mM</td>
<td>77</td>
<td>EGDME 5%</td>
<td>120</td>
</tr>
</tbody>
</table>

* Values are means of two determinations in two independent experiments.

Chemical abbreviations: PCMB: p-chromomercuribenzoate; DMSO: dimethyl sulfoxide; PMSF: phenylmethylsulfonyl fluoride; EGMME: ethylene glycol monomethyl ether; EGDME: ethylene glycol dimethyl ether.

Table 3.2: The effect of inorganic and organic chemicals on the activity of the isoflavone-conjugate-specific β-glucosidase.
Effect of β-Mercaptoethanol on Enzyme Activity and Silver and Mercury Ion Inhibition

The presence of β-mercaptoethanol (2-ME) in the reaction solution containing enzyme and substrate did not affect the enzyme activity at concentrations as high as 500 mM (Figure 3.3A). One molar 2-ME suppressed about 40% enzyme activity, and 2 M of this reducing agent inhibited the enzyme entirely (Figure 3.3A). As described above, the isoflavone-conjugate-specific β-glucosidase was very sensitive to silver ions, which showed 100% inhibition at 1.25 mM (Figure 3.3B). However, this inhibition was reversible in the presence of the reducing agent, 2-ME, at 250 mM. When 2-ME was added to the substrate mixture at 250 mM final concentration, the isoflavone-conjugate-specific β-glucosidase could tolerate silver ion as high as 5 mM, and about 30% activity still remained even at 10 mM of silver ion (Figure 3.3B). A slight increase of activity was observed at higher concentrations of silver ion accompanied by 250 mM 2-ME when compared to the original enzyme preparation (Figure 3.3B).

Enzyme Kinetics and Substrate Specificity

The activity of the isoflavone-conjugate-specific β-glucosidase on a range of glycoside substrates is summarized in Table 3.3. The isoflavone-conjugate-specific β-glucosidase was able to hydrolyze a range of synthetic glycosides, including p-nitrophenyl β-glucoside, β-galactoside, α-mannoside, α-galactoside, o-nitrophenyl β-glucoside, and 4-methylumbelliferyl β-glucoside with relatively low However, this enzyme seems to have a relatively wide substrate range since it has relatively high affinity for 4-methylumbelliferyl β-glucoside and p-nitrophenyl α-galactoside at a 10^-4 M level, and moderate affinity for p-nitrophenyl β-glucoside and o-nitrophenyl β-glucoside with a $K_m$ around 1 mM. On the other hand, as indicated by the low $K_m$ and extremely high $V_{max}/K_m$ values, this enzyme is highly specific for its physiological substrates with a $K_m$ of about 0.1 mM for both DZ1 and DZ2, and 0.3 mM an 0.05 mM
Figure 3.3: The effect of 2-mercaptoethanol (2ME) on the activity of isoflavone-conjugate-specific β-glucosidase (A), and the protection by 250 mM of 2ME from the inhibition of various concentrations of silver nitrate (B). ■ the enzyme treated with silver nitrate alone; □ the enzyme treated with silver nitrate and 250 mM 2-mercaptoethanol.
<table>
<thead>
<tr>
<th>Chemicals</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>Specificity</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(M)</td>
<td>(μmol/min/mg protein)</td>
<td>($V_{max}/K_m$)</td>
<td>(M)</td>
<td>(μmol/min/mg protein)</td>
<td>($V_{max}/K_m$)</td>
</tr>
<tr>
<td>$p$-nitrophenyl $\beta$-glucoside</td>
<td>$1.3 \times 10^{-3}$</td>
<td>0.038</td>
<td>29.23</td>
<td>$2.2 \times 10^{-3}$</td>
<td>51.17</td>
<td>23259.09</td>
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<tr>
<td>$p$-nitrophenyl $\beta$-galactoside</td>
<td>$1.3 \times 10^{-2}$</td>
<td>0.337</td>
<td>25.92</td>
<td>$2.56 \times 10^{-3}$</td>
<td>3.04</td>
<td>1187.50</td>
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<tr>
<td>$p$-nitrophenyl $\beta$-mannoside</td>
<td>N.R.</td>
<td>-</td>
<td>-</td>
<td>N.R.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$p$-nitrophenyl $\alpha$-glucoside</td>
<td>N.R.</td>
<td>-</td>
<td>-</td>
<td>N.R.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$p$-nitrophenyl $\alpha$-galactoside</td>
<td>$8.4 \times 10^{-4}$</td>
<td>0.176</td>
<td>209.52</td>
<td>N.R.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$p$-nitrophenyl $\alpha$-mannoside</td>
<td>$9.9 \times 10^{-3}$</td>
<td>0.037</td>
<td>3.74</td>
<td>Yes$^3$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$o$-nitrophenyl $\beta$-glucoside</td>
<td>$1.4 \times 10^{-3}$</td>
<td>0.505</td>
<td>360.71</td>
<td>$3.9 \times 10^{-3}$</td>
<td>22.23</td>
<td>5700.00</td>
</tr>
<tr>
<td>4-methylumbelliferyl $\beta$-glucoside</td>
<td>$2.3 \times 10^{-4}$</td>
<td>0.019</td>
<td>82.61</td>
<td>$2.3 \times 10^{-3}$</td>
<td>20.92</td>
<td>8821.74</td>
</tr>
<tr>
<td>salicin</td>
<td>$3.5 \times 10^{-3}$</td>
<td>0.076</td>
<td>21.71</td>
<td>$1.1 \times 10^{-2}$</td>
<td>0.37</td>
<td>33.64</td>
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<tr>
<td>esculin</td>
<td>$8.8 \times 10^{-3}$</td>
<td>0.009</td>
<td>1.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>arbutin</td>
<td>$6.1 \times 10^{-3}$</td>
<td>0.054</td>
<td>8.85</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>genistein 7-O-glucoside (GT1)</td>
<td>$3.3 \times 10^{-4}$</td>
<td>1.608</td>
<td>4872.73</td>
<td>$1.1 \times 10^{-2}$</td>
<td>0.29</td>
<td>26.36</td>
</tr>
<tr>
<td>genistein 7-O-glucosyl 6&quot;-O-malonate (GT2)</td>
<td>$5.4 \times 10^{-5}$</td>
<td>1.584</td>
<td>29333.33</td>
<td>Yes$^3$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>daidzein 7-O-glucoside (DZ1)</td>
<td>$9.1 \times 10^{-5}$</td>
<td>1.11</td>
<td>12197.80</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>daidzein 7-O-glucosyl 6&quot;-O-malonate (DZ2)</td>
<td>$1.2 \times 10^{-4}$</td>
<td>1.57</td>
<td>13083.33</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>rutin</td>
<td>N.R.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>isoquercitrin</td>
<td>N.R.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>phloridzin</td>
<td>N.R.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>amygdalin</td>
<td>Yes$^3$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1. Values are means of determinations in three independent experiments. 2. N.R.: No Reaction at 40°C for 30 min.
3. The products were too little to measure or difficult to calculate $K_m$ and $V_{max}$.

Table 3.3 Substrate specificity of the isoflavone-conjugate-specific $\beta$-glucosidase from soybean cv. Williams roots.
for GT1 and GT2, respectively. Furthermore, this enzyme is specific for isoflavone conjugates, but not for flavonol glycosides (rutin and isoquercitrin), chalcone glucoside (phloridzin), and coumarin glucoside (esculin). Amygdalin, a cyanogenic diglucoside, was slightly hydrolyzed using very high concentrations of enzyme and it only yielded very low amounts of product during a longer incubation (60 min). In contrast, the commercial almond β-glucosidase has relatively higher affinity to synthetic substrates, particularly with the extremely high $V_{\text{max}}/K_m$ value for p-nitrophenyl β-glucoside, and low affinity to other substrates such as salicin and GT1. The commercial enzyme did not utilize GT2 as a substrate since only about 12 n mole/ml of product was produced after three hours incubation at 40°C. The values of $K_m$ and $V_{\text{max}}$ assayed at 30°C did not significantly differ from those at 40°C. For example, the $K_m$ and $V_{\text{max}}$ of DZ2 at 40°C are 0.12 mM and 1.57 μmol/min/mg protein, while at 30°C, they are 0.2 mM and 1.15 μmol/min/mg protein, respectively.

**The Hydrolysis of GT2 by the Isoflavone Conjugate Specific β-Glucosidase**

Most β-glucosidases cleave only terminal glucose residues and glucosides where there is no modification on the glucose moiety. To rule out the possibility that the enzyme is a complex composed of esterase and β-glucosidase, a β-glucosidase competitive inhibitor, glucono-δ-lactone, was used to stop the β-glucosidase activity completely. If the enzyme has esterase activity, GT1 should accumulate to a detectable amount, especially in the presence of excess substrate. As shown in Table 3.2, 1 mM of glucono-δ-lactone could not inhibit the β-glucosidase activity. Therefore, a series of concentrations of glucono-δ-lactone was tested to determine which concentration would suppress the enzyme activity completely. This inhibitor exerted 50% inhibition at 24 mM and 99% inhibition at 100 mM (Figure 3.4). Incubating the isoflavone-conjugate-specific β-glucosidase and GT2 along with 100 mM (final concentration) of glucono-δ-lactone for 30 min and 60 min at 30°C, there was no GT1 accumulation in the assay solution, indicating this enzyme most likely hydrolyzed GT2 directly.
Figure 3.4: The effect of glucono-δ-lactone on the activity of the isoflavone-conjugate-specific β-glucosidase from soybean. To inhibit 50% activity, 24 mM of inhibitor is required, and 100 mM inhibitor suppressed enzyme activity almost entirely.

Electrophoresis Under Non-denaturing Conditions and Activity Staining

The isoflavone-conjugate-specific β-glucosidase was found to be on the top of 7.5% gels in which the protein was stained as a fluorescent band by 4-methylumbelliferyl β-glucoside (Figure 3.5A) or as a red band by 6-bromo-2-naphthyl β-glucoside coupled with Fast Blue BB salt (Figure 3.5B). However, the gel could not be stained by esculin and ferric chloride. In addition, the first 2 mm from the top of gel had strongest isoflavone-conjugate-cleaving activity, and the second 2 mm had a minor activity. No further slices from the third to the twenty-third had detectable activity (Figure 3.5C). The partial purified protein solution was treated with SDS alone or SDS and β-mercaptoethanol and then subjected to SDS-PAGE. The protein patterns between with
and without reducing agent were different (Figure 3.6). When the predominant band in Lane A as indicated by arrow was excised and treated with β-mercaptoethanol before loading onto another SDS-PAGE, the original band was split into two identical subunits with half of molecular weight of the original one (Figure 3.6).

Figure 3.5: The isoflavone-conjugate-specific β-glucosidase activity staining of sliced gel electrophoresed under non-denaturing condition. Following native PAGE, the gel was equilibrated with with 200 mM phosphate-100 mM citrate buffer, pH 5.0, for 30 min. The gels then was stained with 4-umbelliferyl β-glucoside (A) and 6-bromo-2-naphthyl β-glucoside coupled with Fast Blue BB salt. Another native PAGE gel was sliced into 2 mm pieces, and equilibrated with the same buffer for 30 min at room temperature. Each segment was then pulverized in 200 µl of isoflavone conjugate substrate and incubated for 2 hours. The reaction was stopped by heating in a boiling water bath, and the activity was measured by HPLC.
Figure 3.6: The effect of β-mercaptoethanol on protein patterns. Partial purified protein solution from native PAGE treated with A) SDS and β-mercaptoethanol; B) SDS only; or C) β-mercaptoethanol only. The largest band as arrow indicated in lane A was excised and pulverized in sample solution containing β-mercaptoethanol for 30 min at room temperature. The protein solution was re-subjected onto SDS-PAGE and ran as mentioned in Materials and Methods (D). The protein band as indicated in lane D was about half of original size when the protein was treated with SDS only.
Glycosyl hydrolases (EC 3.2.1.x) are key enzymes of carbohydrate metabolism found in the three major kingdoms (archaeabacteria, euasbbacteria and eukaryotes). Among them, β-glucosidases (EC 3.2.1.21) are not only involved in cellulose and cellobiose catabolism, as part of the cellulase complex, but also involved in chemical defense against pathogens and herbivores, phytohormone regulation and organ development (see Chapter 1). Due to their ubiquitous presence and variety of functions, some similarities in characteristics among β-glucosidases are not surprising. For example, the pH optima for plant β-glucosidases, including the isoflavone-conjugate-specific β-glucosidase from soybean (Figure 3.2A), are mostly in the neutral to acidic range from 4.5 to 7.5, although some of them are basic proteins with an alkaline pi (Fan and Conn, 1985; Hösel et al., 1978; Simos and Georgatsos, 1988). In contrast, the optimum temperature varies from 30 to 60°C, although most of them are intracellular enzymes. In fact, most temperature optima were found to be 45-50°C, except the coniferin-specific β-glucosidases from lodgepole pine (Dharmawardhana et al., 1995) and chickpea (Hösel et al., 1978; Surholt and Hösel, 1980), and a strictosidine-specific β-glucosidase from Catharanthus roseus (Hamscheidt and Zenk, 1980) which has an optimum at 30°C. The optimum temperature of the isoflavone-conjugate-specific β-glucosidase is also at 30°C with a shoulder at 35 and 40°C (Figure 3.2B). This is quite different from the isoflavone glucoside specific β-glucosidase in chickpea whose optimum temperature is at 45°C. One interesting fact is that the temperature optimum of three extracellular β-glucosidases from a fungus, Schizophyllum commune, are around 70°C, while that of an intracellular enzyme from this organism is 35°C (Lo et al., 1988). The high temperature optima for these enzymes in plants implies that most of the β-glucosidases are either not very active under normal physiological conditions or that other physiological conditions of their environment in planta may influence their temperature optima.
Some enzyme activities are suppressed by metal ions which form stable complexes of low solubility with sulfhydryl groups. Not many cases report that β-glucosidases were influenced by naturally occurring metal ions, including copper, iron, zinc, cobalt, and molybdenum. In fact, only one report for a plant β-glucosidase found in the literature describes inhibition by naturally occurring metal ions. The activities of two β-glucosidases isolated from grape fruits were reduced to 16% and 23% by 10 mM Cu\(^{2+}\) and Ca\(^{2+}\), respectively. In addition, a β-glucosidase purified from a bacterium, *Ruminococcus albus*, was very sensitive to Zn\(^{2+}\) and Cu\(^{2+}\), but this enzyme was involved in cellulose degradation (Ohmiya and Shimizu, 1987). The isoflavone-conjugate-specific β-glucosidase was not influenced by any naturally occurring metal ions at low concentrations, but Zn\(^{2+}\) and Mn\(^{2+}\) diminished the enzyme activity about 23-26% at higher levels (10 mM) (Table 3.2). The mechanism by which divalent ions suppress enzyme activity may be due to the formation of stable chelation complexes with SH groups through a S-metal bond, or because of the catalytic function of these ions for promoting the oxidation of sulfhydryl groups in the active site to form disulfide bonds or other thiol compounds (Jocelyn, 1972; Scopes, 1987).

Apart from those ions mentioned, manganese, calcium, and magnesium ions bind to SH groups as well, but the binding is weak and the latter two ions do not form chelation complexes (Jocelyn, 1972). Inhibition by zinc and manganese ions suggests that sulfhydryl groups may be involved in the isoflavone specific glucosidase activity. Indeed, two other ions, Hg\(^{2+}\) and Ag\(^{+}\), demonstrate the importance of the SH group in the enzyme active site. These two heavy metals are well-known SH group blockers which form covalent bond(s) to form strong complexes with dissociation constants of \(10^{-40}\)-\(10^{-44}\), and \(10^{-20}\), respectively. These complexes are stable over the entire pH range (Jocelyn, 1972). A β-glucosidase purified from barley meal was inhibited completely by 1 mM Ag\(^{+}\), while HgCl\(_2\) and other divalent ions were ineffective at 1 mM (Simos and Georgatsos, 1988). Another β-glucosidase of unknown function from maize was repressed totally by silver and mercury ions at 32 μM and 256 μM, respectively (Esen, 1992). Similarly, the soybean isoflavone-conjugate-specific β-glucosidase was more sensitive to silver ions than to mercury ions at 1 or 10 mM levels (Table 3.2). In contrast, the two other β-glucosidases hydrolyzing
isoflavone glucosides from chickpea and soybean were more sensitive to mercury than to silver ion (Matsuura and Obata, 1993; Hösel and Barz, 1975). The SH groups are also a prerequisite for flavone 7-O-glucoside-specific \( \beta \)-glucosidase activity since the enzyme was inhibited strongly by \( \text{Ag}^+ \), \( \text{Pb}^{2+} \) or \( \text{Hg}^{2+} \), and \( \text{P}-\text{chloromercuribenzoate} \) (PCMB, see below) (Maier et al., 1993).

The inhibition by silver and mercury ions was reversed in the presence of \( \beta \)-mercaptoethanol (Figure 3.3B), suggesting that mercaptoethanol can protect the critical SH group affected by these ions. The protective role of \( \beta \)-mercaptoethanol from the inhibition by silver and mercury ions seems contradictory to the finding of rapid loss of enzyme activity when 5 mM \( \beta \)-mercaptoethanol was present in the buffer system during the purification steps at 4\(^\circ\)C. However, this loss of activity may be due to the quick formation of a disulfide bond between two \( \beta \)-mercaptoethanol molecules; the oxidized \( \beta \)-mercaptoethanol could then accelerate an inactivation process by forming a disulfide bond between \( \beta \)-mercaptoethanol and the SH group of the protein (Scopes, 1987). Interestingly, the isoflavone-conjugate-specific \( \beta \)-glucosidase was insensitive to \( \beta \)-mercaptoethanol until the concentration was as high as 1 M, and for complete enzyme activity loss, 2 M of this reducing agent was needed, suggesting that the disulfide bond linking the two subunits may not be involved in hydrolytic activity, and that the subunit is enzymatically active.

Some other organic substances reacting with sulphydryl groups were also used in this study. For instance, cellular thiols (R-SH) can be alkylated under physiological conditions by iodoacetate and iodoacetamide (R'-Halide) to give sulphides (R-S-R'), and \( \text{P}-\text{chloromercuribenzoate} \) (PCMB), one of univalent organic mercurials, can form reversible mercaptide complexes with free SH groups, therefore altering the function of sulphydryl groups. The experimental data revealed that neither iodoacetate nor PCMB could reduce the isoflavone-conjugate-specific \( \beta \)-glucosidase activity at 1 mM level (Table 3.2). The reason why these SH group modifiers did not influence enzyme activity while \( \text{Hg}^{2+} \) and \( \text{Ag}^+ \) did is probably due either to the different accessibility of the protein SH groups to these compounds or to their different redox potentials (Jocelyn, 1972). It is likely that accessibility is a factor here because only the smallest molecule, \( \text{Ag}^+ \), could inhibit enzyme
activity efficiently. As noted by Esen (1992), the presence of a chaotrophic agent, urea, which partially or completely unfolds proteins facilitated the alkylation of SH groups by treatment with iodoacetate or iodoacetamide, and then resulted in enzyme activity reduction, indicating that the sulfhydryl groups were buried inside the folded protein (Friedman, 1973).

The well-known β-glucosidase inhibitor, glucono-δ-lactone, was not effective except at higher concentrations. As shown in Fig. 20, the concentration for suppressing 50% enzyme activity was 24 mM, while the isoflavone-glucoside specific β-glucosidase in chickpea was very sensitive to this inhibitor with an IC50 of 1 mM (Hösel and Barz, 1975). The $K_i$ of gluconolactone for the non-specific β-glucosidases from soybean with hydrolytic ability towards isoflavone glucosides was 0.5 μM (Matsuura and Obata, 1993). This is additional evidence that our enzyme is distinct from these previously described ones. Although most β-glucosidases are sensitive to glucono-δ-lactone with a $K_i$ in the mM range or even lower, a few cases have been reported in which higher amounts of glucono-δ-lactone were needed to reduce the enzyme activity. The strictosidine-specific β-glucosidases isolated from Catharanthus roseus, for instance, required 10-100 mM to reach 50% inhibition (Hemscheidt and Zenk, 1980). The mechanism of inhibition by glucono-δ-lactone is not clear, but it may involve the lactone reacting with sulfhydryl groups to form a thioester similar to the reaction between β-proprionolactone and glutathione (Jocelyn, 1972). The relative insensitivity of our enzyme to this inhibitor could then be related to the particular potential or location of the sulfhydryl group in our protein as discussed above. Other β-glucosidase inhibitors including castanospermine, nojirimycin, 1-amino-β-glucoside, bromoconduritol, and conduritol β-epoxide, were not included in this study.

Two common esterase inhibitors, phenylmethylsulfonylfluoride (PMSF) and eserine (physostigmine), were used to see if the enzyme also had esterase activity. These inhibitors were not effective; the inhibitory effect of 10 mM PMSF came from the ethanol which was the solvent used to dissolve PMSF (Table 12). Although suggestive, this finding still does not rule out the possibility that the isoflavone-conjugate-specific β-glucosidase is not an enzyme complex composed of both esterase and β-glucosidase since these inhibitors have
different specificities to a certain type of esterases. For example, both eserine and PMSF are very potent irreversible inhibitors of acetylcholinesterase and butyrylcholinesterase (Rakonczay, 1986). Moreover, the isoflavone 7-O-glucoside-6'-malonate malonylesterase from chickpea was also insensitive to any esterase inhibitors including PMSF, eserine, neostigmine, paraoxon and diisopropyl fluoro-phosphate (DFP) (Hinderer et al., 1986). Several tested organic solvents used for dissolving substrates such as ethylene glycol monomethyl ether, ethylene glycol dimethyl ether, and dimethyl sulfoxide (DMSO) were not significantly effective either (Table 3.2).

Usually enzyme kinetics refers to steady-state kinetics where the substrate is much more excessive than the enzyme is, and the concentrations of free substrates and products thus change slowly. In the enzyme-catalyzed reaction, therefore, the enzyme is always bound with the substrate (ES), and the free enzyme molecules are negligible ([E] + [S] → [ES]). In the case of β-glucosidases, there is only one substrate involved in catalysis, and the rate of catalysis by the enzyme is proportional to substrate concentration at low levels and becomes independent at high levels.

The Michaelis constant, $K_m$, is defined as the sum of the rate constant for [ES] dissociation ($K_{-1}$) and the rate constant for product formation ($K_2$), divided by the rate constant for [ES] formation ($K_1$). The rate of catalysis, $v$, is expressed as a function of substrate and enzyme concentration by the well-known Michaelis-Menten equation: $v = \frac{[S]}{V_{max}} / K_m + [S]$. Enzyme kinetic data can be treated graphically by the widely used double-reciprocal Lineweaver-Burke plot where $1/v = (1 + K_m/[S])1/V_{max}$. When $[S] = K_m$, then $v = V_{max}/2$. Thus the $K_m$ is equal to the substrate concentration at which the reaction rate is half of its maximal value. If the concentration of substrate is much higher than the $K_m$, then the velocity is maximal and independent of substrate concentration. In addition, when the rate constant for dissociation from [ES] to [E] and [S] is much greater than the formation rate of product, the $K_m$ is equal to the dissociation constant of the ES complex. Therefore, a higher $K_m$ indicates weak affinity between the substrate and enzyme, while a lower $K_m$ represents strong binding. At very low concentrations of substrates, on the other hand, most of enzyme is in the free form. Consequently, the Michaelis-Menten equation becomes $v =$
\( V_{\text{max}} / K_m [S] \), and \( V_{\text{max}} / K_m \) is the most critical parameter in determining the specificity of an enzyme for a substrate (Stryer, 1986; Creighton, 1993). According to this criterion, the isoflavone-conjugate-specific \( \beta \)-glucosidase is highly specific to its physiological substrates, in particular with GT2, even though this enzyme does have a broad substrate spectrum (Table 3.3). In sharp contrast, the commercial almond \( \beta \)-glucosidase, a classic nonspecific enzyme, has high specificity for synthetic substrates such as \( p \)-nitrophenyl \( \beta \)-glucoside, 4-methylumbelliferyl \( \beta \)-glucoside, and \( o \)-nitrophenyl \( \beta \)-glucoside (Table 3.3).

In the substrate specificity study with our enzyme, the \( K_m \) values for some artificial substrates were low, indicating relatively high affinity to some of these substrates, especially for 4-methylumbelliferyl \( \beta \)-glucoside, which is structurally close to isoflavone-glucosides. However, higher affinity does not infer a more rapid cleaving rate in these cases. According to the definition, \( K_m = (k_1 + k_2)/k_1 \) and \( V_{\text{max}} = k_2 \times [E] \), when substrate specificity is defined by a low \( K_m \) of the enzyme, the \( k_2 \) is low. Consequently, the \( V_{\text{max}} \) cannot be very high since \( V_{\text{max}} \) is proportional to \( k_2 \). Due to this discrepancy, the substrate specificity is best expressed by \( V_{\text{max}} / K_m \) as described above (Selmar et al., 1987). Among the synthetic substrates, the isoflavone-conjugate-specific \( \beta \)-glucosidase shows the highest \( V_{\text{max}} / K_m \) for \( o \)-nitro-phenyl \( \beta \)-glucoside (Table 3.3). This finding is consistent with other \( \beta \)-glucosidases including prunasinase I, IIa, and IIb from blackberry (Kuroki and Poulton, 1987), linamarase from rubber tree (Selmar et al., 1987), coniferin-specific \( \beta \)-glucosidases from chickpea and soybean (Hösel et al., 1978; Hösel and Todenhagen, 1980), and a \( \beta \)-glucosidase from barley meal (Simos and Georatsos, 1988), which have higher relative reaction rates with this substrate when compared to other substrates. As noted earlier, \( \beta \)-glucosidases are usually able to hydrolyze other glycosides such as fucosides (Gus-Mayer et al., 1994) and galactosides (Hösel and Barz, 1975; Kuroki and Poulton, 1986; 1987). Indeed, the isoflavone-conjugate-specific \( \beta \)-glucosidase also uses \( p \)-nitrophenyl \( \beta \)-galactoside and more surprisingly the \( \alpha \)-galactoside as well. Not many plant \( \beta \)-glucosidases are able to hydrolyze glycosides with the \( \alpha \)-conformation, or only at very low reaction rates if they are able. For instance, linustatinase and linamarase, two cyanogenic \( \beta \)-glucosidases, hydrolyzed isomaltose and sucrose with about 1% and 2% relative activity when the
cleaving rate for the corresponding natural substrates was 100% (Fan and Conn, 1985), and a membrane-associated β-glucosidase had specific activities (μmol/min/mg protein) of 0.027 and 0.182 for p-nitrophenyl α-glucoside and arabinofuranoside, respectively (Feldwisch et al., 1994). In addition, a coniferin-specific β-glucosidase and a 4-methylumbelliferyl glucoside β-glucosidase were able to hydrolyze 4-methylumbelliferyl α-glucoside with 1% and 4% relative activity, respectively, when the corresponding enzyme activity for coniferin and 4-methylumbelliferyl β-glucoside was 100%, respectively (Dharmawardhana et al., 1995).

Usually β-glucosidases only remove a terminal glucose which is not covalently linked with other chemicals. The cyanogenic bis-glucosides, for instance, such as amygdalin, linustatin, and neolinustatin are hydrolyzed to remove the first terminal glucose by amygdalin hydrolases and linustatinase, and the resulting β-monoglucoseides are further hydrolyzed by prunasinases and linamarase. Little is known about the effect of esterification of the terminal sugar moiety on enzymatic activity. However, because the isoflavone 7-O-glucoside-6′-malonate malonylsterase and the isoflavone 7-O-glucoside β-glucosidase are distinct enzymes in chickpea, we originally assumed that a separate malonylsterase would also be present in soybean. However, using the isoflavone conjugate mixture during purification we would have detected the esterase if it were a separate enzyme. Such an enzyme would have led to increases in GT1 and DZ1, which was never observed in any extract or at any stage of purification.

The soybean isoflavone-conjugate-specific β-glucosidase has less specificity toward GT1 than GT2. Thus, one of the interesting properties of this particular enzyme is that it not only hydrolyzes the isoflavone glucosides, but also cleaves the glucose modified by a malonyl group at the 6′ position (isoflavone glucoside malonate) with similar V_{max} values. Thus, our enzyme may bypass the need for the esterase in some manner. Three possibilities exist: (1) the glucosidase can simply function directly on the malonylated substrate, (2) the two enzymes form an enzyme complex in which the product of the esterase, isoflavone glucoside, is channeled to the next enzyme, β-glucosidase, to release the free isoflavones, or (3) the monomeric enzyme subunit is a single polypeptide with
multifunctional domains. There are biological advantages for an enzyme complex in which two sequential steps of a pathway are carried out with substrate channeling, because the reaction can be carried out faster and more efficiently (Traut and Jones, 1977). Multiple catalytic domains would provide a similar advantage. However, possibility (2) seems unlikely since the subunits of the enzyme appear to be identical. Moreover, our studies employing the glucosidase inhibitor glucononolactone also suggest that possibilities (2) and (3) are probably not the case for the soybean isoflavone-conjugate-specific β-glucosidase, which may use an even more efficient way to release the aglycones by ignoring the presence of malonyl group on the glucose. Nonetheless, with our current studies it is not possible to absolutely preclude possibilities (2) or (3). Although the reaction in the presence of gluconolactone was carried out with high substrate concentrations, which should displace GT1 from the enzyme and allow its detection as an intermediate, it is still possible that GT1 is unusually tightly bound or even forms a covalent intermediate with the enzyme after cleavage of the malonyl ester. Alternatively GT1 may be inaccessible if substrate channeling were involved.

Thus, although we cannot preclude other mechanisms, it seems most likely that the enzyme is able to directly cleave the esterified glucoside. There is precedence for this type of activity. A flavone 7-O-glucoside-specific β-glucosidase from ligulate florets of Chamomilla recutita (Maier et al., 1993) not only uses apigenin 7-O-glucoside, narigenin 7-O-glucoside and luteolin 7-O-glucoside as substrates, but also can use apigenin 7-O-acetylglucoside as a substrate, although the $V_{\text{max}}$ is reduced dramatically. This is of particular interest since flavones are highly close to isoflavones and acetyl group is one carbon shorter than malonyl group. Ultimately, more detailed kinetic analyses and/or sequence data from the cloned enzymes and mutations in the active site will help resolve this issue with these enzymes.

Activity staining of electrophoresis gels under non-denaturing condition revealed only one β-glucosidase in soybean roots, which was located near the top of the 7.5% resolving gel (Figure 3.5), by treating with 4-methylumbelliferyl β-glucoside or by 6-bromo-2-naphthyl β-glucoside coupled with fast blue BB salt. Although our enzyme
hydrolysed the former substrate, the latter staining agent was not a good substrate for the soybean isoflavone-conjugate-specific β-glucosidase. Moreover, although the isoflavone-conjugate-specific β-glucosidase did utilize esculin as a substrate, the active staining based on esculin as substrate (Kwon et al., 1994) was failed, possibly because this enzyme was not sensitive enough to this substrate. Nonetheless, the enzyme activity found on the top of 7.5% acrylamide gel was confirmed to correspond to the isoflavone glucoside specific hydrolyzing activity by slicing the gel into 2 mm pieces and incubating the smashed gel with isoflavone conjugates. The first 2 mm slice had the highest activity and followed by the second 2 mm slice (Figure 3.5C), which might be due to diffusion that occurred during the gel electrophoresis. No activity was found in any other gel sections. The interested protein in SDS-PAGE as indicated in Figure 3.6 lane A was consisted of two identical subunits linked with disulfide bond, since the treatment of this protein with β-mercaptoethanol resulted in one band with half of original molecular weight. The isoflavone-conjugate-specific β-glucosidase may form aggregates. The formation of aggregates was also found in other β-glucosidas. The linamarase from flax appeared to be an αβ with molecular weight of α and β = 62.5 and 65 kDa, respectively, and more than one band in native gels were found (Fan and Conn, 1985). Similarly, with the Hevea linamarase cut from the alkaline PAGE gels and re-electrophoresed under native conditions, each protein again revealed all four bands. Native porosity gradient gel electrophoresis was also conducted to measure the native molecular weights of aggregates, and activity was stained by using 4-methylumbelliferyl β-glucoside. The results showed that the molecular weights of aggregates ranged from 1675 kDa to 69 kDa, and all aggregates were active (Selmar et al., 1987). High degrees of aggregation were also found in glucosidases from monocotyledonous plants. For instance, the avenacosidase consists of at least 300-350 kDa aggregate and other multimers based on these 300-350 kDa aggregates (Gus-Mayer et al., 1994). The molecular weight of a flavone-glucoside-specific β-glucosidase ranged from 500 kDa by gel filtration to 334 kDa by native PAGE, indicating that the enzyme was either a multimer or aggregate (Maier et al., 1993).
Although previous β-glucosidases were reported to hydrolyze isoflavone-glucosides in chickpea and soybean (Hösel and Barz, 1975; Matsuura and Obata, 1993; Matsuura et al., 1995), the currently purified soybean isoflavone-conjugate-specific β-glucosidase is a distinctive enzyme. First, this enzyme hydrolyzes isoflavone malonylglucosides. In chickpea, the isoflavone 7-O-glucoside-6"-malonate malonylesterase and the isoflavone 7-O-glucoside β-glucosidase are different enzymes, although the authors did not use isoflavone malonylglucosides as substrates to test the enzyme activity. Secondly, our isoflavone-conjugate-specific β-glucosidase is highly specific to isoflavone conjugates with substrate specificities of 168-fold (GT1) to 1000-fold (GT2) over p-nitrophenyl β-glucoside (Table 3.3). On the other hand, the β-glucosidase C isolated from soybean imbibed seeds had a relatively low reaction rate (38%) with isoflavone substrates compared to p-nitrophenyl β-glucoside (100%) (Matsuura et al., 1995). Therefore, the authors did not term this enzyme as an isoflavone-glucoside-specific enzyme, but just an enzyme could hydrolyze isoflavone glucosides during the process of soymilk to produce the objectionable aftertaste. Furthermore, according to our preliminary data, soybean cotyledons or imbibed seeds contain more non-specific β-glucosidases (Chapter 1). Thirdly, the characteristics of these various enzymes differ. The pH optimum for the isoflavone-conjugate-specific β-glucosidase from soybean root is around pH 6, while the enzyme in chickpea is pH 7-7.5, and the one in soybean seeds is pH 4.5. The temperature optima are different as well. The one we studied is at 30°C, whereas the other two are at 45°C. Although all three enzymes are inhibited by silver and mercury ions at 1 mM, the isoflavone-conjugate-specific β-glucosidase is more sensitive to silver than mercury ion, but the others show the reverse responses. In addition, our isoflavone-conjugate-specific β-glucosidase is much less sensitive to glucono-δ-lactone than the other two enzymes, requiring 24 mM to inhibit 50% activity. In contrast, 1 mM is enough to suppress 50% and 92% activity of isoflavone-glucoside-specific β-glucosidase in chickpea and the β-glucosidase C in soybean seeds, respectively. Taken together, we conclude that the β-glucosidase purified from soybean roots is a novel β-glucosidase with high specificity toward the isoflavone conjugates.
Due to the roles of the enzyme products and the characteristics of this enzyme, models (Figure 3.7) are proposed here to describe the involvement of the isoflavone-conjugate-specific β-glucosidase in the interactions between various microbes and soybean. In particular, a model is proposed for their role in the defense mechanisms against one of most devastating soybean pathogens, *Phytophthora sojae*. In soybean, the isoflavone-conjugate-specific β-glucosidase is highly specific for isoflavone conjugates and releases the free aglycones, daidzein and genistein. Both isoflavones serve as chemoattractants and activators of *Nod* genes of *Bradyrhizobium japonicum* (Figure 3.7A). In 7-day-old etiolated soybean seedlings, the malonylated isoflavone glucosides, DZ2 and GT2, are present at about 2000 and 3118 nmole/g fresh tissue, respectively, in cotyledons, while there are about 2000 nmole of DZ2 and only 800 nmole per gram fresh tissue of GT2 in root tips. On the other hand, around 400 nmole/g tissue of free daidzein and 180 nmole/g tissue of free genistein were found in the root tips. Free isoflavones were not abundant in other tissues, except about 250 nmole/g tissue of genistein in cotyledons (Graham, 1991b). The isoflavone-conjugate-specific β-glucosidase may constitutively hydrolyze the pre-formed conjugates and release the free isoflavones into the rhizosphere. Indeed, for instance, about 14 μM of free daidzein was recovered from soybean cv. Williams root tip exudates, suggesting the constitutive release of free isoflavones (Graham, 1991b). At a concentration of about 500 nM, the free isoflavones are able to induce a half maximum expression of *Nod* genes of *B. japonicum* (Kossak et al., 1987). In the rhizosphere, a concentration as low as 10 nM of isoflavones attracts *Phytophthora sojae* zoospores and stimulates the zoospore encystment followed by germination (Morris and Ward, 1992).

With respect to the localization of isoflavone conjugates in soybean, the isoflavone glucosides are found in the cytosol and the malonyl glucosyl isoflavones are in the apoplastic space and vacuoles (unpublished results, T. L. Graham), which is accordance with the finding in chickpea (Mackenbrock et al., 1992). A flavone conjugate, apigenin 7-O-(6-O-malonylglucoside) was also found in vacuoles (Matern et al., 1986). In addition to their occurrence in the apoplast, the malonylated isoflavone conjugates are also secreted into the rhizosphere (Graham, 1991b) and subsequently broken down to release free

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daidzein and genistein by enzymatic reaction. Thus, as shown in Figure 3.7B, when pathogens including some other opportunistic micro-organisms approach the soybean root or start to penetrate, they first encounter the constitutive presence of relatively high amounts of toxic substances, including genistein which is toxic to Phytophthora sojae (Rivera-Vargas et al., 1993), Rhizoctonia solani, Sclerotium rolfsii (Weidenbomer et al., 1990), Cercospora beticola and Monilinia fructicola (Johnson et al., 1976), and daidzein, which is toxic to Fusarium culmorum (Kramer et al., 1984). The localization of genistein, which is normally found in epidermal cells, also is consistent with an antibiotic role in nature (Graham and Graham, 1996a). During penetration or after colonization, the malonylated isoflavone glucosides in the apoplast are hydrolyzed to release additional genistein and daidzein (Graham et al., 1990). The malonylated conjugates in vacuoles may also be shipped out by exocytosis to further increase defense potential. Free daidzein may move back to the cytoplasm by an unknown mechanism to allow the synthesis of glyceollin. This pterocarpan phytoalexin is more toxic to a wider variety of pathogens, since it inhibits the plasma membrane and tonoplast H^+-transporting ATPase (Giannini et al., 1988), and NADH-ubiquinone-oxidoreductase (Boydston et al., 1983). Glyceollin has been demonstrated to be important in disease resistance and plays a secondary role in defense (see Chapter 1, introduction).

The co-existence of substrates and the isoflavone-conjugate-specific β-glucosidase in the apoplast raises a question that the malonylated isoflavone glucosides should be completely hydrolyzed instead of accumulating to levels as high as 2000 or 3000 nmole/ g tissue in soybean organs. There are a couple of possibilities which may explain this phenomenon. Owing to the prerequisite of an SH group for isoflavone-conjugate-specific β-glucosidase function, it seems possible that the enzyme is inactive under normal cellular conditions. The sulfhydryl groups of most extracellular proteins are oxidized due to the absence of glutathione. In wounded or infected tissues, glutathione, which is abundant (> 1mM) in the cytoplasm of leguminous plants (Klapheck, 1988), may activate the enzyme through the reduction of the disulfide bond under physiological conditions. Such a release of glutathione has also been suggested to activate and/or release the wound/HR related
competency factor, CF-1 from cell walls (see below, Dae-Sup Park and Terrence L. Graham, unpublished). Although in a very early experiment, the addition of sulphydryl reagents including glutathione, β-mercaptoethanol, and DTT, in extraction buffer did not improve the enzyme extraction, it is still possible that the intracellular glutathione released during enzyme extraction was sufficient to reduce the disulfide bond and to activate the enzyme activity thereafter. Another possibility is that the malonylated isoflavone conjugates are not accessible under natural conditions. In other words, the plausible recognition site of malonylated isoflavone glucosides, isoflavone backbone and part of glucose moiety, is sequestered by some reasons so that the enzyme can not recognize or bind the substrates. Therefore, no hydrolysis occurs. After pathogen invasion or elicitor challenge, the substrates are released from the compartment and hydrolyzed by the isoflavone-conjugate-specific β-glucosidase.

Both soybeans cultivars with and without Rps genes have constitutive isoflavone-conjugate-specific β-glucosidase activity. However, more rapid and higher amounts of glyceollin only accumulated in proximal cell populations of the incompatible combination, such as P. sojae race 1 and Williams 79 (Graham and Graham, 1991b). Interestingly, the soybean cultivar Williams, which has no known Rps genes, is also fully equipped with the ability to respond to elicitation and express the appropriate defense reactions, except that no hypersensitive response occurs upon the pathogen attack. There may be some regulatory factors lacking in the compatible reaction but existent in the incompatible reaction undergoing hypersensitive response. Competency factors may play this regulatory role in elicitation responses (Graham and Graham, 1994). The wound-associated competency factors, including CF-1, CF-2, and CF-3, have distinct biological functions. CF-1, the response factor, facilitates soybean cell response to much lower glucan concentrations and favors phenolic polymer deposition. CF-2, the gating factor, is responsible for glyceollin accumulation, and CF-3, the elicitor releasing factor, is likely to be the elicitor-releasing β-1,3-glucanase (Graham and Graham, 1994). The unique functions of CF-1 and CF-2 are of particular interest, since they are likely released during hypersensitive cell death under natural conditions. Recently, genistein was found to be strongly correlated with the
accumulation of glyceollin, suggesting a regulatory role of genistein in glyceollin synthesis (Figure 3.7C). In fact, genistein activates a specific peroxidase acting as an NADH oxidase on the membrane, and the latter enzyme may play a role in establishing cell competency in response to elicitation in proximal cell populations (Graham and Graham, 1996b). It is not known whether CF-2 also facilitates the movement of DZ back to cytoplasm (Figure 3.7B) for synthesizing glyceollin. Ultimately, nucleic acid and protein sequence data may allow us to evaluate the regulation of isoflavone-conjugate-specific β-glucosidase in planta and its role in defense mechanism in more detail.
Figure 3.7: Models for the isoflavone-conjugate-specific β-glucosidase functions in (A) soybean-microbe interactions, (B) the release of defensive compounds through enzymatic reaction, and (C) the regulation of competency factors in elicitor-treated soybean cells (from Graham and Graham, 1996b). In Model A, the enzyme products, genistein and daidzein, serve as chemoattractants of both *Bradyrhizobium japonicum* and *Phytophthora sojae* and nod gene activators of bradyrhizobia. In Model B, the involvement of the soybean isoflavone-conjugate-specific β-glucosidase in general defense mechanisms is illustrated. SH is the sulfhydryl group of the enzyme. In Model C, the possible regulatory role of the isoflavone-conjugate-specific β-glucosidase products in defensive responses is described. Genistein has been found to be able to activate the membrane-associated Type II NADH oxidase (NOX II), producing hydrogen peroxide, which in turn facilitates phenolic deposition. On the other hand, NOX II promotes extracellular alkalization, and then stimulates the CF-2 function for glyceollin accumulation. Symbol “+” indicates positive regulation or activation.
Figure 3.7: continued

B  RHIZOSPHERE

TOXIC TO
Phytophthora sojae
other fungi

GT

APOPLAST

β-GLUCOSIDASE

DZ2
GT2

DZ + GT

DZ1
GT1

Vacuole

GLYCEOLLINS

CYTOPLASM

DZ

C  HYPERSENSITIVE CELL DEATH

APOPLAST

COMPETENCY FACTORS

FERULATE ESTERS

GT2

FERULATE

GT

PEROXIDASE

PHENOLIC POLYMERS

Ca^{2+}

O_{2}

H_{2}O_{2}

Plasmalemma

NOXII

GLYCEOLLIN ACCUMULATION

H^{+}

PEROXIDASE ACCUMULATION

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