STRUCTURAL CHANGING OF ACETOHYDROXY ACID ISOMEROREDUCTASE FROM Escherichia coli AS THE EFFECT OF SLOW TIGHT BINDING INHIBITOR IDENTIFIED BY SITE DIRECTED MUTAGENESIS

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STRUCTURAL CHANGING OF ACETOHYDROXY ACID ISOMEROREDUCTASE FROM *ESCHERICHIA COLI* AS THE EFFECT OF SLOW TIGHT BINDING INHIBITOR IDENTIFIED BY SITE DIRECTED MUTAGENESIS

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ABSTRACT

Acetohydroxy acid isomeroreductase (AHIR) is the second enzyme common to the biosynthetic pathway for the branched chain amino acids. Acetohydroxy acid isomeroreductase involved in isomerization and reduction of α-acetolactate which is converted to α, β-dihydroxyisovalerate, or it converts α-aceto-α-hydroxybutyrate to α, β-dihydroxy β-methylvalerate.

The ilvC gene encoding AHIR was subcloned from plasmid DNA pRWB2. The gene and its associated promoter/operator were contained in a Bgl II-Hind III fragment and this fragment was conveniently ligated into pSP72 vector. In order to subclone the coding region of the ilvC gene into an expression vector like pET to get high level expression. The internal NcoI site of this gene was eliminated using a PCR based site directed mutagenesis procedure. The possibility to construct a plasmid that contains a variant of the ilvC gene suitable for subcloning into expression vectors that contain an Nco I site at an appropriate distance from the ribosome binding site has been examined. The ability to express acetohydroxy acid isomeroreductase in this format will allow a straightforward method for the PCR site directed mutagenesis followed by the production of large quantities of the enzyme to facilitate the search for sites in the enzyme that affect the rate of tight binding inhibition.
DEDICATION

I wish to dedicate this thesis to my parents Mr. Somchai and Mrs. Naree Jamonnak. There are no words that could possible express my appreciation, love, and gratitude for years of being understanding, patient, and loving. I love you both.
ACKNOWLEDGEMENTS

I am grateful to my advisor Dr. Kim Calvo for his advice, time, patience and guidance through out my research. I would also like to acknowledge my parents for their love, encouragement, firm support, and extreme understand. To my brothers, Suphanut and Sophanut, thank you for always making me laugh when I needed cheering up. To my cousin, Phiphat and Woraphon, thank you for having faith in me when I felt as though I could not make it. Last but not least I wish to say thank you to the rest of my friends and family for always being there for me.
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<tr>
<td>AHIR</td>
<td>Acetohydroxy acid isomerase reducetase</td>
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<tr>
<td>ALS</td>
<td>Acetolactate synthase</td>
</tr>
<tr>
<td>IpOHA</td>
<td>N-hydroxy-N-isopropylxaminate</td>
</tr>
<tr>
<td>HOE704</td>
<td>2-dimethylphosphinoyl-2-hydroxyacetic acid</td>
</tr>
<tr>
<td>HKIV</td>
<td>3-hydroxy-3methyl-2-oxobutyrate</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate, (oxidized form)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, (reduced form)</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide, (oxidized form)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, (reduced form)</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminoethane</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific broth</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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PEG……………… Polyethylene glycol
MHMOB…………. Methyl-2-hydroxy-2-methyl-3-oxobutyrlate
APS………………. Ammonium persulfate
Kb………………… Kilo base-pairs
Bp………………… Base-pairs
LB………………… Lauria broth
CHAPTER I
INTRODUCTION

Acetohydroxy acid isomeroreductase (AHIR, E.C 1.1.1.86) is the second enzyme in biosynthetic pathway of branched chain amino acids valine, leucine, and isoleucine (1). This enzyme raised strong interest for herbicides and fungicides developments because it is present only in plants and microorganisms but absent in animal (2). The catalysis of this enzyme required two metal ions to facilitate a two-step reaction. Substrate is converted via an alkyl migration follow by the NADPH dependent reduction so that an intermediate is formed during the reaction (3). The compound such as IpOHA and HOE 704 can act as competitive inhibitor of this enzyme to give an inactive complex (4). These compounds bind so tightly but only very slowly that can cause a delay of considerable time for inhibition (5), making this the main reason for any failure in using these compounds as the potential herbicide and fungicide. The better understanding of biochemical and structural characterization of AHIR is needed for developing the inhibition effect and also for designing the new potential inhibitor.

Using knowledge of enzyme catalyze reactions by binding substrates and converting them to products through several intermediate structures. Many studied have been performed in order to gain the better understanding of the conformational
intermediate structures formed during catalysis (6). In case of AHIR, there are five conserved regions that cluster around the active site (7). Using the relative rate of deuterium isotope exchange of peptide NH, these regions of plant enzyme were identified as the site responsible for binding of the inhibitor, NADPH, and two magnesium ions required for activity (8). The catalytic core of this enzyme exits in an open and close conformation. In the open conformation, the active site of enzyme separates enough that substrate or any of cofactor (NADPH or metal ions) molecules can enter and bind. When the active site is occupied, the domains move together into the close conformation. Thus changing this three dimensional structure is one of “induced fit” proposed by Koshland and Neet (9), and brings the NADPH, metal ions and substrate close enough together to induce the isomerization to occur by the assistance of the Mg\(^{2+}\) ion (Mg2). Reduction can be done by using hydrogen from NADPH again by the assistance of the Mg\(^{2+}\) (Mg1). After the reaction has been complete, the presence of the products causes the domain to rotate to an open position from which the product is released. This data may provide important information regarding the conformational changes that accompany tight-binding complex formation. By combining this structural and conformational data for the plant enzyme with the aligned sequences of the plant enzymes and the *E. coli* enzymes, the regions of the bacterial enzyme that have the greatest response to conformational flexibility on forming the Enzyme-metal-NADPH-inhibitor complex could be identified.

My studies have the goal of gaining a better understanding of the relationship between structure and reactivity of the *E. coli* acetohydroxy acid isomeroreductase. In order to provide a molecular basis for designing new potent herbicides targeting this enzyme. Various techniques of DNA cloning, sequencing, and site-directed
mutagenesis have been used with the aim of identifying the amino acid residue in this enzyme which are believed to be involve in controlling the rate of the very slow conformational change that leads to tight binding inhibition by IpOHA and HOE 704. Once the amino acid was targeted, we proposed to engineer a mutant protein, by site direct mutagenesis, which will affect the rate of inhibition induced by a slow-tight binding inhibitor.
CHAPTER II
LITERATURE REVIEW

Branched Chain Amino Acids Biosynthetic Pathway

The branched chain amino acids, valine, leucine and isoleucine, biosynthetic pathway are involved with three important enzymes, acetohydroxy acid syntase, EC 4.1.3.18, acetohydroxy acid isomeroreductase, EC 1.1.1.86 and dihydroxy acid dehydratase, EC 4.2.1.9 (10) (Figure 1). Acetohydroxy acid synthase catalyses condensation of pyruvate with an active acetaldehyde derived from a second molecule of pyruvate to yield α-acetolactate in valine biosynthesis pathway (11). On the other hand in isoleucine biosynthesis pathway, this enzyme is used in condensation reaction of pyruvate and α-ketobutyrate to yield α-aceto-α-hydroxybutyrate (12). α-ketobutyrate is derived from deamination reaction of threonine by catalyzing of the enzyme threonine deaminase which is encoded by the ilvA gene (13). The acetohydroxy acid from the previous step are converted via an alkyl migration (isomerization) followed by a NADPH dependent reduction to give α,β-dihydroxy isovalerate or α,β-dihydroxy-β-methylvalerate in valine and isoleucine biosynthesis respectively. These reactions are catalysed by the enzyme acetohydroxy acid isomeroreductase which is encoded by illvC gene (14). Dehydration of the dihydroxy acids yields α-ketoisovalerate and α-keto-β-methylvalerate which undergo transamination with glutamate as the amino acid donor to produce either valine or
Figure 1. Biosynthesis pathway of branched chain amino acids valine, leucine and isoleucine
isoleucine respectively (15). These reactions are catalyzed by dihydroxy acid dehydratase, which is encoded by the ilvD gene, and transaminase B, which is encoded by the gene ilvE. The valine biosynthesis intermediate, \( \alpha \)-ketoisovalerate, can act as the precursor of the leucine biosynthesis (16)

**Catalytic Properties of Isomeroreductase**

Acetohydroxy acid isomeroreductase catalyzes a two-step reaction which can be distinguished by their bivalent metal ion requirements (Figure 2). The substrates, either \( \alpha \)-acetolactate or \( \alpha \)-aceto-\( \alpha \)-hydroxybutyrate, are converted via an alkyl migration, followed by a NADPH dependent reduction (17). The products are either \( \alpha,\beta \)-dihydroxy-\( \beta \)-isovalerate or \( \alpha,\beta \)-dihydroxy-\( \beta \)-methylvalerate which can act as precursor of valine and leucine, or isoleucine, respectively (18). The alkyl migration catalyzed by this enzyme is unusual in several ways. First, the cobalamin cofactor does not require for this enzyme even though an alkyl migration found in this enzyme is a type found usually in cobalamin-dependent enzymes (19). Second, magnesium ions are absolutely required for an alkyl migration reaction and no other metal ion will substitute effectively (20) while magnesium, manganese (21) or cobalt ions (22) can be used efficiency for the reduction step. In the plant, AHIR exhibits the values correspond to the strongest affinity known between an enzyme and the metal ion. As reported in Dumas et. al. the \( K_d \) and \( K_m \) values of plant enzyme for magnesium ions are 5 \( \mu \)M and 6 \( \mu \)M, respectively (23). Third, initial velocity, steady-state kinetic, and product inhibition studies of the *Salmonella typhimurium* enzyme have shown that AHIR obeys an ordered mechanism in which NADPH bound first in the presence saturating of two bound metal ions, followed by the substrate or inhibitor binding.
Figure 2. Reaction catalyzed by Acetohydroxy acid reductoisomerase. The substrates, (2S)-acetoacetate or (2S)-2-aceto-2-hydroxybutyrate, are converted via an alkyl migration (isomerization) followed by a NADPH dependent reduction to give (2R)-2,3-dihydroxy-3-methylbutanoate or (2R,3R)-2,3-dihydroxy-3-methylpentanoate, respectively.
Figure 3. The ordered mechanism of Acetohydroxy acid reductoisomerase in which NADPH bound first in the presence saturating of metal ions, followed by the substrate or inhibitor binding. The dihydroxy acid product is released first, followed by NADP⁺.
The dihydroxy acid product is released first, followed by NADP+ (24) (Figure 3). Lately, studies on spinach \((Spinacia oleracea)\) (25) and \(Escherichia coli\) (E.coli) enzymes (26) also reported the same (27).

In our group, the catalytic reaction of acetohydroxy acid isomeroreductase has been studying for several years by using the purified \(E. coli\) enzyme. The steady-state and kinetic for all substrates, \(\alpha\)-keto-\(\beta\)-hydroxy carboxylate intermediate, and the reaction of the diol product were determined (28). The data are summarized in table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
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<tr>
<td>(k_{\text{f,AL}}^{\text{cat}})</td>
<td>21 s(^{-1})</td>
<td>(K_{i,NADP})</td>
<td>94 (\mu)M</td>
</tr>
<tr>
<td>(K_{\text{AL}})</td>
<td>36 (\mu)M</td>
<td>(k_{\text{f,HKIV}}^{\text{cat}})</td>
<td>37 s(^{-1})</td>
</tr>
<tr>
<td>(K_{i,\text{AL}})</td>
<td>100 (\mu)M</td>
<td>(K_{\text{HKIV}})</td>
<td>240 (\mu)M</td>
</tr>
<tr>
<td>(K_{\text{NADPH}})</td>
<td>8.4 (\mu)M</td>
<td>(K_{i,\text{HKIV}})</td>
<td>110 (\mu)M</td>
</tr>
<tr>
<td>(K_{i,NADPH})</td>
<td>9.7 (\mu)M</td>
<td>(K_{\text{NADPH}}^{\text{HKIV}})</td>
<td>14 (\mu)M</td>
</tr>
<tr>
<td>(k_{\text{r,diol}}^{\text{cat}})</td>
<td>0.51 s(^{-1})</td>
<td>(K_{i,NADPH}^{\text{HKIV}})</td>
<td>6.3 (\mu)M</td>
</tr>
<tr>
<td>(K_{\text{DIOL}})</td>
<td>6.5 (\mu)M</td>
<td>(D(V/K_{\text{AL}}))</td>
<td>1.07</td>
</tr>
<tr>
<td>(K_{i,\text{DIOL}})</td>
<td>9.4 (\mu)M</td>
<td>(D(V/K_{\text{HKIV}}^{\text{Mg}}))</td>
<td>1.39</td>
</tr>
<tr>
<td>(K_{\text{NADP}})</td>
<td>65 (\mu)M</td>
<td>(D(V/K_{\text{HKIV}}^{\text{Mn}}))</td>
<td>1.60</td>
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\[\begin{align*}
\text{AL} &= \text{HO} - \text{CO}_2^- \\
\text{DIOL} &= \text{HO} - \text{H} - \text{CO}_2^- \\
\text{HKIV} &= \text{HO} - \text{CO}_2^-
\end{align*}\]
According to the higher $K_m$ value of acetolactate as compared to the $K_m$ value of NADPH, the alkyl migration or some conformational change prior to alkyl migration but after NADPH binding is determined as rate limiting step in catalytic reaction of isomeroreductase. The study of deuterium kinetic isotope effects on V/K for each reactant and intermediate also reported the same with more clearly in the presence of manganese ion as compared to magnesium ions. The effects of acid-base on the catalysis of this enzyme were also identified (29) and the mechanism was shown in figure 4. Based on the low value of the V/K for the reaction ($V/K = 5.8 \times 10^5 \, M^{-1}s^{-1}$) and the pH variation of the KIE values, the enzyme seems to follow reverse protonation. From our estimated pKa values of 7.4 and 9.5 for the catalytic groups, base and acid in the catalysis reaction of this enzyme appeared to have pKa as 9.5 and 7.4 respectively. Thus only about 1% of the enzyme is in the correct ionization state at the pH optimum of 8.5. This suggests that the V/K for the correctly ionized enzyme is closer to $6 \times 10^7 \, M^{-1}s^{-1}$.

**Occurrence and Structure Description of Isomeroreductase**

Acetohydroxy acid isomeroreductase (AHIR; also known as Ketol-acid reductoisomerase, KARI) is a second enzyme involved in the branched chain amino acid biosynthetic pathway (30). This enzyme raised strong interest for herbicides and fungicides development because it is presents only in plants and microorganisms but absent in animal (31). The molecular weight of 220,000 of the *E. coli* enzyme was reported with the specific activity of 2 by Umberger et. al. (32). Using the same *E. coli* enzyme Chunduru reported the same molecular weight with a slightly higher specific activity of 2.5 (33). Purification of plant enzyme from the stoma of spinach
Figure 4. Proposed mechanism of reaction for acetohydroxy acid reductoisomerase.

R = CH$_2$CH$_3$ or CH$_3$
(Spinacia oleracea) (34), chloroplast (35), and from the barley (Hordeum vulgare) (36) also reported the same molecular weight. Recently the crystal structure of this plant enzyme complex with cofactor NADPH, magnesium ions and competitive inhibitor (IpOHA, Hoe704) has been reported. The native enzyme could not be crystallized (37). Using a crystallographic study at 1.65 Å resolution, Biou et. al. reported a tetramer structure of identical subunits of this plant enzyme (38). Previous work in the other laboratories also reported the same (39). Each of the four monomers are composed of two sequential domains; one is the N-terminal domain (residue 82-307) and the other is the C-terminal domain (residues 308-595) (40). The N-terminal domain is a mixture of α/β structure with a Rossmann fold that contains most of the NADPH binding site (Figure5). The C-terminal domain is the α-helices structured in a barrel with two functions. First, it contributes formation of the metal ion and substrate/inhibitor recognition sites by binding the substrate or inhibitor via two magnesium ions. This domain is also responsible for the entire dimer formation. The α-helices (A17 and A18) and a loop (residues 422-432) called the dimer loop, forming the interface that relies mostly on polar and hydrophobic interaction (Figure6) (41).

The active site of acetohydroxy acid isomeroreductase (Figure 7) sits at the interface between two domains of each monomer. This active site is deeply buried inside the protein core and only the adenine moiety of NADPH is on the surface (42). Inner the active site is composed of binding pocket region with a clear hydrophobic content of acidic and apolar protein residues where aliphatic carbons of the inhibitor and substrate fit (43). Two divalent cations (Mg1 and Mg2) have bipyramid coordinated with the carboxyl groups of acidic residues Glu/Asp, three oxygen atoms
Figure 5. N-terminal domain (residue 82-307) is built around a 10-stranded. It is part of the sheet being antiparallel with four strands, followed by sets of two and five parallel strands respectively. The connecting helices are above and below the sheet plane. The five outer strands (strands B5–B4–B6–B7–B8) form the dinucleotide diphosphate-binding site, with a βαβ motif (B4-A2-B5) corresponding to the canonical diphosphate-binding motif. (Adapted from reference 45)
Figure 6. C-terminal domain (residues 308-595) is composed of six-α-helices core like cable threads around each other, thus forming a bundle. Helices involved in formation of this domain are A11-12, A13-14-15, A17, A20, A21-22-23 and A25-26-27. The other helices in the domain are at the surface of the monomer, being either in contact with the other monomer or accessible to the solvent. (Adapted from reference 46)
Figure 7. Schematic representation of inhibitor and magnesium ions binding in the catalytic pocket of acetohydroxy acid isomeroreductase (Adapted from reference 47).
of the inhibitor (IpOHA), five oxygen atoms of water, and one oxygen atom of NADPH nicotinamide ribose (44). The inhibitor can be bound only in the present of cofactor. There is no direct interaction between NADPH and IpOHA. The only interaction between these two molecules is mediated by water molecules and metal ions in active site (48).

**Sequences and Conserved Regions**

The gene encoding acetohydroxy acid isomeroreductase (*ilvC* gene) from plants (49), fungi (50), and bacteria (51) have been recently examined. The results show some ordinary features as well as some important differences. There are only 34 amino acid residues conserved among the acetohydroxy acid isomeroreductase sequences from the difference sources. All of these are located in five conserved regions which are designated as region I-V, cluster closely around active site and play role to be the ligand binding residues (52). The function of these regions has been identified by site directed mutagenesis, exhibiting the typical GXGXX(G/A)XXX (G/A) motif involves in the NADPH binding of region I (53) and the two metal ions binding involved in region III and IV (54) while the region II and V play the important role in the structure stabilizing (55). For the region III and IV that involved with two Mg$^{2+}$ ions binding required two steps of the reaction. M1 was found to bind only on region III at Glu319, Asp315, whereas M2 bind both on region III at Asp315 and region IV at Glu 492 and Glu 496 residues via water molecules. These two Mg$^{2+}$ ions interact tightly with competitive inhibitor IpOHA (56).
Structural Changing of Acetohydroxy Acid Isomeroreductase Induced by the Ligand Binding

Halgand et. al. used a deuterium isotope exchange of peptide NH combined with ESI-MS to characterize the conformational changing of plant enzyme influence by the ligand binding (57). They reported that binding of only Mg\(^{2+}\) or NADPH result in the decreasing of the deuterium accessibility (decrease in mass) especially at the dimer loop residue 423-431. The significant mass lost was also detected in region I especially in the part of the \(\alpha/\beta\) Rossmann fold structure of A7-B7-A8-B8 as the effect of NADPH binding and also in the region IV as the effect of the Mg\(^{2+}\) binding. Thus, binding of each cofactor leading to the conformational modification in the specific region of the active site of this enzyme and also induces long range modifications in each of the N- and C-terminal domain. Binding of both Mg\(^{2+}\) and NADPH, lead to a synergistic effect compared to the effect of each cofactor alone. Mass decreases in some additional segment were found especially in region III. Decreasing in solvent access also found at \(\alpha\)-helix A5 and large decrease in mass still observed at dimer loop. This further mass decrease can be used to explain the ordered mechanism of ligand binding. The effect of both cofactor binding help to initiate the conformational change at the active site of the two domains of each monomer, leading to change in structure of the enzyme in which NADPH and Mg\(^{2+}\) bind to promote the conformational change that facilitate inhibitor or substrate binding. Binding of the competitive inhibitor such as IpOHA to the enzyme-Mg\(^{2+}\)-NADPH complex which is the real intermediate of the ligand binding process, further decrease in solvent accessibility within the active site and overall structure were observed. Clearly the conformational change in both active site and dimer interface were
detected as the effect of ligand binding, especially in the dimer loop which accounts for 25% of the dimer interface interactions also affected greatly by decreasing about 50% in mass in all case of the Mg2+ ions, or NADPH binding alone, together, or complexed with IpOHA.

Models for Slow Tight Binding Inhibition

The conformational flexibility highly effects the association of a slow, tight binding inhibitor with an enzyme. Depending on the binding model, the final tight binding state may require a conformational change and if the binding is slow, this conformational change must also be slow (half-life on the order of minutes). There are 2 basic kinetic schemes (Figure 8) to describe the slow tight binding inhibitor process (58). In scheme 1, there is a single, slow association (k₃) of the inhibitor with the enzyme. The dissociation rate of the inhibitor is fairly large (k₄), so that the inhibition constant is relatively small (Kᵢ = k₄/k₃ = 1 μM or less). The rate of association is small (k₃[I]) relative to k₁[S] and k₇ because there are multiple conformations of the enzyme, only one of which (in very low concentration) will bind the inhibitor. The second scheme is more general. In this case, the inhibitor forms an initial complex with the enzyme (EI) which then undergoes a thermodynamically favorable, slow conformational change to give the tight binding complex (EI*). In this case, k₅[EI] (rate for isomerization to EI*) and k₆[EI*] (rate for isomerization to EI) are both small relative to k₁[S], k₂, k₇, k₃[I], and k₄. In both of these models, if the [I] ≤ [E] we have slow, tight binding inhibition.
Figure 8. Basic kinetic models of the slow tight binding inhibitor process
Inhibitors of Acetohydroxy Acid Isomeroreductase

The compounds such as \(N\)-hydroxy-\(N\)-isopropylxamate (IpOHA) and 2-dimethylphosphinoyl-2-hydroxyacetic acid (Hoe704) (Figure 9) can act as the competitive inhibitors of plant (59) and bacteria (60) acetohydroxy acid isomeroreductase. These compounds exhibit herbicidal activity because they are the transition state analogue of both bacterial and plant AHIR enzyme with respect to the substrate (61). Surprisingly, although both IpOHA and Hoe704 bind extremely tightly to the enzyme these compounds show only very poor herbicidal action (62). As reported in the pioneer works on the \textit{E. coli} enzyme. Even the competitive inhibitor (IpOHA) binds so tightly to the enzyme with the \(K_i\) value of 22 pM, that they effectively never dissociate. The herbicidal effects of this inhibitor are only observed at an application rate of 2 kg/ha, leading to the fact that the formation of the inactive complex occurs only very slowly. The \(K_m\) value of \(5.9 \times 10^4 \text{M}^{-1}\text{S}^{-1}\) was also supported the slowly binding of this compound to the bacteria enzyme (63). Dumas \textit{et al.} also reported the similarly effect of this compound on the plant enzyme. IpOHA behaved as nearly irreversible inhibitor and performed such a very slow complex formation with the association rate of \(1.9 \times 10^3 \text{M}^{-1}\text{S}^{-1}\) to the spinach (\textit{S. Oleracea}) enzyme, exhibiting a time needed to achieve substantial inhibition (64). Parallel kinetic behavior of Hoe704 for the plant enzyme was also reported (65). Hoe704 exhibited similarly effect with higher herbicidal activity at \(K_i\) value of 0.82 \(\mu\text{M}\) and \(K_m\) value of \(2.2 \times 10^4 \text{M}^{-1}\text{S}^{-1}\). Since IpOHA and Hoe704 bind very slowly to the enzyme. The buildup in concentration of the substrate which can overcome the initial loose binding of the inhibitors to the enzyme was allowed, causing the main reason for any failure in using these compounds as the potential herbicide.
Figure 9. $N$-hydroxy-$N$-isopropylxamate (IpOHA) (A) and 2-dimethylphosphinoyl-2-hydroxyacetic acid (Hoe704) (B)
CHAPTER III
MATERIALS AND METHODS

Materials

Chemicals, Bacterial Strains and Plasmids

Ultra pure DNA grade agarose was purchased from Bio-Rad. Agar
granulated, sodium chloride, potassium phosphate dibasic anhydrous, and EDTA were
obtained from Fisher scientific. Acrylamide, ammonium persulfate, N-N methylene-
bis-acrylamide, ampicillin sodium salt, streptomycin sulfate, and TEMED were
obtained from Sigma-Aldrich. Bacto yeast extract was obtained from Difco
laboratories. Ammonium sulfate was purchased from Schwanz/Mann Biotech.
Sodium dodecyl sulfate (SDS) was purchased from ICN biomedical. Isopropyl-beta-
D-thiogalactopyranoside was purchased from Acros organics. Lambda DNA Hind III
digest used as the molecular weight marker was obtained from Sigma-Aldrich. NA-
45 DEAE membrane used in extraction of DNA from agarose gel was purchased from
Schleicher and Schuell. The restriction enzymes used for cloning were obtained from
Promega, Novagen, Sigma, and New England BioLabs. The QIAprep Spin Miniprep
Kit used for plasmid DNA isolation and purification was purchased from QIAGEN.
The BugBuster 10X for protein extraction was purchased from Novagen. Mutagenic
oligonucleotide primers used in site directed mutagenesis were obtained from
Retrogen. The QuikChange® II site-directed mutagenesis kit including
deoxynucleotide mix, Pfu Turbo® DNA polymerase, and DpnI restriction enzyme were purchased from the Stratagene. Platinum Taq high fidelity and PCR optimizer™ kit were obtained from Invitrogen. T4 DNA Ligase was obtained from New England BioLabs. The Dalton Mark VI for SDS gel electrophoresis and bacto-tryptone were purchased from Sigma-Aldrich. The bacterial strain which was a generous gift from Dr. Ron Wek of the University of California, Irvine was maintained as the frozen glycerol stocks at -80°C. The bacterial strains, plasmid vectors and their sources are listed in Table1.

Instrumentation

The nucleic acid concentrations were determined by using a Perkin-Elmer Lambda-3 double beam spectrophotometer. Bacterial cells were disrupted using a Branson Sonifier 250 (VWR Scientific). A Mini-Sub™ DNA electrophoresis cell from Bio-Rad was used for electrophoresis. A model Galaxy 14D microcentrifuge from VWR Scientific and model J-21C or J2-21 centrifuge from Beckmen were used for centrifugation. SDS-PAGE gel electrophoresis was done using the Mini-PROTEAN II electrophoresis cell from Bio-Rad. The enzymatic analyses were performed on the Olis modernized Cary spectrophotometer. The Polymerase Chain Reaction (PCR) process was performed on MJ Research thermal Mini-cyclers. Bacterial cultures were incubated with shaking by using Magni Whirl (Blue-M) shaker and Blue-M stable-therm dry type bacteriological incubator.
Table 2: Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/Plasmids</th>
<th>Phenotype/Characteristic</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRWB 2</td>
<td>A 4.4 Kb XhoI fragment, containing ilvA, ilvY and ilvC gene inserted into the SalI site of plasmid PUC8. The orientation of the insert is such that the lacZ gene is transcriptionally fused to the ilvA gene</td>
<td>Dr. Ron Wek</td>
</tr>
<tr>
<td>DH5α</td>
<td>F‘φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(ρ−,MK+)phoA supE44 thi-1 gyrA96 relA1 λ- end A1, recA1, gyrA96, thi, hsdR17, (ρ−,MK+), relA1, supE44, Δ(lac-proAB), [F’, traD36,proAB, lacIqZ ΔM15]</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>JM109</td>
<td>end A1, recA1, gyrA96, thi, hsdR17, (ρ−,MK+), relA1, supE44, Δ(lac-proAB), [F’, traD36,proAB, lacIqZ ΔM15]</td>
<td>Promega</td>
</tr>
<tr>
<td>pJL3</td>
<td>A 2360 bp DNA circular with high copy tac expression vector</td>
<td>Dr. Todd Porter</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Genotype; F’ ompT, hsdS8(ρB− mB+) dcM Tetr gal (DE3) endA Hte. This host has the advantage of being deficient in the lon and ompT proteases.</td>
<td>Novagen</td>
</tr>
</tbody>
</table>
### Table 2: Bacterial strains and plasmids (continued)

<table>
<thead>
<tr>
<th>Strain/Plasmids</th>
<th>Phenotype/Characteristic</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLR(DE3)</td>
<td>Genotype; F' ompT hsdSβ (rB mB) gal dcm Δ(srl-recA)306::Tn10(tefR)(DE3). This is a recA derivative of BL21 that improves plasmid monomer yields and may help stabilize target plasmids containing repetitive sequences.</td>
<td>Novagen</td>
</tr>
<tr>
<td>PSP72</td>
<td>This vector contains the SP6 and T7 RNA polymerase promoters flanking a unique multiple cloning region, which includes restriction sites for HindIII and BglII. It also contains the β-lactamase coding region which confers ampicillin resistance.</td>
<td>Promega</td>
</tr>
<tr>
<td>PET11-d</td>
<td>A 5.7 Kb expression vector containing an N-terminal T7 ● Tag sequence and Neol/HindIII cloning site. Target genes are initially cloned using host that do not contain the T7 RNA polymerase gene. Target protein expression is initiated by transferring the plasmid into an expression host, BL21(DE3), containing a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control.</td>
<td>Novagen</td>
</tr>
<tr>
<td>PET21-d</td>
<td>A 5.4 Kb expression vector containing an N-terminal T7 ● Tag sequence</td>
<td>Novagen</td>
</tr>
</tbody>
</table>
Methods

Growth of Bacterial Strain Containing the Plasmid DNA

The bacterial strains carrying the plasmid DNA pRWB2 was a generous gift from Dr. Ron Wek of the University of California, Irvine. This plasmid DNA containing the \textit{ilvA}, \textit{ilvY}, \textit{ilvC} gene was grown in Terrific Broth (TB) media prepared by adding 12 g tryptone, 24 g yeast Extract and 4 ml glycerol to 1 liter of distilled water. The solution was adjusted pH to 7.0 and sterilized by autoclaving. When the temperature dropped to 60°C or less, 100 μg/ml of ampicillin and 100 ml of a solution containing KH$_2$PO$_4$ (0.17 M), K$_2$HPO$_4$ (0.72 M) were filter-sterilized into the media. The TB media (10 ml) was inoculated with a frozen glycerol stock of bacteria with a sterilized pasteur pipette and incubated at 37°C with shaking overnight in a Magni Whirl (Blue-M) shaker water bath.

Isolation and Purification of Plasmid DNA

The media containing an overnight culture of bacteria was transferred into a 1.5 ml microcentrifuge tubes. The tubes were centrifuged at 14,000 rpm for 15 minutes at 0°C in a microcentrifuge. The supernatant was discarded leaving the bacterial pellet as dry as possible under vacuum. The pellet was resuspended in 100 μl of an ice cold solution containing glucose (50 mM), Tris.HCl (25 mM pH 8.0), EDTA (10 mM pH 8.0). A 200μl of freshly prepared solution containing NaOH (10 N) and SDS (20%) was added to this suspension. The tube was tightly capped and gently inverted 4 or 5 times to mix. The tube was place on ice for 2 minutes. A 150 μl ice cold solution (pH 4.8) containing 100 ml potassium acetate (5M), 11.5 ml glacial acetic and 28.5 ml water was added to this tube. The tube was vortexed for a
few seconds in an inverted position and stored on ice for 2 minutes. The tube was centrifuged at 14,000g for 15 minutes at 4°C in a microfuge. The supernatant was transferred to a fresh tube without disturbing the pellet. RNase A (boiled for 10 minutes to inactivate DNase) was added to a final concentration of 20 μg/ml. The solution was incubated at 37°C for 20 minutes. An equal volume of phenol:chloroform (v/v) saturated with TE buffer was added and the tube was vortexed for 1 minute and centrifuged at 14,000g for 3 minutes. The upper aqueous phase was transferred to a fresh tube and mixed with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). This mixture was vortexed for 1 minute and centrifuged again at 14,000g for 3 minutes. The upper aqueous phase was transferred to a fresh tube and 2.5 volumes of absolute ethanol were added. The solution was mixed by gently turning the tube upside down for 4-6 times and allowed it to sit at room temperature for 2 minutes. The solution was centrifuged at 14,000g for 10 minutes at 4°C in a microcentrifuge. The supernatant was discarded and the pellet was dried under vacuum. The dried pellet was resuspended in 25 to 50 μl of autoclaved water. The DNA sample obtained by this procedure was pure and could be used for subsequent restriction endonuclease digestion.

The QIAprep Miniprep System was also used as higher quality plasmid DNA was required for screening purposes. The media containing an overnight culture of bacteria was transferred into a 1.5 ml microcentrifuge tube. The tube was centrifuged at 14,000g for 15 minutes at 4°C in a microcentrifuge. The supernatant was discarded leaving the bacterial pellet as dry as possible under vacuum. The bacterial pellet was purified according to the procedure of QIAprep Spin Miniprep Kit using a microcentrifuge as an outline in the QIAprep Miniprep Handbook.
Determination of Nucleic Acid Concentration

A Perkin-Elmer Lambda-3 double beam spectrophotometer was used to determine the concentration of single and double stranded DNA. The spectrophotometer was calibrated with distilled water, and the optical density of the DNA solution was determined at 260 nm. The concentration of DNA (μg/ml) was calculated by the formula (absorbance at 260 nm X extinction coefficient of DNA X dilution factor). The extinction coefficient of double stranded DNA is 50 μg/ml/A_260 and that of single stranded DNA is 40 μg/ml/A_260. The purity of DNA was determined by taking the ratio of A_260/A_280. Values of this ratio ranging from 1.7 to 2.0 indicated pure DNA.

Restriction Endonuclease Digestion

For large scale digests 30-50 μg of DNA was digested by adding 30-50 units of the appropriate enzyme/enzymes, appropriate 10X buffer and sterile water so that the final concentration of the buffer was 1X. Reactions were performed in polypropylene microfuge tubes and were incubated in hot water bath at 37°C overnight. When long period of incubation were required, the tube were spun occasionally for 1-2 seconds at 12,000g to collect evaporated water that condensed on the tube cap. For elevated temperatures the reactions were performed under paraffin oil. When DNA was digested with more than one restriction enzyme it was important to maintain the required salt concentration or else the enzyme would exhibit star activity. The DNA fragments obtained from the restriction endonuclease digestion were separated by using electrophoresis of 1% agarose gel in TBE buffer containing 0.5 μg/ml ethidium bromide. The agarose gel was prepared as explained earlier.
Extraction of DNA from Agarose Gel

The DEAE cellulose membrane NA45 was used for binding and recovery of DNA from agarose gel. To increase the binding capacity, membrane was washed for 10 minutes in 10mM EDTA pH 7.6, for 5 minutes in 0.5 M NaOH, followed by several rapid washed in distilled water. Membranes could be stored in water at 4°C for a week for later use. After the electrophoretic separation of DNA in the mini-sub™ DNA electrophoresis cell, an incision of about 2mm wider than each side of the band was made directly in front of the band of interest. A strip of NA 45 was placed in an incision. Electrophoresis was continued until binding was completed as judged by the ethidium bromide fluorescence using long wave UV. The membrane was then washed several times in 5-10 ml of low ionic strength NET buffer (0.15 N NaCl, 0.1 mM EDTA, 20mM Tris pH 8.0) to remove the agarose residual. The wet strip of NA 45 membrane was placed in a 1.5 ml microfuge tube. A 200 μl volume of high ionic strength buffer (1.0 M NaCl, 0.1 mM EDTA, 20mM Tris pH 8.0) was added to the tube. The content was incubated at 65°C for 45-60 minutes to elute DNA from the membrane. The solution was transferred into a fresh microfuge tube, and the strip was rinsed with another 50 μl of high ionic strength buffer and incubated it again at 65°C for 45-60 minutes. The combination of 200 μl and 50 μl solution was extracted with 3 volumes of water-saturated n-butanol to remove the ethidium bromide residual. The tube was vortex for 1 minute to remove n-butanol (top layer). Then the ice cold 2.5 volumes of ethanol were added. After adding ethanol the solution was stored at -20°C overnight before centrifuging out the DNA. After centrifugation the DNA pellet was dried under vacuumed for 35-45 minutes to remove the ethanol. This pellet DNA was ready to use in the polymerization and ligation steps.
Ligation

To each tube containing DNA pellet of different kind of DNA template from agarose gel extraction 5 μl of autoclave water was added. After adding water the tubes were incubated at room temperature for 25 to 30 minutes. These tubes were combined and the solution was slowly pipetted up and down to mix. The tube containing these templates DNA of interested was kept on ice for 15 to 20 minutes and 2 μl of 10X synthesis buffer (0.4 mM each dNTP, 0.75 mM ATP, 17.5 mM Tris-HCl, pH 7.4, 3.75 mM MgCl₂ and 21.5 mM DTT), 7 μl of autoclave water were added to the tube. The tube was vortexed for a few second and was centrifuged at 14,000g for 1 minute at 4°C in a microfuge. The tube was kept on ice for 2 minutes and 400 units of T4 DNA ligase were added. The solution was slowly pipetted up and down to mix and was incubated overnight at the room temperature. After incubating this mixture was kept freezing at -20°C and then is used for subsequent transformation.

Gel Analysis of the Reaction Products by Agarose Gel Electrophoresis

Before transfecting the competent cells with the ligated DNA, 10 μl of the ligation mixture is run on a 1% agarose gel containing 0.5 μg/L ethidium bromide. The ethidium bromide binds to the covalently close circular (ccc) relaxed DNA (RF-IV DNA) and causes positive supercoils. Since this DNA binds less ethidium bromide than the negative supercoil (RF-I DNA) it often migrates faster than RF-I DNA, but the two forms migrate very close together. The DNA synthesis reaction results in the formation of RF-IV DNA, hence a band migrating slightly ahead of RF-I DNA indicated the formation of the RF-IV DNA.
Growth of Bacterial Strains

The bacterial strains carrying an F’ episome (e.g., JM109, DH5α) and bacterial strains lacking of the lon and ompT proteases (e.g., BL21[DE3], BLR[DE3]) maintained as frozen glycerol stocks were streaked out on glucose minimal medium agar plates and grown overnight at 37°C. The glucose minimal medium solution was prepared by adding 12.8 g Na₂HPO₄, 3.0 g KH₂PO₄, 0.5 g NaCl to 200 ml of deionized water. For preparing plates 30 ml of the glucose minimal medium solution was added to 2.3 g of bacto-agar in 118 ml water and autoclaved. The solutions of 0.3 ml of 1 M MgSO₄, 0.02 ml of 1 M CaCl₂, 0.08 ml of 0.1 M thiamine.HCl and 1.5 ml of 20% glucose were filtered-sterilized separately. When the agar-media solution was cooled down to 40°C, 1.0 g NH₄Cl and the above sterile filtered solutions were added and mixed by swirling. The media was poured into plates and allowed to solidify. Bacteria from frozen stock were streaked on these plates and incubated at 37°C for 24-30 hours. The plaques started appearing in 14-16 hours. Cells grown by this procedure can be used to make the competent cells and can be stored at 4°C for periods up to 1 week.

Preparation of Competent Cells

The autoclaved LB media 10 ml (0.1 g bacto-tryptone, 0.05 g bacto-yeast extract, 0.1 g NaCl, pH 7.0) was inoculated with a single well isolated colony picked from the glucose minimal agar plates. The culture was incubated at 37°C for 8-10 hours with shaking in a Magni Whirl (Blue-M) shaker water bath. It is important to grow these cells for 8-10 hours, as the cells tend to lose the F’ episome when they reach their stationary phase. Cells grown by this procedure can be used to make the
competent cells and can be stored as the glycerol stocks at -80°C for later used. To prepare the competent cells, 100 ml of LB media was inoculated with 1 ml of the 8-10 hours shaking culture. This inoculated media was incubated at 37°C with shaking at 210-230 rpm. Rates of growth were measured every 30 minutes at 600 nm by using Bausch & Lomb spectronic 20. When the OD$_{600}$ reached 0.3-0.35 the flask was placed on ice for 2 hours before cells were centrifuged down at 5,000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 5 ml of 1X washing buffer (2.5 ml of dilution buffer, 2.5 ml of 2X wash buffer). The suspension was slowly pipetted up and down to mix and was placed on ice for 40 minutes. The tube was centrifuged at 3,000 rpm for 10 minutes at 4°C and the supernatant was discarded. The pellet was resuspended in 5 ml of 1X competent buffer (2.5 ml of dilution buffer, 2.5 ml of 2X competent buffer) and was slowly pipetted up and down to mix. The cells are very delicate and hence should be handled with care. The 200 μl competent cells were placed into each 1.5 ml microcentrifuge tube and kept at -80°C for later use in transformation.

**Transformation**

The sterile 1.5 ml microcentrifuge tube was chilled on ice for 10-15 minutes. The frozen competent cells was brought out of -80°C refrigerator and placed on ice until it just thawed. The thawed competent cells were gently mixed by flicking the tube. The 100 μl of thawed competent cells was transferred to the chilled sterile microcentrifuge tube and 1-50 ng of DNA was added. The competent cells control DNA 1 μl (0.1 ng) per 100 μl of competent cells can be used for determining transformation efficiency. After adding the DNA the tube was quickly flicked several
times and immediately returned to ice for 10 minutes. The tube was removed from ice then heat shocked the cells for 45-50 seconds by immersing the tube in a water bath at exactly 42°C then the tube was immediately placed back on ice for 2 minutes. The cells are very delicate and hence should be handled with care and do not shake. After cells were chilled 900 µl of ice cold (4°C) SOC medium was added. The SOC media was prepared by adding 2.0 g bacto-tryptone, 0.5 g bacto-yeast extract, 1 ml of 1M NaCl, 0.25 ml of 1 M KCl to 97 ml of distilled water and autoclaved. The solution of 1 ml Mg²⁺ (1M of MgCl₂ ⋅ 6H₂O, 1M of MgSO₄ ⋅ 7H₂O), 1 ml of 2M glucose were filter-sterilized separately. When the autoclaved solution was cooled down to 40°C, the above sterile filtered solutions were added and mixed by swirling. After adding the SOC medium the tube was incubated with shaking at 37°C for 1 hour. The mixture was centrifuged at 3,000 g for 2 minutes in a microfuge. The top 700 µl of the supernatant was removed and the pellet was resuspended in the remaining 200 µl solution. The solution was plated on LB/ampicillin plates. After transfection of competent cells with the ligated DNA the plates were incubated upside down at 37°C for a maximum 10 hours. The plaques started appearing in 6-8 hours.

Subcloning of ilvC gene into pSP72 Vector

The plasmid pRWB2 containing the ilvC gene was obtained from Dr. Ron Wek and Dr. G.W. Hatfield of the University of California, Irvine. The ilvC fragment was excised from the pRWB2 vector using BglII and HindIII restriction enzymes. The resulting 1.946 Kbp fragment was subcloned into the BglII and HindIII sites of pSP72 vector as method discussed earlier. The ligation mixture was transformed into JM109 competent cells. The transformed cell suspension was plated on LB/ampicillin.
(100μg/ml) plates and incubated at 37°C overnight. The single colonies were grown overnight with shaking in 10 ml LB media containing 100 μg/ml ampicillin at 37°C. The resulting plasmid was isolated from the cultures using QIAGEN isolation method and was labeled as pSPNJ1. The clone was confirmed by determining the restriction enzymes pattern.

**Designing Oligonucleotide Primers for Mutagenesis**

Oligonucleotides were designed using the program Oligomutantmaker version (1.0). The oligonucleotides were designed to change one, two, or three bases, to either create or delete a unique restriction endonuclease site on the template DNA. The restriction site created/deleted was used to screen for the mutants. The oligonucleotides were checked to see if it might have any significant homology to any other region of DNA. Finally the oligonucleotides were selected to have a T<sub>m</sub> in the range 60 to 80°C. The T<sub>m</sub> was calculated by the formula T<sub>m</sub> = 4*(GC) + 2*(AT).

**Phosphorylation of the primer**

The oligonucleotide was resuspended in water at 125 pmol/μl. The mutagenic oligonucleotides were phosphorelated as described in Zoller & Smith. One μl oligonucleotide was incubated with 3 μl of 1 M Tris, pH 8.0, 1.5 μl of 0.2 M MgCl<sub>2</sub>, 1.5 μl of 0.1 M DTT, 13 μl of 1 mM ATP, pH7.0 and 10 μl of sterile water. To the tube, 5 units of T4 polynucleotide kinase were added. The contents of the tube were mixed and the tube was incubated at 37°C for 45 minutes. The reaction was stopped by heating the tube at 65°C for 10 minutes. The oligonucleotide was diluted to 6 pmol/μl with TE. The oligonucleotide was stored at -20°C until use.
Silent Mutation of the *ilvC* Gene

The plasmid pSPNJ1 carrying the *ilvC* gene was conducted under the site directed mutagenesis using oligodeoxynucleotide primer to produce the silent mutation in the *ilvC* gene. This oligodeoxynucleotide primer has one base change relative to the wild type *ilvC* gene sequence. The *ilvC* gene has two NcoI restriction sites; one is present at the initiation ATG of the gene and the other is present within the reading frame of the gene at nucleotide number 4142 (Appendix C). The silent mutation of *ilvC* gene was performed in order to remove the internal NcoI restriction site by replacing the ACC codon of threonine with ACT codon of the same amino acid. The parential methylated and hemimethylate of the silent mutant gene containing plasmids were digested with the DpnI. Then the none-methylated silent mutant gene containing plasmids were transformed into JM109 competent cell. The transformed cell suspension was plated on LB/amplcillin (100 μg/ml) plates and incubated at 37°C overnight. The single colonies were grown overnight with shaking in 10 ml LB media containing 100 μg/ml ampicillin at 37°C. The resulting plasmid was isolated from the cultures using QIAGEN isolation method and was labeled as pSPNJ2A. The clone was confirmed by determining the NcoI and HindIII restriction enzymes pattern.

Subcloning of Silent Mutant Gene into pET11d Expression vector

The silent mutant gene was cloned into the NcoI and HindIII site of pET11d expression vector and was used to make all the subsequent mutants. The wild type and silent mutant fragments were excised from the pSPNJ2A clones using NcoI and HindIII restriction enzymes by the method discussed earlier. The pET11d vector was
similarly digested and extracted form the agarose gel. The wild type and silent mutant genes were ligated with the resulting 5.35 Kbp fragment of pET11d vector then were transformed into JM109 competent cells. The transformed cell suspension was plated on LB/ampicillin (100μg/ml) plates and incubated at 37°C overnight. The single colonies were grown overnight with shaking in 10 ml LB media containing 100 μg/ml ampicillin at 37°C. The resulting plasmid was isolated from the cultures using QIAGEN isolation method. The wild type gene containing plasmid was labeled as pET11NJ1A. The silent mutation gene containing plasmid was named as pET11NJ2A and was used to make all the subsequent wild type and mutants DNA.

Generating of the Bacterial Strain Carrying the Wild Type and Mutant Expression Vectors

The silent mutation gene containing plasmids, pET11NJ2A was resuspended in 25 μl of autoclave distilled water and directly transformed to the BL21(DE3) bacterial strain as the method explained before. The transformed cell suspension was plated on LB/ampicillin (100μg/ml) plates and incubated at 37°C overnight. The single colonies were grown overnight with shaking in 10 ml LB media containing 100 μg/ml ampicillin at 37°C. The resulting plasmid was isolated from the cultures using QIAGEN isolation method. This plasmid was labeled as pET11NJ2AB and was designated as the wild type expression vector. The pET11NJ2AB plasmid was conducted under the site directed mutagenesis using oligonucleotide primer to produce the single mutants of \( ilvC \) gene before transformed to JM109 competent cells using the method discussed earlier. Later the QIAGEN isolation method was used to
isolate the resulting plasmid. This plasmid was designated as the mutant expression vector and was labeled as pET11NJ2B.

**Growth of the Bacterial Strain Carrying the Wild Type and Mutant Expression Vectors**

The LB media (10ml) containing 100 μg/ml of ampicillin was inoculated with a frozen glycerol stock of bacterial strain, BL21(DE3) carrying either the wild type or mutant plasmids. This solution was incubated at 37°C with shaking overnight in Magni Whirl (Blue-M) shaker water bath. This overnight culture was used to inoculate 250 ml of fresh LB broth containing 100 μg/ml of ampicillin. The culture was continued growing under the aeration at 37°C until the OD$_{600}$ reached 0.8-1.0, IPTG was added to a final concentration of 0.4 mM. The bacteria were allowed to grow for another 2-3 hours with aeration at 37°C and then centrifuged at 0°C for 30 minutes at 15,000 rpm. The supernatant was discarded and the pellet was purified and then analyzed using SDS/PAGE and protein estimation method.

**Purification of Mutant and Wild Type Reductoisomerases**

The bacterial pellet was resuspended in the ice cold of 25 mM Tris, pH 8.0 containing 4 mM MgCl$_2$, 3 mM β-mercaptoethanol, 0.5 mM EDTA and 20 μg/ml phenylmethanesulfonfyl fluoride. The pellet was carefully mixed with this buffer and then sonicated at 50% power for 2 minutes. This crude cell lysate was cooled for 2 minutes on ice and the sonication was repeated. After this is done for 45 minutes to an hour the cell lysate was centrifuged by using 5415 centrifuge from Brinkmann model JA-20 at 15,000 rpm for 30 minutes at 0°C. All subsequent steps were carried
out at 4°C. To the supernatant a 1/10\textsuperscript{th} volume of 5\% (w/v) streptomycin sulfate was added. The solution was sitting on ice for 5 minutes then stirred for 10 to 15 minutes. The solution was centrifuged at 15,000 RPM for 30 minutes at 0°C. The cell pellet was discarded and a 1/10\textsuperscript{th} volume of 2\% (w/v) cetyl trimethyl ammonium bromide was slowly added with stirring to the supernatant. The suspension was immediately centrifuged at 15,000 rmp for 30 minutes. Cetyl trimethyl ammonium bromide is insoluble in water at room temperature, hence it is necessary to warm it prior to use. After centrifuged the cell pellet was discarded and the supernatant was made 40\% saturating with ammonium sulfate (enzyme grade). The pH of mixture was adjusted to 8.0 by using 0.5 M NaOH. After the solution was sit at the room temperature for 45 minutes the solution was centrifuged at 15,000 rpm for 30 minutes to discard the cell pellet. The supernatant was then made 65\% saturated with ammonium sulfate under pH 8.0 and the solution was sit at room temperature for another 45 minutes. The solution was centrifuged at 15,000 rpm for an hour at 0°C then the cell pellet and supernatant was frozen at -20°C for subsequent protein analysis method.

**Preparation of Sodium Dodecyl Sulfate Polyacrylamide Gel**

Sodium dodecyl sulfate polyacrylamide gel was performed according to Laemmli buffer system using discontinuous polyacrylamide gel (66). This gel system consists of a separating (lower) gel and a stacking (upper) gel. The separating gel solution was made from 3.35 ml of distilled water, 2.5 ml of Tris-HCl (1.5 M, pH 8.8), 100\textmu l of 10\% (w/v) SDS, 50 \textmu l of 10\% (w/v) ammonium persulfate prepared fresh daily, 5\textmu l of 0.05\% TEMED, and 4.0 ml of 30\% acrylamide/bis. The 30\% of acrylamide/bis was prepared by using 29.2 g/100 ml of acrylamide and 0.8 g/100 ml
of N’N’-bis-methylene-acrylamide in 300 ml distilled water filter and store at 4°C in the dark. Acrylamide/bis solution was degas for 15 to 20 minutes before use and can be stored at 4°C up to 1 month. The separating gel solution was prepared by combining all of the above reagents except ammonium persulfate (APS) and TEMED then deaerated under vacuum for at least 15 minutes. After deaerate APS and TEMED were added to the solution. A comb was placed into the assembled gel sandwich. Then the mark was made on the glass plate 1 cm below the teeth of the comb. The separating gel solution was poured into the assembled gel sandwich using a glass pipet and bulb until the solution level reach the mark. After pouring the separating gel solution was overlay immediately with water-saturated isobutanol or t-amyl alcohol. The solution was allowed to sit at room temperature for 45 minutes to 1 hour. After 45 minutes the overlay solution was completely rinsed off with distilled water. The area above the separating gel was dried with filter paper then the stacking gel solution was poured to the assembled gel sandwich.

The stacking gel solution was prepared from 6.1 ml of distilled water, 2.5 ml of Tris-HCl (0.5 M, pH 6.8), 100 μl of 10% (w/v) SDS, 50 μl of 10% ammonium persulfate (prepared fresh daily), 5 μl of 0.1% TEMED, and 1.3 ml of 30% Acrylamide/bis (The 30% Acrylamide/bis stock solution from the separating gel solution preparation). All of the above reagents except APS and TEMED were combined then the solution was deaerated under vacuum at least 15 minutes. After deaerate APS and TEMED were added to the solution. A comb was place in the gel sandwich and tilt to make a slight ~10° angle to prevent air from being trapped under the comb teeth while the solution was poured. The solution was poured to the gel sandwich using the same method with separating gel solution. After all of the comb
teeth was covered by solution the gel was allowed to sit for 30 to 45 minutes to polymerize. Then the comb was slowly pulled straight up from the gel and the well was completely rinsed with distilled water. The gel is now ready to be attached to the inner cooling core, the sample loaded and the gels run.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS/PAGE)**

The protein sample (40 μg) was diluted 1:2 with sample buffer containing 4.0 ml distilled water, 1.0 ml Tris-HCl (0.5 M, pH 6.8), 0.8 ml glycerol, 1.6 ml of 10% (w/v) SDS, 0.4 ml β-mercaptoethanol, and 0.2 ml of 0.05% (w/v) bromophenol blue. The mixture was loaded into the wells. The Dalton Marker VI (20-50 μg) from Sigma was used as the molecular weight marker. Then the electrophoresis was carried out at 158 volts for 45 to 50 minutes in a Mini-PROTEAN II dual slab cell form Bio-Rad using diluted 1:4 of 5X running buffer with distilled water. 5X running buffer (pH 8.3) was prepared by adding 9 g Tris base, 43.2 g glycine, 3 g SDS into 600 ml distilled water. This solution can be stored at 4°C and was warmed to 37°C before use if precipitation occurs. The gel was fixed in 40% methanol, 10% acetic acid solution for 15 minutes with coumassie blue R250 (0.1% in the fixing solution). The gel was destained with the same fixing solution, transferred to Whatmann 3 MM filter paper and dried on a slab gel drier at 80°C for 15 to 30 minutes.

**Protein Estimation Method**

Proteins have an absorption spectrum in the ultraviolet region. If the extinction coefficient of the protein is known and the ultraviolet absorption at 280 nm is measured then the concentration of the protein can be estimated. However many
other compounds will absorb in the 280 nm region, especially nucleic acids, which have a maximum absorbance at 260 nm. Warbaug and Christan (67) developed a method of correcting for the presence of nucleic acid by measuring the ratio of the absorbance at 280 nm to that at 260 nm. This ratio is used to estimate the amount of protein present when nucleic acids are also present. Pure proteins have a ratio $(A_{280}/A_{260})$ greater than 1.7.

**Enzyme Assay**

The activity of reductoisomerase during purification from *E.coli* was monitored by following the change in absorbance at 340 nm of the Olis Modernized Cary UV-Visible Spectrophotometer. The assay mixture contained 1 M of acetolactate, 0.2 mM NADPH, 10 mM KH$_2$PO$_4$, tris-buffer (pH 8.0) and varying amounts of enzyme. Acetolactate (1 M) was prepared by adding 92 μl of 2.22 M NaOH into 25.85 μl of 7.7 M methyl-2-hydroxy-2-methyl-3-oxobutyrate (MHMOB). The initial yellow color of this 2 layers solution can be observed. The solution was vortex for 1-2 minutes to mix. The yellow color fades away in about 10 to 15 minutes after mixed. The mixture was allowed to sit at room temperature for 2 hours then 80.1 μl of 0.9 M Tris-HCl was added. Acetolactate can be stored at -20°C for later use. Tris-containing buffer (100 ml, pH 8.0) for the enzyme assay contained 25 mM Tris-HCl, 4 mM MgCl$_2$, 0.5 mM EDTA, and 3 mM β-mercaptoethanol.
Subcloning of *ilvC* Gene into pSP72 Vector

The *ilvC* fragment (Appendix A) was excised from the pRWB2 vector using BglII and HindIII restriction enzymes (Appendix C). The resulting 1.946 kb fragment was subcloned into the BglII and HindIII sites of pSP72 vector as method discussed earlier. The resulting 4.408 kb plasmid was labeled as pSPNJ1 and transformed into the *E.coli* strain JM109 using ampicillin selection. The restriction map of pRWB2 vector is shown in figure 10 and construction of plasmid pSPNJ1 is shown in figure 11. As the size of pSP72 vector is 2.462 kb and that of *ilvC* gene is 1.946 kb. The presence of the clone was confirmed by isolating the pSPNJ1 DNA and digesting it with the restriction enzymes BglII and HindIII. The digested of pSPNJ1 DNA yielded two bands of size, 2.322 kb and 1.900 kb respectively after electrophoresis in 1% agarose gel. The undigested of pSPNJ1 DNA yielded a single band at approximately 4.4 kb (figure 12). This pattern confirms that the isolated plasmid DNA pSPNJ1 had the *ilvC* gene inserted into pSP72 vector.

Silent Mutation of the *ilvC* Gene

The plasmid pSPNJ1 carrying the *ilvC* gene was subjected to site directed mutagenesis to produce the silent mutation in the *ilvC* gene. The *ilvC* gene has two
NcoI restriction sites; one is present at the initiation ATG of the gene and the other is present within the reading frame of the gene at nucleotide number 1124 (Appendix A). The oligodeoxynucleotide primer used in site directed mutagenesis has one base change relative to the wild type gene sequence. This primer destroyed the internal NcoI site by replacing the ACC codon of threonine with the ACT codon of the same amino acid. The resulting plasmid was labeled as pSPNJ2A (figure13). To confirm the presence of the desired mutation, the replicative forms of pSPNJ1 and pSPNJ2A were isolated and digested with NcoI and HindIII restriction enzymes. pSPNJ1 has two NcoI restriction sites in its sequence, and pSPNJ2A should have only one NcoI site. Digestion of pSPNJ1 with restriction enzyme NcoI and HindIII followed by 1% agarose gel electrophoresis yielded three bands at 2.583 kb, 1.125 kb and 0.664 kb respectively, while the digestion of pSPNJ2A with the same enzymes yielded two bands at 2.583 kb and 1.789 kb (figure14). It was important to electrophorese both these plasmids on the same agarose gel in order to accurately compare fragment sizes. This restriction pattern confirms that the internal NcoI site of ilvC gene has been eliminated in pSPNJ2A.

**Subcloning of the Silent Mutant ilvC Gene into pET11d Expression Vector**

As the attempt to obtain a gene cassette that allowed efficient subcloning and expression. We created the silent mutant ilvC gene containing an eliminated internal NcoI site. This gene cassette was subcloned into the NcoI and HindIII site of pET11d expression vector and was used to make all the subsequent mutants. Digestion of pSPNJ2A with NcoI and HindIII restriction enzymes followed by 1% agarose gel electrophoresis yielded two bands at 2.583 kb and 1.789 kb respectively.
Figure 10. Restriction endonuclease map of $ilvA$, $ilvY$ and $ilvC$ in the plasmid pRWB 2.
Figure 11. Construction of plasmid pSPNJ1 (4408 bp). The *ilvC* gene (1946 bp) was excited from the pRWB2 vector and subcloned into the BgIII and HindIII restriction sites of the vector pSP72 (2462 bp).
Figure 12. Restriction analysis of pSPNJ1. MW: Lambda Hind III marker (23130, 9414, 6557, 4341, 2322, 1900)

Picture A. Lane 1 to 5: pSPNJ1 digested with restriction enzymes Bgl II and Hind III.

Picture B. Lane 2 to 7: Undigested pSPNJ1
Using Quick change site direct mutagenesis by doing PCR to amplify a specific DNA sequence from a complex mixture of pSPNJ1 primer *NulF* 5’ GAA CTG GCG TTC GAA ACT ATG GTC GA 3’

dNTP mix and PfuUltra High-Fidelity DNA Polymerase

Wild type *ilvC* gene 5’ GAA ATG GCG TTC GAA ACC ATG GTC GAT 3’

Silent mutant *ilvC* gene 3’ CTT TAC CGC AAG CTT TGC TAC C 5’

Figure 13. Construction of plasmid pSPNJ2A containing Silent Mutation of the *ilvC* Gene.
Figure 14. Restriction analysis of pSPNJ2A compared with pSPNJ1.

Lane 7. Lambda Hind III marker (23130, 9414, 6557, 4341, 2322, 1900)

Lane 5 and 6. Digestion of pSPNJ1 with restriction enzyme NcoI and HindIII yielded three bands at 2.583 kb, 1.125 kb, and 0.664 kb respectively.

Lane 1 and 2. Digestion of pSPNJ2A with NcoI and HindIII yields two bands at 2.583 kb and 1.789 kb.

Lane 3. Undigested of pSPNJ1.

Lane 4. Undigested of pSPNJ2A.
The 1.789 kb fragment was extracted from the agarose gel by the method discussed earlier. The pET11d vector was similarly digested and extracted from the agarose gel. The 1.789 kb silent mutant gene fragment was ligated with the resulting 5.674 kb fragment of pET11d vector. The resulting 7.463 bp plasmid was labeled as pET11NJ2A and transformed into the *E.coli* strain JM109 using ampicillin selection. The construction of plasmid pET11NJ2A is shown in figure12 (Appendix E). As the size of pET11d vector is 5.674 kb and that of silent mutant *ilvC* gene is 1.789 kb. The presence of the clone was confirmed by isolating the pET11NJ2A DNA and digesting it with the restriction enzymes NcoI and HindIII. The digested of pET11NJ2A DNA yielded two bands of approximately size, 5.5 kb and 1.8 kb respectively after electrophoresis in 1% agarose gel. The undigested of pET11NJ2A DNA yielded a single band at approximately 7.5 kb. This pattern confirms that the isolated plasmid DNA pET11NJ2A had the silent mutant *ilvC* gene inserted into pET11d vector.
Figure 15. Construction of plasmid pET11NJ2A by subcloning the silent mutant gene into pET11d expression vector
CHAPTER V
DISCUSSION AND SUMMARY

The choice of target amino acids in my research were chosen based on the relationship between structure and reactivity of acetohydroxy acid isomeroreductase from various organisms. A variety of different computer based techniques including multiple sequence alignment, secondary structure prediction, and comparison with known protein motif were used and the isomeroreductase sequences were aligned by the CLUSTAL V program. In plant isomeroreductase enzyme, a position that was believed to be highly involved in a conformational changing of the enzyme as an effect of ligands binding is called the “dimer loop”. As reported in Halgand et. al. using a deuterium isotope exchange of peptide NH combined with ESI-MS to characterize the conformational changing of plant enzyme influence by the ligand binding. The dimer loop (amino acid residues 423-431) was greatly affected by the ligand binding resulting in decreasing of the deuterium accessibility. This position was accounts for 25% of the dimer interface interactions of the enzyme and also affected greatly by decreasing about 50% in mass in all case of the Mg2+ ions, or NADPH binding alone, together, or complexed with IpOHA. Using E. coli acetohydroxy acid isomeroreductase, the amino acid tryptophan residue 320 (Appendix A) which resemble to the position in the dimer loop of the plant enzyme
was chosen. This amino acid residue was planned to be replaced by a small amino acid glycine as the effort to control the rate of the very slow conformational change that leads to tight binding inhibition. As an endeavor to examine the chosen amino acid residue we constructed a plasmid that contains a variant of the ilvC gene that suitable for subcloning into expression vectors contain an Nco I site at an appropriate distance from the ribosome binding site. Using the PCR site directed mutagenesis followed by the production of large quantities of the enzyme, this method will facilitate the examination of targeted amino acid residue and also the search for sites in the enzyme that affect the rate of tight binding inhibition.

In doing so the subcloning of the ilvC gene from pRWB2 has been accomplished. The gene and its associated promoter/operator were contained in Bgl II-Hind III fragment. This fragment was conveniently ligated into pSP72 vector. The intervening gene sequence could be determined by sequencing using primers complementary to the SP6 promoter and the T7 promoter. The sequence confirmed the orientation of the gene and the presence of the ilvY promoter. This promoter is positively activated by the enzyme substrate, acetolactate. We were unable to obtain high level expression of AHIR when bacteria containing this plasmid were grown in the presence of acetolactate. In order to effect high level expression, we believed we had to clone the ilvC gene into an expression plasmid such as pET.

There are a number of pET vectors that contain a T7 promoter followed by a Shine Delgarno sequence and an ATG codon. This ATG codon is an initial start codon for proteins in E. coli. The ilvC gene has such a start codon as part of an Nco I restriction site. Unfortunately, there is an Nco I site internal to the ilvC gene. In order
to subclone the coding region of the *ilv*C gene into an expression vector like pET, we had to eliminate the internal Nco I site.

Using a PCR based site directed mutagenesis procedure, we can eliminated the internal Nco I site without leading to a change in the amino acid sequence of the enzyme. Careful restriction analysis and sequencing confirmed the fidelity of the change in nucleotide sequence. This gave us plasmid pSPNJ2A. Using a pET11d vector, plasmid pET11NJ2A confirmed that it was possible to subclone the *ilv*C gene into a pET vector. Using a straightforward method for the PCR site directed mutagenesis followed by the production of large quantities of the enzyme, this plasmid will facilitate the search for sites in the enzyme that affect the rate of tight binding inhibition.
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APPENDIX B

RESTRICTION SITE ANALYSIS OF ilvC GENE

Alu I (AGCT) :
Sites: 39, 48, 98, 717, 873, 1179, 1314, 1339, 1742
Fragment Sizes: 9, 25, 39, 50, 135, 156, 306, 403, 595, 619

Tha I (CGCG) :
Sites: 74, 94, 227, 229, 376, 1012, 1024, 1354, 1433, 1481, 1704, 2282
Fragment Sizes: 2, 12, 20, 48, 55, 74, 79, 133, 147, 223, 330, 578, 636

Hae III (GGCC) :
Sites: 71, 158, 961, 1551, 1614, 1756, 1991, 2191
Fragment Sizes: 63, 71, 87, 142, 146, 200, 235, 390

Hha I (GCGC) :
Sites: 32, 94, 139, 227, 229, 347, 376, 381, 459, 861, 1026, 1354, 1433,
1481, 1483
Fragment Sizes: 2, 5, 29, 32, 45, 48, 62, 78, 79, 88, 118, 165, 328, 402,
854

Hpa II (CCGG) :
Sites: 319, 535, 593, 838, 941, 1001, 1136, 1321, 1345, 1615, 1631,
1661, 1947
Fragment Sizes: 15, 16, 24, 30, 58, 60, 88, 135, 185, 216, 245, 270, 286,
319, 390

Mnl I (CCTC GAGG) :
Sites: 1501, 1508, 1979, 2002, 2089, 2138
Fragment Sizes: 7, 23, 49, 87, 199, 471, 1501

Mae II (ACGT) :
Sites: 498, 1229, 1455, 1901, 2262
Fragment Sizes: 75, 226, 361, 446, 498, 731

Mae I (TTAA) :
Sites: 260, 308, 1473, 1579, 1765, 2119, 2150, 2267, 2322
Fragment Sizes: 15, 31, 48, 55, 106, 117, 186, 260, 354, 1165

Nla III (CATG) :
Sites: 168, 564, 888, 1128, 1405, 1842, 1886, 2105, 2255, 2313
Fragment Sizes: 24, 44, 58, 150, 168, 219, 240, 277, 324, 396, 437

Rsa I (GTAC) :
Sites: 270, 353, 493, 512, 732, 1060, 1076, 1219, 1255, 1570, 1629, 1929,
2175
Fragment Sizes: 16, 19, 36, 59, 83, 140, 143, 162, 220, 246, 270, 300, 315,
328

Sau 3a (GATC) :
Sites: 284, 297, 425, 543, 735, 881, 1780, 1841, 1885, 2206
Fragment Sizes: 13, 44, 61, 118, 128, 131, 146, 192, 284, 321, 899

69
Taq I (TCGA) :
Sites: 182, 410, 710, 1064, 1118, 1130, 1654, 1774, 2160
Fragment Sizes: 12, 54, 120, 177, 182, 228, 300, 354, 386, 524

Alw I (GGATC GATCC) :
Sites: 279, 305, 420, 538, 730, 1775, 1880
Fragment Sizes: 26, 105, 115, 118, 192, 279, 457, 1045

Ava II (GGWCC) :
Sites: 822, 1966
Fragment Sizes: 371, 822, 1144

Bbv I (GCAGC GCTGC) :
Sites: 48, 339, 573, 1166, 1301, 1409, 1619, 1809, 1812
Fragment Sizes: 3, 48, 108, 135, 190, 210, 234, 291, 525, 593

Dde I (CTNAG) :
Sites: 1250, 1985
Fragment Sizes: 352, 735, 1250

Eco RII (CCWGG) :
Sites: 152, 232, 468, 577, 985, 1328, 1540, 1675, 2176
Fragment Sizes: 80, 109, 135, 152, 161, 212, 236, 343, 408, 501

Fnu 4H (GCNGC) :
Sites: 37, 72, 328, 377, 584, 587, 804, 1027, 1180, 1183, 1315, 1398, 1434, 1608, 1702, 1823, 1826, 1871
Fragment Sizes: 3, 3, 3, 39, 35, 36, 37, 45, 49, 83, 94, 121, 132, 153, 174, 207, 217, 223, 256, 466

Fok I (GGATG CATCC) :
Sites: 655, 2033
Fragment Sizes: 304, 655, 1378

Hga I (GACGC GCGTC) :
Sites: 218, 324, 525, 1201
Fragment Sizes: 106, 201, 218, 676, 1136

Hinf I (GANTC) :
Sites: 24, 172, 619, 759, 1132, 1150, 1165, 1392, 1500, 2198
Fragment Sizes: 15, 18, 24, 108, 139, 140, 148, 227, 373, 447, 698

Hph I (GGTGA TCACC) :
Sites: 315, 432, 523, 592, 774, 801, 1104, 1485, 1679, 1688
Fragment Sizes: 9, 27, 69, 91, 117, 182, 194, 303, 315, 381, 649

Mae III (GTNAC) :
Sites: 598, 1258
Fragment Sizes: 598, 660, 1079

MboII (GAAGA TCTTC) :
Sites: 289, 499, 736, 929, 1006, 1159, 1725, 1790, 1790
Fragment Sizes: 0, 65, 77, 153, 193, 210, 237, 289, 547, 566

Nci I (CCSGG) :
Sites: 535, 638, 1322, 1947
Fragment Sizes: 303, 390, 484, 535, 625

Ple I (GAGTC GACTC) :
Sites: 1158, 1494
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Fsp I (TGCGCA) :
Sites: 346
Fragment Sizes: 346, 1991

Hae I (WGGCCW) :
Sites: 1551
Fragment Sizes: 786, 1551

Hae II (RGCGCY) :
Sites: 382
Fragment Sizes: 382, 1955

Kpn I (GGTACC) :
Sites: 734
Fragment Sizes: 734, 1603

Nco I (CCATGG) :
Sites: 1124
Fragment Sizes: 1124, 1213

Nde I (CATATG) :
Sites: 908
Fragment Sizes: 908, 1429

Nla IV (GGNNCC) :
Sites: 474, 732, 890, 1623, 1665, 1757
Fragment Sizes: 42, 92, 158, 258, 474, 580, 733

Nru I (TCGCGA) :
Sites: 2282
Fragment Sizes: 55, 2282

Nsi I (ATGCAT) :
Sites: 2257, 2311
Fragment Sizes: 26, 54, 2257

Nsp I (RCATGY) :
Sites: 168, 2255, 2313
Fragment Sizes: 24, 58, 168, 2087

Pvu II (CAGCTG) :
Sites: 39, 48, 873
Fragment Sizes: 9, 39, 825, 1464

Sca I (AGTACT) :
Sites: 1060
Fragment Sizes: 1060, 1277

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APPENDIX C

NUCLEIC ACID SEQUENCE OF AND PROTEIN CODED BY pRWB2 GENE

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V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V V
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V      V         V         V         V         V
V      V         V         V         V         V
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2102
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2222
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V      V         V         V         V         V
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2882
V      V         V         V         V         V
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2942
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3002
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140

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180

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200

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V V V V V V

gagctgataccgctcgcagccgaacgaccgagcgcagcgagtcagtgagcgaggaag

7202

cggaaga

7209
APPENDIX D

RESTRICTION SITE ANALYSIS OF pRWB2 GENE

Aat II (GACGTC) :
Sites: 1656, 5276
Fragment Sizes: 3589, 3620

Acc I (GTMKAC) :
Sites: 731
Fragment Sizes: 7209

Afl III (ACRYGT) :
Sites: 1629, 7083
Fragment Sizes: 1755, 5454

Aha II (GRCGYC) :
Sites: 266, 656, 908, 1653, 3333, 4969, 5273, 5655
Fragment Sizes: 252, 304, 382, 390, 745, 1636, 1680, 1820

Apa LI (GTGCAC) :
Sites: 550, 5026, 5523, 6769
Fragment Sizes: 497, 990, 1246, 4476

Ase I (ATTAAT) :
Sites: 46, 105, 6020
Fragment Sizes: 59, 1235, 5915

Asu II (TTCGAA) :
Sites: 1211, 1691, 1814, 1841, 4135
Fragment Sizes: 27, 123, 480, 2294, 4285

Ava I (CYCGRG) :
Sites: 235
Fragment Sizes: 7209

Bam HI (GGATCC) :
Sites: 240
Fragment Sizes: 7209

Ban I (GGYRCC) :
Sites: 130, 415, 837, 1324, 1783, 2294, 3489, 3747, 3905, 4968, 6242
Fragment Sizes: 158, 258, 285, 422, 459, 487, 511, 1063, 1097, 1195, 1274

Bcl I (TGATCA) :
Sites: 623, 974
Fragment Sizes: 351, 6858

Bgl II (AGATCT) :
Sites: 2741
Fragment Sizes: 7209

Bsm I (GAATGC GCATTC) :
Sites: 2008
Fragment Sizes: 7209

82
Bsp HI (TCATGA) :
  Sites: 5250, 5355, 6363
  Fragment Sizes: 105, 1008, 6096

Bsp MI (ACCTGC GCAGGT) :
  Sites: 456, 4421, 4803
  Fragment Sizes: 382, 2862, 3965

Bsp MII (TCCGGA) :
  Sites: 408, 1413, 4361
  Fragment Sizes: 1005, 2948, 3256

Bss HII (GCGCGC) :
  Sites: 441, 443, 1221, 1244, 1712, 2802, 3242, 4496
  Fragment Sizes: 2, 23, 440, 468, 778, 1090, 1254, 3154

Cfr10 I (RCCGGY) :
  Sites: 447, 2108, 2267, 3609, 4017, 4631, 6110
  Fragment Sizes: 159, 488, 614, 1342, 1479, 1546, 1661

Cla I (ATCGAT) :
  Sites: 2175, 2621
  Fragment Sizes: 446, 6763

Dra I (TTTAAA) :
  Sites: 3278, 5617, 6309, 6328
  Fragment Sizes: 19, 692, 2339, 4159

Eae I (YGGCCR) :
  Sites: 35, 2280, 2810, 4815, 5802
  Fragment Sizes: 530, 987, 1442, 2005, 2245

Eco47 III (AGCGCT) :
  Sites: 1046, 1941
  Fragment Sizes: 895, 6314

Eco RI (GAATTC) :
  Sites: 230, 1693, 2958, 3096, 3948, 4700
  Fragment Sizes: 138, 752, 852, 1265, 1463, 2739

Eco RV (GATATC) :
  Sites: 3203, 3455, 4608
  Fragment Sizes: 252, 1153, 5804

Eco RV (GATATC) :
  Sites: 3203, 3455, 4608
  Fragment Sizes: 252, 1153, 5804

Eco RV (GATATC) :
  Sites: 3203, 3455, 4608
  Fragment Sizes: 252, 1153, 5804

Fsp I (TGCGCA) :
  Sites: 2041, 3363, 4949, 5972
  Fragment Sizes: 1023, 1322, 1586, 3278

Hae I (WGGCCW) :
  Sites: 1956, 4568, 6609, 7061, 7072
  Fragment Sizes: 11, 452, 2041, 2093, 2612

Hae II (RGCGCY) :
  Sites: 333, 889, 1048, 1255, 1943, 2758, 3399, 4972, 6843, 7213
  Fragment Sizes: 159, 207, 329, 370, 556, 641, 688, 815, 1573, 1871

Hgi AI (GWGCWC) :
  Sites: 408, 554, 5030, 5527, 5612, 6773
  Fragment Sizes: 85, 146, 497, 844, 1161, 4476

Hinc II (GTYRAC) :
  Sites: 732, 2302, 2887
  Fragment Sizes: 585, 1570, 5054
Hind III (AAGCTT) :
Sites: 4804
Fragment Sizes: 7209

Kpn I (GGTACC) :
Sites: 3751
Fragment Sizes: 7209

Nar I (GGCGCC) :
Sites: 4969
Fragment Sizes: 7209

Nco I (CCATGG) :
Sites: 1779, 3016, 4141
Fragment Sizes: 1125, 1237, 4847

Nde I (CATATG) :
Sites: 3925, 5021
Fragment Sizes: 1096, 6113

Nla IV (GGNNCC) :
Sites: 132, 242, 417, 839, 1235, 1326, 1785, 2272, 2296, 3491, 3749, 3907, 4640, 4682, 4774, 4970, 5308, 5898, 6109, 6150, 6244, 7016, 7055
Fragment Sizes: 24, 39, 41, 42, 91, 92, 94, 110, 158, 175, 196, 211, 258

Nsp I (RCATGY) :
Sites: 3185, 5170, 7087
Fragment Sizes: 1917, 1985, 3307

Pst I (CTGCAG) :
Sites: 4800
Fragment Sizes: 7209

Pvu I (CGATCG) :
Sites: 806, 4930, 5826
Fragment Sizes: 896, 2189, 4124

Pvu II (CAGCTG) :
Sites: 54, 3056, 3065, 3890, 4899
Fragment Sites: 9, 825, 1009, 2364, 3002

Sal I (GTCGAC) :
Sites: 730
Fragment Sizes: 7209

Sca I (AGTACT) :
Sites: 4077, 5714
Fragment Sizes: 1637, 5572

Sdu I (GDGCHC) :
Sites: 408, 554, 5030, 5527, 5612, 6773
Fragment Sizes: 85, 146, 497, 844, 1161, 4476

Sma I (CCCCGGG) :
Sites: 237
Fragment Sizes: 7209

SnaB I (TACGTA) :
Sites: 1745
Fragment Sizes: 7209

Ssp I (AATATT) :
Sites: 424, 5390
Fragment Sizes: 2243, 4966
Sst II (CCGCGG) :
Sites: 1539
Fragment Sizes: 7209

Stu I (AGGCCT) :
Sites: 1956, 4568
Fragment Sizes: 2612, 4597

Sty I (CCWWGG) :
Sites: 1779, 3016, 4141
Fragment Sites: 1125, 1237, 4847

Xho II (RGATCY) :
Sites: 240, 2441, 2741, 3314, 3442, 5548, 5565, 6333, 6345, 6431, 6442
Fragment Sizes: 11, 12, 17, 86, 128, 300, 573, 768, 1007, 2106, 2201

Bst EII (GGTNACC) :
Sites: 640, 1404, 1467, 2569, 3615
Fragment Sizes: 63, 764, 1046, 1102, 4234

Dra II (RGGNCCY) :
Sites: 690, 3173, 5215
Fragment Sizes: 2042, 2483, 2684

AlwN I (CAGNNNCTG) :
Sites: 2733, 2757, 3056, 3164, 3176, 3377, 6674
Fragment Sizes: 12, 24, 108, 201, 299, 3268, 3297

Dra III (CACNNNGTG) :
Sites: 621
Fragment Sizes: 7209

Tth 111 I (GACNNNGTC) :
Sites: 1971
Fragment Sizes: 7209

Xmn I (GAANNNNTTC) :
Sites: 2846, 4408, 5595
Fragment Sizes: 1187, 1562, 4460

Bgl I (GCCNNNNNGGC) :
Sites: 320, 586, 2818, 3600, 4733, 4959, 6077
Fragment Sizes: 226, 266, 782, 1118, 1133, 1452, 2232

PflM I (CCANNNNNTGG) :
Sites: 858, 3056, 3595
Fragment Sizes: 539, 2198, 4472

Bst XI (CCANNNNNNTGG) :
Sites: 1453
Fragment Sizes: 7209
APPENDIX E

NUCLEIC ACID SEQUENCE OF AND PROTEIN CODED BY pET11NJ2A GENE

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V V V V V V V ttcttgagagcagaaagggcctcgtgatatgcctattt 36
V V V V V V V tttataggtaatgctcatgataaataatggtttcttagacgtcaggtggcacttttcggtggg 96
V V V V V V V aaatgtggcgggaaccctctatgttatattaattttctaaacatttcatcatagttatgatctcgcct 156
V V V V V V V catgagacaataaccctgataaatgctcataataatattgaaaaaggaagatagataatgat 216
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APPENDIX F

RESTRICTION SITE ANALYSIS OF pET11NJ2A GENE

**Alu I (AGCT):**
Sites: 643, 706, 806, 1327, 1584, 1630, 1720, 1946, 2229, 2248, 2297, 2308, 2365, 3273, 3676, 3735, 3799, 3892, 4007, 4431, 4711, 5355, 5364, 5414, 6033, 6189, 6595, 6630, 6655, 7085, 7105, 7121
Fragment Sizes: 9, 11, 16, 19, 25, 46, 47, 49, 50, 57, 59, 63, 64, 90, 93, 100, 115, 135, 156, 226, 257, 280, 283, 306, 403, 403, 424, 521, 619, 644, 659, 908

**Tha I (CGCG):**
Sites: 105, 437, 930, 1260, 1841, 2184, 2287, 2289, 2358, 2728, 2825, 2947, 2973, 3118, 3128, 3257, 3323, 3384, 3389, 3416, 3545, 3660, 3822, 3824, 4055, 4079, 4261, 4296, 4342, 4490, 4497, 4586, 4602, 4620, 4691, 4741, 4778, 4892, 5223, 5390, 5410, 5543, 5545, 5692, 6328, 6340, 6670, 6749, 6797, 7020

**Hae III (GGCC):**
Sites: 18, 605, 872, 952, 1410, 1844, 1873, 2415, 2917, 3101, 3314, 3371, 3422, 3443, 3532, 3816, 3914, 4283, 4629, 4764, 4986, 5050, 5058, 5182, 5387, 5474, 6277, 6867, 6930, 7072
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**Hha I (GCGC):**
Sites: 105, 437, 774, 867, 1260, 1369, 1543, 1643, 1710, 1980, 2013, 2156, 2186, 2289, 2635, 2718, 2908, 2944, 3006, 3157, 3416, 3547, 3587, 3662, 3759, 3824, 3852, 4057, 4079, 4081, 4088, 4095, 4263, 4296, 4497, 4586, 4620, 4876, 4906, 5034, 5088, 5148, 5169, 5348, 5410, 5455, 5543, 5545, 5663, 5692, 5697, 5775, 6177, 6342, 6670, 7249, 6797, 6799

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**Mnl I (CCTC GAGG):**
Sites: 29, 622, 828, 976, 1057, 1457, 1706, 1781, 1989, 2253, 2283, 2465, 2502, 2560, 2892, 3060, 3087, 3125, 3186, 3390, 3488, 3574, 3823, 4148, 4630, 4975, 5194, 5284, 6817, 6824
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Eco RII (CCWGG) :
Sites: 1723, 1736, 1857, 2917, 3300, 3852, 3909, 4449, 4764, 5468, 5548, 5784, 5893, 6301, 6644, 6856, 6991
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Fnu 4H (GCNGC) :

Fok I (GGATG CATCC) :
Sites: 557, 844, 1025, 2198, 2339, 2525, 2603, 2665, 3314, 3359, 4433, 4442, 5971
Fragment Sizes: 9, 45, 62, 78, 141, 186, 287, 649, 1074, 1173, 1529, 1723

Hga I (GACGC GCGTC) :
Sites: 464, 1194, 1772, 2190, 2347, 2979, 3129, 3373, 3405, 4261, 4267, 4496, 4514, 4940, 5179, 5534, 5640, 5841, 6517
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Hph I (GGTGA TCACC) :
Sites: 269, 304, 510, 926, 1153, 2260, 2269, 2844, 3065, 3863, 3896, 4318, 4763, 4836, 5139, 5184, 5631, 5748, 5839, 5908, 6090, 6117, 6420, 6801, 6935, 7004
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Mae III (GTNAC) :
Sites: 335, 523, 676, 734, 1065, 1348, 1464, 1527, 2136, 2231, 2444, 2528, 2551, 3211, 3478, 4306, 4829, 5914, 6574
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Sites: 18, 214, 324, 401, 1156, 1227, 2018, 2771, 3363, 3634, 4003, 4342, 4513, 4858, 5108, 5605, 5815, 6052, 6245, 6322, 6475, 7041

96
Nci I (CCSGG) :
Sites: 460, 811, 1507, 2208, 2243, 2549, 2877, 3103, 3717, 4062, 4871, 4922, 5047, 5851, 6154, 6638
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Ple I (GAGTC GACTC) :
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Sau 96I (GGNCC) :
Sites: 16, 632, 854, 871, 950, 2413, 2600, 2879, 2921, 3100, 3224, 3473, 3561, 3913, 3937, 4281, 4282, 4627, 5056, 5385, 5472, 6138, 6275, 6928, 7071
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Scr FI (CCNGG) :
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Sfa NI (GATGC GCATC) :
Sites: 295, 544, 735, 1787, 2007, 2047, 2085, 2220, 2441, 2503, 2581, 2677, 2897, 2929, 3224, 3386, 3711, 4123, 4126, 4314, 4455, 5165, 5177, 6131, 6464, 6792
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Aat II (GACGTC) :
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Acc I (GTMKAC) :
Sites: 2116
Fragment Sizes: 7137

Afl III (ACRYGT) :
Sites: 1884, 4488
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Aha II (GRCGYC) :
Sites: 74, 456, 3155, 3850, 4533, 5032, 5146, 5167, 5632
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Apa I (GGGCC) :
Sites: 4285
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Apa LI (GTGCAC) :
Sites: 324, 1570, 2070, 4508
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Ase I (ATTAAT) :
Sites: 821, 3746, 5229
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Asu II (TTCGAA) :
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Ava I (CYCGRG) :
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Bal I (TGGCCA) :
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Sites: 4285, 5098, 5112
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Bcl I (TGATCA) :
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Bgl II (AGATCT) :
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Bsm I (GAATGC GCATTC) :
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Bsp HI (TCATGA) :
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Bss HII (GCGCGC) :
Sites: 4077, 5541, 6795
Fragment Sizes: 1254, 1464, 4419

Cfr10 I (RCCGGY) :
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Cla I (ATCGAT) :
Sites: 7111
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Dra I (TTTAAA) :
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Fragment Sizes: 21, 24, 35, 35, 39, 41, 42, 43, 49, 90, 92, 94, 114, 121, 126, 130, 158, 173, 211, 258, 279, 287, 318, 432, 463, 590, 622, 733, 745, 772

Nru I (TCGCGA) :
Sites: 3389
Fragment Sites: 7137

Nsp I (RCATGY) :
Sites: 1888, 2255, 2547, 5021, 5484
Fragment Sizes: 292, 367, 463, 2474, 3541

Pst I (CTGCAG) :
Sites: 754, 7099
Fragment Sizes: 792, 6345

Pvu I (CGATCG) :
Sites: 627
Fragment Sizes: 7137

Pvu II (CAGCTG) :
Sites: 2297, 3799, 3892, 5355, 5364, 6189
Fragment Sites: 9, 93, 825, 1463, 1502, 3245

Sca I (AGTACT) :
Sites: 515, 6376
Fragment Sizes: 1276, 5861

Sdu I (GDGCHC) :
Sites: 328, 413, 1574, 2074, 2898, 3189, 4285, 4512, 4996, 5098, 5112
Fragment Sizes: 14, 85, 102, 227, 291, 484, 500, 824, 1096, 1161, 2353

Sna I (GTATAC) :
Sites: 2115
Fragment Sizes: 7137

Sph I (GCATGC) :
Sites: 5021
Fragment Sites: 7137

Ssp I (AATATT) :
Sites: 191
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Sty I (CCWWGG) :
Sites: 2990, 5315, 6440
Fragment Sites: 1125, 2325, 3687

Xba I (TCTAGA) :
Sites: 5276
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Xho II (RGATCY) :
Sites: 349, 366, 1134, 1146, 1232, 1243, 2692, 3712, 4924, 5210, 5613, 5741
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Fragment Sites: 42, 7095

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Fragment Sizes: 12, 108, 201, 2936, 3880

Tth 111 I (GACNNNGTC):
Sites: 2142
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Sites: 396, 2330, 6707
Fragment Sizes: 826, 1934, 4377

Bgl I (GCCNNNNNGGC):
Sites: 878, 3197, 3431, 5899, 7032
Fragment Sizes: 234, 983, 1133, 2319, 2468

PfM I (CCANNNNNTGG):
Sites: 2996, 3045, 4913, 5355, 5894
Fragment Sizes: 49, 442, 539, 1868, 4239

Bst XI (CCANNNNNNTGG):
Sites: 4442, 4565, 4694
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