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Enzyme immobilization by adsorption of phenylbutyramidinated protein on porous polymer beads

Song, Yeqing, Ph.D.
The Ohio State University, 1989
ENZYME IMMOBILIZATION BY ADSORPTION OF
PHENYLBUTYRAMIDINATED PROTEIN ON POROUS POLYMER BEADS

Dissertation

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

By

Yeqing Song, B.S., M.S.

* * * * *

The Ohio State University

1989

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Approved by

Advisor
Department of Biochemistry
To

My Family

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1. IMMobilIZED ENZYME

1.a Definition

An enzyme is termed "immobilized" if its mobility has been restricted by chemical or physical means. The term "immobilized enzymes" was recommended at the first Enzyme Engineering Conference at Henniker, New Hampshire in August 1971. Immobilized enzymes are defined as "enzymes which are physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" (Chibata, 1978). Before that time, various terms were used, such as "water insoluble enzyme," "trapped enzyme," "fixed enzyme," "matrix-supported enzyme," and "insolubilized enzyme." It should be noted that an essential criterion for the definition is that human manipulation has to be involved.

1.b Advantages

The advantages of immobilizing enzymes are as follows (Messing, 1985; Kilara and Shahani, 1985; Hartmeier, 1988; Bailey 1986):

1
1. Immobilization allows for multiple or repetitive use of a given batch of enzyme.

2. Immobilized enzymes are better for process control, because they can be used in a continuous operation, and can be retained in a reactor.

3. In some cases, by immobilization, the enzyme properties (activity and stability) can be altered to be more suitable for conditions used for industrial processes which may involve a wide range of temperatures, pH values, and flow rates.

4. Immobilization makes heterogeneous catalysis possible, in which reactant is easily separable from products. Therefore, it will reduce the cost in product processing and effluent handling.

5. Immobilized enzymes may retain their activity longer than those in solution and have predictable decay rates.

6. Immobilization makes it possible to achieve and maintain a high catalytic activity in a small volume, as volumetric productivity (yield per unit of space) is a vital criterion in industrial processes.

7. An immobilized enzyme may be fixed in position near other enzymes involved in a catalytic sequence, thereby increasing the catalytic efficiency for multiple enzymatic conversion reactions.
8. Immobilization can help to avoid protein contaminations of the processed solution, which is important in protein sequence and structural determinations to avoid the interferences, also important in food and pharmaceutical applications to avoid immune responses in the body.

9. Immobilized enzymes provide good experimental and theoretical models for the study of bound enzymes in living systems.

1.c History of immobilized enzymes

One of the earliest reports of immobilized enzymes was that yeast invertase (β-D-fructofuranosidase) adsorbed on active charcoal, and on alumina retained enzymatic activity to break down sucrose (Nelson and Griffin, 1916). However, the research was not explored until after World War II. Those early pioneering efforts have been reviewed by Messing (1975) and Kennedy and Cabral (1983).

In the late 1960's and 1970's a great deal of enthusiasm developed for research and industrial applications of immobilized enzymes. Immobilized glucose isomerase used in commercial production of high fructose corn syrup is one of a few early successes. More recently, rapid and widespread developments in biotechnology, resulting from the revolutionary advances in molecular genetics and related disciplines, have stimulated and expanded interest in
immobilized enzymes and their applications in industrial, medical, pharmaceutical, diagnostic and analytical fields.

1.d Classification of immobilized enzymes

There is no one ideal general immobilization method for all the applications of immobilized enzymes. Many methods have been reported for enzyme immobilization. The classification of these immobilized enzymes was recommended for the first time at the first Enzyme Engineering Conference. The initial distinction was made between bound and encapsulated enzymes. Each group was then further subdivided according to the immobilization method. Figure 1 shows a basic classification of immobilized enzymes.

The classification was originally intended for immobilized enzymes. With the development of immobilization technology, however, it has been extended to apply to more complex biocatalysts such as immobilized organelles or whole cells (Hartmeier, 1988).

The classification on the basis of the immobilization method covers the most important, although by no means all, known forms of immobilized enzymes. In fact, the more recent developments in the field of immobilization involve the combination of two or more types, which can no longer be fitted into the simple scheme shown in Figure 1. Sometimes, efficient immobilization can be achieved by a combination of
Figure 1. Classification of immobilized enzymes.

E enzyme unit; C Carrier unit. Adapted from Hartmeier, 1988.
two or more methods. For example, the immobilization method described in this dissertation is a combination of chemical modification and adsorptive binding.

2. INTRODUCTION TO THE PROJECT

2.a Immobilization by adsorption after phenylbutyramidination

A new procedure for attaching enzymes and other biologically active proteins to organic polymer beads has been developed in this laboratory (Ampon and Means, 1988). Two major steps are involved as shown in figure 2: phenylbutyramidination and adsorption. Protein is first modified by a soluble, hydrophobic imidoester. The resulting hydrophobic protein derivative then adsorbs spontaneously onto organic polymer beads.

Immobilization of enzymes through simple adsorption is usually very mild and very effective. However, in most cases, the binding forces between protein and support are relatively weak. It seems that high adsorption and high enzyme activity is difficult to attain at the same time. And in general, when the amount of adsorption increases, the enzymatic activity decreases (Kamei et al., 1987). Also this phenomenon is found in covalent attachment of enzymes to the solid phase: upon increase of the number of enzyme-carrier binding, the enzymatic activity decreased while
Figure 2. Immobilization of enzyme by adsorption of phenylbutyramidinated protein.

The figure is not proportional to the actual sizes of compounds. MPBI: methyl phenylbutyrimidate.
protein coupling increased (Koch-Schmidt and Mosbach, 1977).

A great number of attempts have been made to achieve high adsorption and high activity. Since the 1960's, it has been clear that, in general, the level of activity of an immobilized enzyme depends on the degree of hydration of the polymer matrix (Manecke, 1962). One approach has been to modify or synthesize organic homopolymer or copolymer as better supports (Hofstee, 1976; Butler, 1975; Carleysmith et al., 1980a), which usually is costly and not very efficient. A recent study by Kamei et al. (1987) have described a procedure to synthesize a copolymer with hydrophilic-hydrophobic heterogeneous structure.

The method developed in this laboratory involved a new and unconventional approach: instead of "modifying" polymer beads, protein molecules have been modified to increase their hydrophobicity (Ampon and Means, 1988). The procedure involved the amidination of the proteins by reaction with a soluble imidoester, methyl 4-phenylbutyrimidate (MPBI), under mild conditions to increase the hydrophobicity without changing the net charges of the proteins, followed by simple adsorption of the resulting hydrophobic enzyme derivatives onto the polymer beads.
2. Importance of this new method

Although many new methods are reported each year, only a few have been commercialized due to a combination of economic and technological factors. One of the most significant reasons is that the economics of immobilized enzyme applications (immobilization cost, carrier cost, catalytic efficiency, etc.) are not yet attractive enough for commercial use. Another important reason is that the development and optimization of the immobilized enzyme system can be expensive because effective methods must be established for each enzyme. These methods are usually complicated and are not applicable to other enzymes.

Nevertheless, in these aspects, the method described by Ampon and Means offers some considerable advantages over other methods reported. The comparison of our method with other general methods is listed in Table 1.

First, the method is very effective: there is no significant loss of enzyme or enzymatic activity during immobilization. After phenylbutyramidination, catalytic activity remains unchanged, and the enzyme derivatives can be adsorbed to the organic beads almost completely. And after immobilization, most enzymatic activity can be retained without significant loss if enzyme loading levels are not too high.
<table>
<thead>
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<th>Crosslinking</th>
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<th>Ionic Binding</th>
<th>Covalent Binding</th>
<th>Our Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation</td>
<td>Intermediate</td>
<td>Simple</td>
<td>Simple</td>
<td>Difficult</td>
<td>Simple</td>
</tr>
<tr>
<td>Binding force</td>
<td>Strong</td>
<td>Weak</td>
<td>Intermediate</td>
<td>Strong</td>
<td>Strong</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td>Low</td>
<td>Intermediate</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Regeneration of carrier</td>
<td>Impossible</td>
<td>Possible</td>
<td>Possible</td>
<td>Rare</td>
<td>Possible</td>
</tr>
<tr>
<td>Cost of immobilization</td>
<td>Intermediate</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Stability</td>
<td>High</td>
<td>Low</td>
<td>Intermediate</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>General applicability</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
The second advantage is its simplicity. Basically, only four steps are involved, phenylbutyramidination, dialysis, adsorption and washing the beads.

In addition to being effective and simple, the cost of the carrier material is unusually low. Amberlite XAD-8 and other similar beads are commercially available and inexpensive as compared to other organic polymer beads. The general properties and the prices of those beads are listed in Table 2. Also, the beads can be regenerated if necessary (Rohm and Haas Inc., 1981).

The mechanical strength and chemical durability of a carrier during use is also important. Amberlite polyacrylate beads are spherical with a rigid structure, and can be expected to handle high flow rate with minimal pressure drops. Moreover, it is stable over a wide range of pH values, ionic strengths, solvent conditions and temperatures. In addition, it is not readily biodegradable. So, the carriers are suitable for various applications with the possibility for scale-up.

With this method, a high volumetric productivity, high yield per unit of space and time, potentially could be achieved because Amberlite XAD-8 beads and several related supports have very high capacity for modified enzymes, and can adsorb an amount of enzyme up to its own weight without significant leakage.
Table 2. The Amberlite beads employed.

<table>
<thead>
<tr>
<th>Amberlite beads</th>
<th>XAD-7&lt;sup&gt;a&lt;/sup&gt;</th>
<th>XAD-8&lt;sup&gt;a&lt;/sup&gt;</th>
<th>XAD-71&lt;sup&gt;b&lt;/sup&gt;</th>
<th>XAD-2&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical structure</td>
<td>Acrylic ester</td>
<td>Acrylic ester</td>
<td>Acrylic ester</td>
<td>poly- styrene</td>
</tr>
<tr>
<td>Ave. pore diam. Å</td>
<td>90</td>
<td>225</td>
<td>140</td>
<td>90</td>
</tr>
<tr>
<td>Bead size µ</td>
<td>250-425&lt;sup&gt;c&lt;/sup&gt;</td>
<td>250-425&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50-80</td>
<td>250-425&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Skeletal density g/ml</td>
<td>1.24</td>
<td>1.23</td>
<td>1.25</td>
<td>1.09</td>
</tr>
<tr>
<td>Surface area m²/g</td>
<td>450</td>
<td>160</td>
<td>550</td>
<td>300</td>
</tr>
<tr>
<td>Porosity volume %</td>
<td>55</td>
<td>52</td>
<td>61.7</td>
<td>42</td>
</tr>
<tr>
<td>Price per pound</td>
<td>$17.00</td>
<td>$28.90</td>
<td>N/A</td>
<td>$29.14</td>
</tr>
</tbody>
</table>

<sup>a</sup>: As reported by the manufacturer.
<sup>b</sup>: Known also as Amberchrom XAD-71, kindly provided as a gift by Dr. Robert Albright of Rohm and Haas Inc.
<sup>c</sup>: Except when indicated otherwise; As selected using U.S. Standard Sieves.
2.c Objectives

Enzyme immobilization is a complex process which involves the combination of various chemical and physical phenomena. In order to optimize our method and expand its application, we have to understand the effects of each individual process and the combined effects of multiple processes as much as possible. Therefore the chemical modification, adsorption performance, the processes of immobilization, diffusion, activity decay and denaturation, and so on, have been studied separately. By dividing the process into several compounds, we could examine the effect of each phenomenon on the overall results of a given immobilization procedure. These basic phenomena are also often encountered in other immobilization methods, so this kind of study could also benefit other research in the enzyme immobilization field.

In this study, we have conducted several investigations into the effects of phenylbutyramidination, adsorption and other related factors on the activities of immobilized enzymes by using trypsin and α-chymotrypsin as model enzymes. Trypsin and α-chymotrypsin have been extensively studied in other immobilization procedures (Miron and Wilchek, 1985; Koch-Schmidt and Mosbach, 1977; Mauz et al., 1984; Royer and Uy, 1973). Both enzymes are monomers with low molecular weights. Their catalytic mechanisms are relatively simple and neither
requires cofactors or activators. Reaction rates of both can be determined by continuously monitoring the amounts of titrant added in order to maintain a given pH using a pH stat even when the reaction system is heterogenesis. Also these two enzymes have potential applications as they have been widely used for various medical purposes, beer haze removal, and meat tenderization (Luong, Male and Nguyen, 1988). A disadvantage of their use is that they can be autolyzed. However, our results have shown that autolysis is not significant after immobilization. The soluble enzymes can also be stabilized in the presence of Ca++ or by maintaining a pH below 4.

In this study, first, we investigated each individual process involved in the immobilization procedure which included: 1. the extent of chemical modification and its effect on the catalytic activity before and after enzyme immobilization; 2. adsorption of modified enzymes, as well as their substrates on different organic polymer beads; 3. the effects of adsorption on enzyme activity, and its efficiency under different circumstances. Then, some physical and chemical phenomena existing after enzyme immobilization have been studied, such as, the diffusion patterns of various amounts of protein on the beads with different pore sizes, and the correlation of the amount of protein with observed catalytic activities. Finally, EPR
has been employed to study the active site conformation of an immobilized, spin labeled derivative of chymotrypsin before and after immobilization.
MATERIALS

Bovine pancreatic trypsin (type III), bovine pancreatic α-chymotrypsin (type II), benzoyl-L-arginine ethyl ester (BAEE), benzoyl-L-tyrosine ethyl ester (BTEE), Sucrose, 2,2,6,6,-tetramethyl-4-amino-1-piperidinyloxy (4-amino-Tempo), 3-(Fluorosulfonyl) benzoyl chloride, ascorbic acid, potassium ferricyanide K₃Fe(CN)₆, and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were obtained from Sigma Chemical Co. Naphthol blue black was purchased from Kodak Laboratory Chemicals. p-Nitrophenyl-m-guanidino benzoate (NPGB) was a product of Nutritional Biochemical Co. Amberlite XAD-2, XAD-7 and XAD-8 were purchased from Polysciences Inc. Amberlite XAD-71 was a gift from Dr. R. L. Albright of Rohm and Haas Inc. Methyl phenylbutyrimidate hydrochloride was synthesized as described by Ampon and Means (1988).
CHAPTER I. PHENYL BUTYRAMIDINATION OF PROTEINS

1. Introduction

Imidoesters have several desirable characteristics as reagents to increase the hydrophobicity of an enzyme. First, imidoesters react selectively with only the ε-amino groups of lysyl residues and the terminal α-amino group of proteins to form amidines. Since they will not react with sulfhydryl groups, phenolic groups, imidazole groups or peptide bonds, some undesired reactions will be eliminated (Wofsy and Singer, 1963).

Furthermore, the reaction does not change the net charge of an amino group although the loci of those positive charges are displaced by 1.5 Å (Kapmeyer and Pfleiderer, 1977). Structural alterations, if any, should therefore be minimal and changes in biological function should also be minimal except in those cases where amino groups are directly involved in the active sites (Wofsy and Singer, 1963).

It is also important to note that amidination can be achieved under mild, slightly alkaline conditions at 0°C. and that many imidoesters are easily synthesized from a variety of inexpensive nitriles. So, a series of
imidoesters could be screened to find a more favorable one for a specific purpose.

2. Methods

2.a Phenylbutyramidination of enzymes

The amino groups of trypsin and chymotrypsin were modified by reaction with methyl phenylbutyrimidate hydrochloride (MPBI), by a procedure similar to that described by Ampon and Means (1988) with minor modifications. Trypsin or α-chymotrypsin, 20 mg (60-70% active enzyme), were dissolved in 2.5 to 10 ml of 0.02 M CaCl$_2$, 0.10 M dimethylaminoethanol/HCl buffer, pH 9.0 (adjusted at room temperature), and cooled to about 0°C in an ice bath. To the enzyme solution, 20 mg of MPBI was added slowly with stirring in small increments over 2-3 hours. MPBI could also be dissolved in dioxane (15 mg/ml) in the stock solution, because it has been reported that dioxane can reduce the rate of hydrolysis of imidoesters (Kaard, Kostner and Simer, 1986). The reaction was then terminated by adjusting the pH to about 3.5 with 30% acetic acid. Under such conditions about 60-75% of the amino groups were modified. It is critical to keep the reaction solution soluble to achieve extensive modification. The hydrolysis of methyl phenylbutyrimidate to methyl phenylbutyrate (favored below about pH 9) or
phenylbutyramide (favored above pH 9) (Kaard, Kostner and Simer, 1986) could accelerate the precipitation of the partially modified enzyme, as both are very insoluble in water. This could be minimized by adding the imidoester to the reaction solution in small increments. The addition of MPBI should be stopped when the solution becomes cloudy. Cloudiness after completion of the reaction was eliminated by dialysis against 6 mM acetic acid.

2.b Different extent of modification

To achieve different degrees of modification, reactions were stopped at different times, from 0 to 20 hours. The reaction solutions, 0.2-0.5 ml were then passed through a TSK column by FPLC (Pharmacia). The column was washed with 0.02 M Tris/HCl buffer, pH 5.4, with a flow rate of 1 ml/min., and a methanol gradient from 0 to 10% during a 10-minute washing cycle. Instead of FPLC, a column of Sephadex G-25 (2.5 x 10 cm), equilibrated and eluted with 10^{-3} M HCl also can be used. After dialysis or passage through the column, the extent of modification, for each sample was determined from the number of amino groups detected, using 2,4,6-trinitrobenzenesulfonic acid, TNBS, (Fields, 1972). The protein concentrations of each sample were determined by UV absorbance at 280 nm, using
E$_{280}^{1%}$ = 20.4 for α-chymotrypsin, and 14.3 for trypsin (Worthington Enzymes, Enzyme Reagents Related Biochemicals). This method was consistent with amino acid composition analysis after hydrolyzing the trypsin derivatives according to Ampon and Means (1988).

2. C Activity assay

Tryptic activities were determined from rates of benzoyl-L-arginine ethyl ester (BAEE) hydrolysis. Chymotryptic activities were determined from rates of benzoyl-L-tyrosine ethyl ester (BTEE) hydrolysis using a Radiometer automatic titrator (Ampon and Means, 1988; Schwert, et al., 1948). For a typical assay, about 10 to 20 mg (dry weight) of Amberlite beads to which enzyme was immobilized were added to 3.2 ml of 0.001 M triethanolamine buffer containing 0.02 M KCl and 0.005 M CaCl$_2$, pH 7.9. For soluble enzymes, 5-20 μl of native or modified enzyme was used for each assay. The substrate, 10 μl of 0.3 M BAEE or BTEE in methanol, was then added to the rapidly stirred reaction vessel at pH 8.0 which was maintained by the addition of 0.01 M NaOH. When the reaction vial was closed, the interference of atmospheric CO$_2$ in the air with the assay was not noticeable. If the amount of the enzyme was too low, nitrogen gas should be used to fill the top space in the vessel (Royer, 1975).
After each assay, the beads were collected, dried at 32°C overnight, then weighed and discarded. The activities of both enzymes are expressed in μMoles NaOH/min.

2.d Solubility of modified enzymes

After dialysis or passage through a column of Sephadex G-25, the solubilities of different enzyme derivatives was determined by light scattering according to Nishikawa Becker (1968) at 320 nm using a Cary 118 spectrophotometer.

3. Results

Amino groups of trypsin and chymotrypsin were readily modified by methyl phenylbutyrimidate. The previous study showed that this compound has the best activity retention after the modification among other imidoesters with different bulk structures and hydrophobicities (Ampon and Means, 1988).

\[
\text{C}-(\text{CH}_3)_3-\text{C}^\equiv\text{NH}_2\text{Cl} \quad \text{OCH}_3
\]
3.a Extent of modification

The extent of chemical modification could be controlled by varying the amount of imidoester added and the reaction time. More than 80% of amino groups were phenylbutyramidinated within 5 hours. Figure 3 shows the time course for the phenylbutyramidination of trypsin. During the reaction time, the imidoester was added in small increments until the reaction solution turned cloudy.

3.b Effect of phenylbutyramidination on activity

Our result showed that the activities of trypsin and chymotrypsin were unchanged if phenylbutyramidination was less than 60-70% (Figures 4 and 5). However when amino groups are more extensively modified with MPBI (more than 80%), the resulting bulky phenylbutyramidine groups decreased the solubility of those derivatives in aqueous solution and their apparent activities. The absorbance at 320 nm which reflects light scattering at high levels of modification thus correlated with decreases in the activities of both phenylbutyramidinated enzyme derivatives (see Figures 4 and 5).
Trypsin solution: 30 mg enzyme in 10 ml buffer. During the entire reaction period, 40 mg MPBI was added in small increments according to Ampon and Means (1988). After additional hours shown in the figure, the reaction was terminated.
Figure 4. Changes in the activity and solubility of chymotrypsin with increasing extent of phenylbutyramidination.

Activities against BTEE (●), and solubilities as determined from light scattering at 320 nm (▲) of chymotrypsin are shown at different levels of amino groups modification by MPBI. For activity assay, 5 μl of enzyme solution (1.5 mg/ml) was added to 3.2 ml of assay buffer.
Figure 5. Changes in the activity and solubility of trypsin with increasing extent of phenylbutyramidination.

Activities against BAEE (●), and solubilities as determined from light scattering at 320 nm (▲) of trypsin are shown at different levels of amino groups modification by MPBI. For activity assay, 5 μl of enzyme solution (1.2 mg/ml) was added to 3.2 ml of assay buffer.
3.c Effects of phenylbutyramidination on apparent activity after immobilization

The effects of phenylbutyramidination on enzymatic activity after immobilization, are shown in Figures 6 and 7. After modification to different extents, chymotrypsin and trypsin were immobilized as described by Ampon and Means (1988). Slightly more activity was observed for phenylbutyramidinated chymotrypsin after adsorption on Amberlite XAD-8 beads than in the case of the native chymotrypsin (Figure 6). The increase due to modification was even more pronounced in the case of trypsin than chymotrypsin. The highest activities immediately after immobilization were obtained at about 70% modification (Figure 7). After long storage times, however, higher apparent activities were obtained at somewhat higher levels of modification (about 85%), as shown in the case of trypsin in Figure 7.

With different numbers of amino groups phenylbutyramidinated, both trypsin and chymotrypsin were considerably more stable after immobilization. The activity of unmodified trypsin on Amberlite XAD-7 decreased rapidly with the time, while the extensively phenylbutyramidinated enzyme appeared more stable as shown in Figure 8.
Figure 6. Activity recovery of immobilized chymotrypsin with different degree of phenylbutyramidination.

The enzyme activities were assayed 1 day (□), 10 days (☑) and 130 days (☒) after immobilization. The concentration of immobilized chymotrypsin in Amberlite XAD-8 was 10 mg/g. The beads were stored in a cold room at 7±1°C.
Figure 7. Activity recovery of immobilized trypsin with different degree of phenylbutyramidination.

The enzyme activities were assayed 5 days (○), and 130 days (▲) after immobilization. The concentration of immobilized trypsin on Amberlite XAD-7 was 23±2 mg/g. The beads were stored in a cold room at 7±1°C.
Figure 8. Effect of modification on apparent storage stabilities of immobilized trypsin

The amino groups of trypsin have been modified: 87% (▲); 21% (●); and without any modification (■). The loading level of immobilized trypsin on Amberlite XAD-7 is 23±1 mg/g. The beads were stored in a cold room at 7°C.
α-Chymotrypsin, known to be less resistant to high temperature than trypsin, was immobilized on XAD-8 by the same procedure. The stabilities of immobilized but unmodified and immobilized phenylbutyramidinated chymotrypsin at 50°C are quite different as shown in Figure 9.

4. Discussion
4.a The effect of modification on soluble enzymes

The amino groups of trypsin and chymotrypsin were modified by methyl phenylbutyrimidate to increase the hydrophobicity while still maintaining their enzymatic activity. This easily prepared covalent modification did not alter the net charges of the proteins.

\[
\text{(I-1)}
\]

Uniquely, most other chemical modification methods which alter the electrostatic charge, amidination does not significantly decrease the activity of many enzymes (Coggins, 1978; Wofsy and Singer, 1963). It has been reported that the activity of trypsin is unchanged after modification by methylacetimidate (Nureddin and Inagami,
Figure 9. Effect of modification on stability of immobilized chymotrypsin at 50°C.

Chymotrypsin with about 60% of its amino groups modified (●) and without modification (♦), was adsorbed on Amberlite XAD-8 10 mg/g. The beads were then incubated with 0.05 M triethanolamine/HCl buffer, containing 0.02 M CaCl₂, pH 7.5, at 50°C. The initial activity of the immobilized enzyme modified on about 60% of its amino groups was defined as 100%.
1969). It also has been shown that the activities of trypsin and chymotrypsin increase about 20% after conjugation to polymeric methyl imidoester (Zaborsky, 1974).

In our experiments, as shown in Figures 4 and 5, the activities of the trypsin and chymotrypsin modified by methyl phenylbutyrimidate did not change if the enzyme solution stayed soluble (amino groups were modified less than 60-70%). However, extensive modification (more than 80%) tended to decrease both the solubility and the apparent activities and should be avoided. The decrease seemed to correlate with the solubility of the hydrophobic enzyme derivatives and presumably reflects its decreased accessibility to substrate in that condition and perhaps, some degree of denaturation.

After storage, both soluble phenylbutyramidinated trypsin and chymotrypsin derivatives gave higher activity than native enzyme solutions, due to the higher population of active enzyme resulting from the lower autolysis after modification. It has been shown in previous reports that amidination of trypsin could significantly slow down autolysis of trypsin derivatives in solution (Ampon and Means, 1988; Nureddin and Inagami, 1969 and 1975).
As extensive modification decreases solubility and apparent activity, it is crucial to avoid excessive modification. Precipitation could also be reduced by limiting the hydrolysis of the imidoester to insoluble methyl phenylbutyrate, by controlling the pH above 8 (Makoff and Malcolm, 1981 and Kaard, et al. 1985), and by controlling the total amount of imidoester in each addition.

4. b The effect of modification on immobilized enzymes

Trypsin and, to a lesser extent, chymotrypsin retained more activity upon immobilization after phenylbutyramidination of 60-90% of their amino groups (Figures 6 and 7). Trypsin with less than 60% of its amino groups modified could be more mobile in the polymer beads, and tend to diffuse to internal porous surfaces where it would have poorer access to the substrate. Autolysis does not account for the observed decreases, as our experiments showed autolysis was not significant after immobilization even for unmodified enzyme as will be described in Chapter IV. So, the more extensively phenylbutyramidinated trypsin exhibited a greater activity retention after immobilization especially in long term range, maybe, because of its lesser mobility as compared with unmodified but immobilized trypsin.
It has been reported that the apparent stabilities of immobilized enzymes are related to the number of protein-carrier bonds (Martinek, et al. 1977). Our experiments show that modification with MPBI will increase the apparent stability of the immobilized enzymes. As shown in Figure 8, with 87% of the amino groups modified, the apparent activity of immobilized trypsin is virtually unchanged after 130 days in a cold room at 7°C, while native trypsin has less than 50% of its original activity under the same conditions. It has been reported that trypsin can be stabilized against autolysis by acetamidination of 11 lysyl residues (Nureddin and Inagami, 1969 and 1975). However, in the present case, stabilization is not only due to the blocking of lysyl residues, but since unmodified trypsin is also relatively stable after immobilization (see Chapter IV), it may also be due to its lower mobility after modification.

The effect of phenylbutyramidination on enzyme stability at 50°C was studied because chemical denaturants, urea or guanidine salts, might significantly interfere with the adsorption forces involved in the immobilization procedure. α-Chymotrypsin, known to be less resistant to heat than trypsin, was chosen for the investigation. After immobilization, modified chymotrypsin exhibits improved stability at 50°C (Figure 9).
Multipoint attachment of enzymes, either covalent or noncovalent, to carriers has been shown to increase their thermal stability (Martinek, Klibanov and Goldmacher 1977). Adsorption to polyacrylate carriers, which may involve multiple attachment, might increase its thermal stability. In Figure 9, the observed enhancement in thermostability of immobilized phenylbutyramidinated chymotrypsin might therefore be due to the stronger binding between the enzyme and the polyacrylate beads resulting from multiple attachment after phenylbutyramidination. As shown in Chapter IV an increase in the number of amino groups modified also decreases enzyme mobility inside the beads (see Chapter IV).
CHAPTER II. ADSORPTION ON AMBERLITE POLYMER BEADS

1. Introduction
1.a Adsorption

The process of adsorption involves the separation of a substance from one phase accompanied by its accumulation or concentration at the surface of another. Unlike absorption, a process in which material transfers from one phase to interpenetrate the second phase to form a "solution," adsorbate will localize to a specific location on adsorbents.

The adsorption of an enzyme onto an insoluble support without covalent bonding is the oldest, simplest and most economical method of enzyme immobilization (Woodward, 1985). It is accomplished by bringing an aqueous solution of the enzyme into contact with the adsorbent surface.

Although the adsorption process is simple, the forces involved are complex. Even so called "inert" carriers always have some surface activity. Basically, when using the procedure of Ampon and Means (1988), the enzyme derivatives are held to the surface of the beads by hydrophobic interactions. However, additional forces may
also be involved, principally hydrogen bonds and ionic interactions (Giordano, 1980).

Immobilization by adsorption offers several advantages over other immobilization methods. The adsorption process is simple to carry out under mild conditions. Generally speaking, adsorption has less effect on catalytic activity than the unphysiological coupling conditions and/or chemicals usually necessary in other procedures. Also, the method is suitable for the immobilization of multiple enzymes simultaneously, and the carriers usually are regenerable.

Unfortunately, there is an inherent disadvantage in most noncovalent adsorption procedures in that the binding forces between the protein and the support are often relatively weak and reversible. Adsorbed enzymes in such cases are therefore easily desorbed under experimental or normal operational conditions. The adsorption may be affected by the presence of certain substrates, at certain pH values, ionic strengths, temperatures, and certain types of solvents.

However, in the method described by Ampon and Means (1988), the protein becomes highly hydrophobic after the modification of amino groups with a bulky imidoester. Phenylbutyramidinated enzymes will be partitioned between hydrophobic beads and aqueous bulk solution. Under the
experimental conditions, the adsorption can be virtually irreversible.

There are four distinct techniques for immobilization of enzymes by physical adsorption (Messing, 1985): (i) the unstirred static procedure, which is the least efficient and requires the most time; (ii) electrodeposition, in which enzyme will be adsorbed to a carrier by the force of a nearby electrode; (iii) a reactor loading process, which is employed most frequently for commercial preparation; and, (iv) the dynamic batch process. the latter is used most frequently in laboratory preparations, and it was also used in our studies. The adsorption procedure was accomplished by slow end to end rotation in a close vial.

1. b Adsorbents

Synthetic polymers are widely used for enzyme immobilization owing to their favorable physical and chemical characteristics. Two families of commercial non-ionic adsorbents have been developed and are widely used: polystyrene and polymethacrylate. Historically, polystyrene was the first organic polymer employed for immobilization. Recently the interest in polystyrene as a support has been renewed, mainly because of its low cost and ready availability (Kennedy and Cabral, 1983).
However, polyacrylate types are now among the most used synthetic polymers in the field of immobilization.

In this study, we have used several inexpensive, commercially available synthetic polymer beads, such as Amberlite XAD-2, Amberlite XAD-7, Amberlite XAD-8, and some related beads have also been examined. The crosslinked structures of these beads are shown in Figure 10 (Fox, 1985). Amberlite XAD-2, a nonpolar polystyrene bead, is more hydrophobic than Amberlite XAD-7, a polyacrylic ester with intermediate polarity, whereas XAD-8 has a similar polarity to XAD-7 but with a larger pore diameter.

2. Methods

2.a Bead pretreatment

Amberlite beads were air dried at room temperature, if the products were moist. Standard sieves were used to separate the beads into different particle sizes. The beads were washed with methanol in a Soxhlet extractor for 1-3 days (Junk et al., 1978). Then a fritted glass filter funnel was used while washing the beads 10 times with methanol and 20 times with distilled water (500 ml of washing solution was used for 10 g beads in each washing). The beads were washed thoroughly with 0.02 M triethanolamine buffer containing 0.02 M CaCl₂, pH 7.5, and were stored in the same buffer with a trace of chloroform
Crosslinked polystyrene structure; Amberlite XAD-2.

Crosslinked polymethacrylate structure; Amberlite XAD-7.

Crosslinked polymethacrylate structure; Amberlite XAD-8.

Figure 10. Chemical compositions of several Amberlite adsorbent beads. From Sleiko, 1985.
or sodium azide to prevent the growth of microorganisms. Prior to the immobilization process, the beads should be stored in the buffer with the appropriate pH, ionic strength, activator and cofactor for a length of time sufficient for preconditioning. Before each use, the beads were washed with the buffer again to remove any fine beads in the supernate.

2.b Adsorption equilibrium of modified enzymes on polymer beads

Various amounts of dialyzed phenylbutyramidinated derivatives of chymotrypsin and trypsin, ranging from 0.0001 to 16 mg of protein, in 10 ml solution with final concentration of 0.05 M triethanolamine/HCl, 0.02 M CaCl$_2$, 0.01 M KCl, pH 7.5 were mixed by end to end inversion in a closed 20 ml vial with approximately 10 mg of preconditioned Amberlite beads for up to 24 hours in a cold room. After mixing was stopped, and the beads were settled, the supernatants were collected to determine the concentration of any remaining protein by both UV absorbance at 280 nm and by enzyme assay using a pH stat. These two criteria were consistent with data obtained by determining the amino acid content of the beads after acid hydrolysis. After washing, the beads were dried in an oven at 32°C, weighed, and then discarded.
2.c Adsorption rates of substrates on the polymer beads

BAEE and BTEE were employed as the substrates for trypsin and α-chymotrypsin. Their adsorption onto the beads was investigated under conditions similar to that of enzyme activity assay. To 3.2 ml of 0.001 M triethanolamine/HCl buffer, containing 0.02 M KCl, 0.005 M CaCl$_2$, pH 8.0, 10 μl of 0.3 M BAEE or BTEE was added. About 8 mg of Amberlite XAD-8 and XAD-2 beads were added to the substrate solution, respectively, then the absorbance change versus time was recorded while the solution was stirred briefly every five minutes. The rate of adsorption was measured by monitoring the decrease of UV absorbance of the supernatant. For BAEE, the UV absorbance was recorded at 270 nm, and for BTEE, at 280 nm.

2.d Adsorption equilibrium of BAEE and BTEE on Amberlite XAD-8

Different amounts of 0.3 M BAEE or BTEE, 10 to 80 μl, were added to 8 ml of 0.001 M triethanolamine/HCl buffer, containing 0.02 M KCl and 0.005 M CaCl$_2$, pH 8.0. The UV absorbance of each sample was measured at 270 nm for BAEE and at 280 nm for BTEE. Then about 10 mg of Amberlite XAD-8 beads were added to each solution, mixing by end to end inversion in a closed vial for 24 hours in a cold room. After the mixing, the beads were settled, and the
supernatant was collected to determine the remaining substrate concentration by UV absorbance at the appropriate wavelength.

3. Results

Adsorption results from a combination of several complicated factors, having to do with the chemical and physical structure of the adsorbent and adsorbate molecules. Since it is not easy to predict the adsorption, we investigated the adsorption of the modified enzymes as well as their substrates on different polymer beads, as the adsorption of substrate might also have effects on the activity of an immobilized enzyme.

Phenomenologic differences among adsorption processes are illustrated graphically in Figure 11 according to Weber (1985). The terms $C_e$ and $q_e$ represent the equilibrium "concentration" of a substance in each of two phases: $q_e$ is the amount of adsorbate associated with a unit weight of solid adsorbent, here it is in units of mg enzyme or substrate/mg Amberlite beads, while $C_e$ is the residual concentration in the liquid phase, in mg/ml. Curves I and III indicate the nonlinear relationships characteristic of "favorable" and "unfavorable" adsorption respectively, whereas curve II is a linear adsorption which usually can be seen when the concentration of adsorbate is low.
Figure 11. Three basic types of equilibria adsorption phenomena. From Sleiko, 1985.

$q_e$ is the amount adsorbed and $C_e$ is the amount in solution.
3.a. Adsorption capacity for phenylbutyramidinated enzymes

The adsorption capacity was investigated by determining the adsorption curves of phenylbutyramidinated enzymes on different synthetic Amberlite polymer beads. The quantitative partitioning of amidinated enzymes between the bulk solution and the surface of the Amberlite beads was determined after 24 hours adsorption at which time it was either at equilibrium or was, for other reasons, no longer changing with time.

The adsorption curves of phenylbutyramidinated chymotrypsin derivatives on Amberlite beads (Figure 12), in mg/mg \((q_e)\), versus the initial concentration \(C_0\), in mg/ml, show the amount of adsorbed chymotrypsin versus its initial concentration in solution. As indicated, the adsorption of the enzyme on the beads is close to complete in this case, even when the amount of protein is equal to the amount of the adsorbent \((w/w)\).

Several equations can be used to describe such adsorption equilibria. The Freundlich equation, which is used most often, also fits our data better than other models.
Figure 12. Adsorption curves of modified chymotrypsin on Amberlite beads.

Chymotrypsin on XAD-2 (▲), XAD-7 (●) and on XAD-8 (♦). Before adsorption, about 60% of the amino groups were modified with MPBI. Protein derivative solution, 10 ml, was mixed with 10 mg Amberlite beads for 24 hours in a cold room. The amount of the enzyme adsorbed was determined by subtracting the amount of protein in the suspension from the initial concentration.
where \( q_e \) is the amount of protein derivatives per unit weight of beads after 24 hr adsorption (mg/mg), and \( C_e \) is the amount of protein in the solution (mg/ml) at equilibrium. \( K_F \) and \( 1/n \) are characteristic constants, but are difficult to attach rigorous physical significance (Weber, 1985). However, \( K_F \) can be taken as a relative indicator of adsorption capacity, and \( n \) is related to the adsorption energy. If adsorption is favorable, then \( n > 1 \).

The results of the partition after the adsorption were analyzed by the Equation II-1 and shown in Figure 13.

The linearized Freundlich equation in logarithmic form is employed:

\[
\log q_e = \log K_F + \frac{1}{n} \log C_e \quad (\text{II-2})
\]

for convenience of data fitting and parameter evaluation as shown in Figure 14.

In Figure 12, all three types of beads demonstrate good adsorption capacities in the experimental concentration range. The amount of enzyme derivatives adsorbed on the beads could be up to their own weight, especially for Amberlite XAD-8 and XAD-2, at which stage less than 5% of initial protein remained in the solution at equilibrium.
Figure 13. Adsorption isotherm of phenylbutyramidinated enzymes on several Amberlite beads.

Trypsin on XAD-7 (●), trypsin on XAD-8 (■), chymotrypsin on XAD-2 (△), and chymotrypsin on XAD-8 (♦). Before adsorption, about 60% of amino groups in proteins were modified with MPBI. Protein derivative solution, 10 ml, was mixed with 10 mg Amberlite beads for 24 hours in a cold room. The concentration of modified enzymes was determined by UV and activity assay. \( q_e \) is the amount of adsorbate adsorbed into Amberlite beads (mg/mg), while \( C_e \) is the final concentration in the solution, mg/ml.
Figure 14. Freundlich model for the adsorption of modified enzymes on Amberlite beads

Chymotrypsin on XAD-8 (♦), chymotrypsin on XAD-2 (★), trypsin on XAD-8 (■), trypsin on XAD-7 (●). Before adsorption, about 60% of amino groups in proteins were modified with MPBI. Protein derivative solution, 10 ml, mixing with 10 mg Amberlite beads for 24 hours in a cold room at 7°C.
3.b Adsorption capacity of BAEE and BTEE on Amberlite XAD-8 beads

Adsorption isotherms for BAEE and BTEE on Amberlite XAD-8 were shown in Figure 15.

The experimental conditions for substrate adsorption are very similar to those for the adsorption of amidinated enzymes on the Amberlite beads. Plots of the linearized Freundlich equation shown in Figures 14 and 15 indicate that the phenylbutyramidinated protein had much better adsorption than BTEE and BAEE. The K_p values, indicating relative adsorption capacity, for BTEE and BAEE are smaller than those of the amidinated enzymes (K_p values for phenylbutyramidinated enzymes are about 10±2 mg/mg depending on different beads, for BTEE about 2 mg/mg and for BAEE about 0.02 mg/mg).

3.c Adsorption rates for the substrates

The experimental data show that BAEE and BTEE had different adsorption rates on different polymer beads under our assay conditions. In Figure 16, the adsorption rates of those two substrates onto Amberlite XAD-8 and XAD-2 are compared. BTEE is a quite hydrophobic compound, while BAEE, having a net positive charge, is more hydrophilic. There were substantial differences in the rates of adsorption between the two compounds. BAEE had poor
Figure 15. Freundlich model for the adsorption of substrate compounds on Amberlite beads

BTEE on XAD-8 (+), and BAEE on XAD-8 (x). Substrate solutions, 10 ml, were mixed with 10 mg Amberlite beads for 24 hours in a cold room at 7°C. Concentration were determined by UV absorbance.
Figure 16. Adsorption rates of BAEE and BTEE on Amberlite beads.

BAEE on XAD-8 (——), BAEE on XAD-2 (---), BTEE on XAD-2 (···), and BTEE on XAD-8 (···). The figure shows typical adsorption rates in 3.2 ml of a substrate solution containing 10 µl of 0.3 M of BAEE or BTEE, mixing with 12 mg Amberlite beads. The rate of adsorption was monitored at 275 nm for BTEE, and 265 nm for BAEE.
adsorption on both XAD-8 and XAD-2, while BTEE had a much higher adsorption rate especially on XAD-8 beads.

4. Discussion

4.a Adsorption of phenylbutyramidinated protein

The experimental data in Figure 12 showed that Amberlite XAD-2, XAD-7 and XAD-8 all had high adsorption capacities. So, in general, the phenylbutyramidinated enzymes can be adsorbed without much limit on the Amberlite non-polar adsorbent beads. Of those three beads, XAD-7 and XAD-8 are more suitable for enzyme activity because, perhaps, they have both hydrophobic and hydrophilic parts.

It has been reported that when the hydrophilicity of the beads increase, which is important for the retention of enzymatic activity, the protein-carrier adsorption will decrease. Amberlite XAD-8, which is less hydrophobic than XAD-2 but with a bigger pore size, exhibits an even greater adsorption of chymotrypsin than XAD-2 as shown in Figure 13. The reason for Amberlite XAD-2 and XAD-7 having slightly less adsorption here may be that their pore size are quite small (average pore diameter 90 Å) as compared to the size of both enzymes.
With this immobilization procedure, the amount of protein adsorbed on Amberlite beads is greater than that on the other polymers reported, such as a series of copolymer synthesized by Kamei et al. with different ratios of hydrophilic parts, 2-hydroxyethyl methacrylate, and hydrophobic parts, styrene, (Kamei et al., 1986 and Kamei et al., 1987). Their results showed that when the hydrophilic poly(2-hydroxyethyl methacrylate) content increased from 0 to 100 (mole %) which increased the retention of activity, the maximum adsorption of trypsin decreased from 4.3 mg/m² to 2.5 mg/m², or roughly from about 60 mg to 30 mg/g beads. In our experiment, Amberlite XAD-7 and XAD-8 adsorbed phenylbutyramidinated enzyme derivatives in amounts greater than their own weight while still retaining a considerable amount of activity.

4.b Adsorption isotherms for enzymes and substrates

Amberlite polystyrene and polyacrylate beads are commercial adsorbents used primarily for water treatment and similar applications. Adopted for enzyme immobilization, the "adsorption isotherms" for phenylbutyramidinated enzymes and their substrates shown in Figures 12, 13, 14 and 15 have provided some very valuable information for enzyme immobilizations.
The plot also gives another valuable information: $K_F$ values which are related to the weight pickup or the concentration of the adsorbate on the adsorbent. The adsorption capacity for modified enzymes is higher than that of BTEE and BAEE under the same condition. In Figure 15, the $K_F$ values for both BAEE and BTEE curves are smaller than those of the phenylbutyramidinated enzymes.

In addition, the isotherm curve also indicated the strength of the affinity force and the amount of desorption at assumed equilibrium, which can be seen in Figure 13. In order to prevent leakage of the immobilized enzymes from carriers, we must control the amount of proteins per unit adsorbent.

Moreover, slopes of the plots in Figures 14 and 15 indicate how sensitively the adsorption equilibrium responds to the adsorbate concentration change. A smaller $n$, a steep slope could mean that the adsorption capacity changes drastically with a small change in the concentration. This also has implications for the conditions of application of immobilized enzymes.
CHAPTER III. IMMOBILIZATION OF PHENYLBUTYRAMIDINATED ENZYMES

1. Introduction

Although enzyme immobilization offers several advantages and can be very profitable, the restriction of enzymes to a particular microenvironment may change their conformations and alter both their kinetic and other enzyme properties. As a result of such alterations, enzyme activity is usually reduced due to the following factors (Trevan, 1980):

1. Conformational and steric effects. A decrease of enzymatic activity could occur on binding to solid supports by either covalent bonds or adsorption by two different mechanisms: the binding and/or the enzyme-carrier interactions might alter the three-dimensional structure of the immobilized enzyme; the binding may also introduce additional steric hindrance so as to impose new limits on its accessibility to substrate.
2. Partitioning effects. These effects are related to the chemical nature of the support, arising particularly from electrostatic or hydrophobic interactions between the matrix and low molecular weight species in the solution. Partitioning effects cause different local concentrations of charged species, substrates, products, inhibitors, hydrogen ions, hydroxyl ions, and so on, by their attraction to or repulsion from the carriers in the absence of mass transfer effects.

3. Internal and external mass transfer effects. When an enzyme is immobilized on or within a solid carrier, mass transfer effects may exist because the substrate must diffuse from the bulk solution to the active site of the immobilized enzyme, and the product must diffuse in an opposite direction. The diffusional resistance during the translocation may be classified as an internal mass transfer effect when the enzyme is immobilized in a porous support. External mass transfer effects occur between the bulk solution and the outer surface of the enzyme-carrier particle.

In this section, phenomena related to the above factors, such as the activity retained after immobilization at different protein loading levels, using beads with different composition, and different particle size have been investigated individually. The adsorption
of modified enzymes and their substrates, which is involved in the partitioning effect, have been examined in a previous section. The phenomena related to internal mass transfer will be described in the next chapter.

2. Methods

2.a Immobilization of phenylbutyramidinated enzymes

An equal volume of dialyzed protein solution and 0.1 M triethanolamine/HCl buffer, containing 0.04 M CaCl$_2$, 0.2 M KCl, pH 7.5 were mixed with the porous polymer beads by end-to-end inversion in a cold room from 2 to 24 hours depending on the relative amounts of the enzymes and the beads (0.002-500 mg/g). At loading levels less than 50 mg/g, two hours of adsorption were sufficient. After the adsorption, the beads were then washed 3 times with 1 M KCl in 0.02 M CaCl$_2$, 0.05 M triethanolamine-HCl buffer, pH 7.5, and 3 times with 40% ethylene glycol in the same buffer without KCl. Finally, the beads were washed thoroughly with the buffer and stored in a cold room. The enzymatic activity on the beads was assayed with a pH stat and compared with that of the free enzyme.
2.b Immobilization on beads of different size

Amberlite XAD-8 beads were separated into three size groups using US Standard Sieves: 100-250 μm, 250-420 μm and 420-800 μm. The phenylbutyramidinated enzymes were immobilized on these groups of XAD-8 beads at various loading levels.

2.c Immobilization on beads of different hydrophobicity

Although differing in hydrophobicity, both polystyrene beads and polyacrylate beads have excellent adsorption capacity. Amberlite XAD-2 and XAD-8, a typical polystyrene bead and a typical polyacrylate bead, were employed for the immobilization. Enzyme activity recovered after immobilization was studied with α-chymotrypsin, using a hydrophobic substrate, BTEE, which adsorbed onto both beads very easily.

3. Results

3.a Activity retention after immobilization

The effect of immobilization on catalytic activity was investigated by comparing phenylbutyramidinated soluble enzyme with immobilized phenylbutyramidinated enzyme. In Figure 17, phenylbutyramidinated chymotrypsin was immobilized on the different Amberlite beads at 0.002 - 2 mg of enzyme per gram of beads. Except polystyrene beads,
Figure 17. Activity of phenylbutyramidinated chymotrypsin on different beads as compared to soluble modified enzyme.

The activity of soluble modified chymotrypsin (♦), and modified chymotrypsin one day after immobilization on Amberlite XAD-2 (♦), XAD-7 (■), XAD-8 (●) and XAD-71 (x) are shown per mg of polymer beads. Chymotrypsin was modified with MPBI on about 60% of its amino groups.
XAD-2, all three polyacrylate beads, XAD-7, XAD-8 and XAD-71 showed a very good activity recovery for immobilized chymotrypsin. Our results showed that the activity retention is related to enzyme loading levels. At lower loading levels, the difference between free and immobilized enzyme was less than that at higher loading levels, as the availability of the substrate for the each immobilized enzyme molecule decreased with increasing concentrations of enzyme in the beads (Trevan, 1980). The activities of several other enzymes on polyacrylate beads were also very efficient (Ampon and Means, 1988).

3.b Activity retention on the beads with different particle size

The influence of bead size was also investigated. α-Chymotrypsin was immobilized on Amberlite XAD-8 beads of different size, 100-250 μm, 250-425 μm and 425-800 μm. The phenylbutyramidinated enzyme was immobilized on these three different sized beads at various loading levels. Figure 18 shows that the levels of enzyme activity are greatest on the small beads and appears to be related to their external surface areas (Table III). External surface areas were calculated according to the apparent density and the median size of the bead fractions (Gregg and Sing,
Figure 18. The effect of bead size on the activity of immobilized chymotrypsin.

Activity for different amounts of chymotrypsin are shown as determined one day after immobilization on Amberlite XAD-8 100-250 μm (○), 250-425 μm (■) and 425-800 μm (▲). Before immobilization, about 60% of amino groups of chymotrypsin was modified with MPBI.
Table 3. Comparison of different sizes of Amberlite XAD-8 beads.

<table>
<thead>
<tr>
<th>Dry bead diameter (μm)</th>
<th>425-800</th>
<th>250-425</th>
<th>100-250</th>
</tr>
</thead>
<tbody>
<tr>
<td>External surface area (cm²/g)</td>
<td>160-300</td>
<td>300-510</td>
<td>510-1280</td>
</tr>
<tr>
<td>Estimated external surface area °f wet bead (cm²/g)</td>
<td>130-240</td>
<td>240-410</td>
<td>410-1020</td>
</tr>
<tr>
<td>Activity (μmoles/mg)</td>
<td>0.016</td>
<td>0.0238</td>
<td>0.0412</td>
</tr>
<tr>
<td>Activity^a (μmoles/cm²)</td>
<td>0.087</td>
<td>0.074</td>
<td>0.058</td>
</tr>
</tbody>
</table>

a: estimated median external surface of wet bead was used for calculation.
where $A$ is the external surface area ($m^2/g$), $p$ is the apparent density and $l$ is the diameter of the beads.

Activities of immobilized chymotrypsin appeared to be greatest at loading levels from 5-20 mg/g beads, and declined somewhat at slightly higher levels as shown in Figure 18. This will be discussed in the next chapter.

Another experiment comparing Amberlite XAD-7 with XAD-71 also showed smaller beads like Amberlite XAD-71 exhibited more activity than Amberlite XAD-7 (Figure 19).

3.c Immobilization on beads of different hydrophobicity

The influence of bead compositions and structures on the activity has also been investigated. Amberlite XAD-8, a polymethacrylate ester, was compared with XAD-2, a polystyrene-divinylbenzene polymer of similar particle size, therefore similar external surface area, but slightly different porosity. Both have excellent adsorption capacities for the phenylbutyramidinated enzymes. Ampon and Means (1988) have reported that trypsin after immobilization on Amberlite XAD-2, retained little enzymatic activity. Because the substrate of trypsin was BAEE, a positively charged ester, it was not clear whether
Figure 19. Comparison chymotrypsin activity on Amberlite XAD-7 and XAD-71.

Chymotrypsin on Amberlite XAD-7 (●), and on XAD-71 (▲). Before the immobilization, about 60% of amino groups of chymotrypsin were modified with MPBI. Immobilized chymotrypsin was incubated at 70°C after 8 days.
the enzyme was still "active" but with very poor access to the hydrophilic substrate, or was inactivated due to strong interactions with the very non-polar polymer surface. In order to clarify this, chymotrypsin and its hydrophobic substrate, BTEE, were employed. The experimental data showed that the initial activity of chymotrypsin immobilized on XAD-2 was less than 25% of that on Amberlite XAD-7 or XAD-8 (Figure 17). Moreover, activities decreased with time, such that after 35 days, the activity was undetectable. This decline was much faster than that due to autolysis of soluble phenylbutyramidinated chymotrypsin (Figure 20).

4. Discussion

4.a Activity retention after immobilization

Since no enzymatic activity was lost during chemical modification, and the phenylbutyramidinated proteins are almost completely adsorbed, the effects of immobilization are important for the efficiency of this method. As shown in Figure 17, considerable enzyme activity was recovered on all three polyacrylate beads especially on XAD-8 and XAD-71. At low levels, activities were only slightly lower than obtained with the soluble enzyme. The activities increased with the amount of enzyme adsorbed, but with decreasing specific activities at higher loading levels.
Figure 20. Activity of immobilized chymotrypsin decreases with time on Amberlite XAD-2 beads.

Activities of soluble modified chymotrypsin at 3 mg/ml (♦) and immobilized at 10 mg/g on Amberlite XAD-2 (♦) and on XAD-8 (○). About 60% of amino groups of chymotrypsin was pre-modified with MPBI. The beads were stored in a cold room at 7±1°C.
Such decreasing specific activity are commonly observed with immobilized enzyme system and can be explained by the following intrinsic microenvironment problems of enzyme immobilization (Trevan, 1980). First, the local concentration of the enzyme increases dramatically after the immobilization, and the availability of substrates for each enzyme molecule may therefore be reduced significantly. Secondly, substrate depletion and product accumulation may take place inside the porous beads due to diffusional limitations.

Enzyme-carrier interactions, are not likely to account for decreases in specific activity observed only at high enzyme loading levels. It has been reported, in some cases that for the immobilization involving unfavorable enzyme-carrier interactions, the recovery of activity is actually greater at higher enzyme loading levels (Kamei et al., 1987). In our case, however, immobilization by spontaneous adsorption on Amberlite XAD-7, XAD-8 and XAD-71 does not depress the enzymatic activity significantly at low enzyme loading levels. Structural studies of the phenylbutyramidinated enzyme before and after immobilization after spin labelling using electron spin resonance will be discussed separately later.
4.b Activity retention on beads of different size

As shown in Figure 18, more activity has been recovered in the fraction of 100-250 µm Amberlite XAD-8 beads than larger bead fractions, more or less in proportion to its external surface area per unit of beads. Since small beads will have a greater ratio of external to internal surface, more enzyme should be immobilized on external surface in such cases. As substrates usually have a higher concentration and products a lower concentration at external as compared to the internal surfaces due to the diffusional limitations, smaller beads should be more efficient enzyme supports.

4.c The effect of bead composition

Although differing in hydrophobicity, both polystyrene beads and polyacrylate beads have excellent adsorption capacity. Amberlite XAD-2, a typical polystyrene bead and XAD-7 and XAD-8, typical polyacrylate beads, of similar size were employed for immobilization. Enzyme activities recovered were studied with α-chymotrypsin, using a hydrophobic substrate, BTEE which adsorbed to both types of beads very easily.
As shown in Figure 17, phenylbutyramidinated chymotrypsin had less activity when immobilized on XAD-2 than it did when immobilized on XAD-7 or XAD-8, although its hydrophobic substrate, BTEE, has high affinity for each of those polymer beads according to our previous adsorption rate studies (Figure 16). The low activities and rapid inactivations on Amberlite XAD-2 (Figure 20) were therefore probably not due to poorer accessibility of the substrate but due to conformational changes in the enzyme after immobilization according to ESR experiments. When spin labeled chymotrypsin was immobilized on XAD-2, a conformational change in the active site was observed in EPR spectra (discussed in the appendix A).

5. CONCLUSION

In using methyl 4-phenylbutyrimidate to increase the hydrophobicity of enzymes, the compatibility of high adsorption and good activity retention became possible in this simple, inexpensive enzyme immobilization procedure. When used as adsorbents, Amberlite XAD-7, XAD-8, and XAD-71 have excellent capacities for phenylbutyramidinated enzymes. In general, the adsorption will not be limiting in this procedure, because these organic polymer beads can adsorb enzymes up to their own weight. Enzyme activities did not to decrease upon phenylbutyramidination. Activity
retention after adsorption were also very high, and there is no significant loss in the extremely low protein loading levels. Smaller beads with larger radio of external surface to internal surface appeared to show higher activity per unit weight after immobilization. The effects of the enzyme loading levels, internal diffusion of adsorbed enzyme, and study on the active site conformational change with EPR, using spin labeling, will be discussed in the appendix A.
CHAPTER IV. ENZYME ACTIVITIES AT DIFFERENT LOADING LEVELS AND THEIR CHANGES WITH TIME

1. INTRODUCTION

A large number of studies on enzyme immobilization have been published. High catalytic activity in a small volume is a highly desirable attribute for most immobilized enzyme systems as volumetric productivity is important for industrial processes (Hartmeier, 1988). The effects of different enzyme loading levels on solid supports have only rarely been reported. Several papers have only briefly mentioned the enzyme loading influences (Woodward, 1985; Trevan, 1980; Lasch and Koelsch, 1978). The difficulty in these kind of studies is that the amount of enzyme immobilized on the carrier is limited by the impairment of coupling method for the immobilization. As mentioned before, the porous organic polymer beads, Amberlite XAD-7 and XAD-8 can adsorb phenylbutyramidinated enzymes in various amount and even exceeding their own weight.
In this study, the influence of loading levels on the activities of immobilized chymotrypsin and trypsin and changes in activity with time are evaluated. In this case activities are decisively affected by the amount and the initial distribution of those enzyme in the porous beads. The activities of immobilized enzymes also change with time, probably due to "diffusion" of the adsorbed enzyme in the porous beads.

2. Methods

2.a Scanning electron micrographs of the immobilized enzyme

Amberlite XAD-7 and XAD-8 beads with different amounts of immobilized enzyme were cut in half using a razor blade. The cut beads were fixed with OsO$_4$ for 24 hours and then sputter-coated with gold using a Technics Hummer III. The pictures were kindly taken by Mr. David Stutes using a scanning electronic microscope (Hitachi Model S 500) fitted with a Polaroid camera.

2.b Staining proteins immobilized on Amberlite XAD-8 beads

Amberlite XAD-8 beads with different amounts of immobilized enzyme were stained with 0.1% (w/v) naphthol blue black in 3% (w/v) aqueous trichloroacetic acid for 1 hour (Ampon, 1987; Carleysmith et al., 1980b). Excess dye
was removed by rinsing the beads with 2% aqueous solution of acetic acid, then with 50% ethanol. The wash cycle was repeated several times until the washes were colorless.

Beads were split using a razor blade to obtain cross sections. The pictures were taken using a Nikon FX-35 camera attached to a Nikon Optiphot microscope. Magnification on the microscope was 50 X. Exposure compensation indicator was set at 0, or ±1 stop, using a Nikon AFX-II control box.

2. c Bead grinding

Trypsin- and chymotrypsin-containing Amberlite XAD-7 or XAD-8 beads in a drop of appropriate buffer were ground in polypropylene reaction vials by applying pressure with a broad flat stainless steel spatula. Under such pressure, the beads were reduced to a large number of very fine fragments.

2. d Active site titration of immobilized trypsin

Active site titrations were done by a modification of the method described by Chase and Shaw (1967). Amberlite XAD-8 beads to which trypsin was immobilized were ground as described before. About 50 mg of ground beads were mixed with 4 ml of 0.1 M Barbital buffer, containing 20 mM CaCl₂, pH 8.3. To the solution, 20 μl of 0.01 M p-nitrophenyl-m-
guanidinobenzoate (NPGD) was added, and mixed for 2 minutes. After low speed centrifugation for 3 minutes, 3 ml of supernatant was collected, and the absorbance at 420 nm was recorded using a Cary 118c.

3. RESULTS

The polyacrylate beads employed in this study were all porous with different sized micropores, and particle sizes are listed in Table 2. The micropores in the beads are continuous and interconnected.

3.a Activity-loading level curve

As shown in Figures 21 and 22, an interesting activity-loading level curve for immobilized enzymes was observed: as the loading levels of immobilized enzyme on the polymer beads increased, in the range of about 0-20 mg/g beads, the activities increased. This is the most efficient enzyme concentration range for the activity recovery. Activities then decreased from about 20 to 50 mg per gram beads. And after about 100 mg/g beads, again began to rise gradually.
Figure 21. Activity comparison for trypsin between crushed beads and intact beads.

Trypsin was immobilized on Amberlite XAD-8 (250-425 μm) at day 1. Intact beads (○), and crushed beads (■). About 60% of amino groups were modified with MPBI.
Figure 22. Activity comparison for chymotrypsin between crushed beads and intact beads.

α-Chymotrypsin was immobilized on XAD-8 (250-425 μm) at day 1. Intact beads (●), and crushed beads (■). About 60% of amino groups were modified with MPBI.
3.b Activity curve of ground beads

If the beads were crushed before each assay, a different activity-loading level curve was obtained (Figures 21 and 22). Crushing, which causes the beads to shatter into much smaller pieces, greatly increases the amount of external surface area as well as the ratios of external to internal surface area. Unlike activity-loading curves with intact beads, activities steadily increase with increasing amounts of enzyme to a somewhat higher limiting value.

3.c Changes in activity-loading curves with time

There were substantial changes in activity-loading curves after immobilization.

For α-chymotrypsin, the changes in activity curves are very interesting. The high activity at about 20 mg/g at day 1 declined gradually for several days and disappeared after about 2 weeks. Activities at higher loading levels, > 50 mg/g beads, however actually increased slightly after incubation at 7°C (Figure 23). Similar results have also been observed on Amberlite XAD-71 as shown in Figure 24.
Figure 23. The loading effect on activities of chymotrypsin immobilized on Amerlite XAD-8 assayed at different times.

α-Chymotrypsin was immobilized on Amberlite XAD-8 for 1 day (●), 11 days (▲), and 50 days (■). About 60% of amino groups were modified with MPBI. The immobilized enzyme was stored in a cold room at 7°C.
Figure 24. The loading effect on activities of chymotrypsin immobilized on Amberlite XAD-71 assayed at different times.

α-Chymotrypsin was immobilized on Amberlite XAD-71 for 1 day (○), 16 days (■), and 50 days (▲). About 60% of amino groups were modified with MPBI. The immobilized enzyme was stored in a cold room at 7°C.
Somewhat different changes in activity were observed with trypsin on Amberlite XAD-8 as shown in Figure 25. The high activity initially observed at about 20 mg/g of trypsin also declined, but unlike immobilized chymotrypsin, the activity at all levels declined.

3.d Changes in activity-loading curve of ground beads

Although the activity-loading level curves of trypsin and chymotrypsin changed with time, little change was observed in the case of chymotrypsin, when the beads were crushed before assay for up to three months (Figure 26). Similar results were obtained for trypsin but with a slight decreases at higher loadings, above 200 mg/g beads (Figure 27).

3.e Activity changes for immobilized chymotrypsin and trypsin

As indicated in Figures 23 and 24, the activity-loading curves of immobilized enzymes on intact beads changed after immobilization. The activity changes of immobilized chymotrypsin with time are shown in Figure 28 at three different loading levels. These figures suggest that the decrease in activities of immobilized chymotrypsin with time is not serious at most loading levels.
Figure 25. The loading effect on activities of trypsin immobilized on Amerlite XAD-8 assayed at different times.

Trypsin was immobilized on Amberlite XAD-8 for 1 day (●), 8 days (▲), and 130 days (■). About 60% of amino groups were modified with MPBI. The immobilized enzyme was stored in a cold room at 7°C.
Figure 26. Activity of immobilized chymotrypsin in ground beads with respect to load.

α-Chymotrypsin was immobilized on Amberlite XAD-8 for 2 days (▲), and for 117 days (●). About 60% of amino groups were modified with MPBI. The immobilized enzyme was stored in a cold room at 7°C. The beads were ground before the activity assay.
Figure 27. Activity of immobilized trypsin in ground beads with respect to load.

Trypsin was immobilized on Amberlite XAD-8 for 2 days (▲), and on XAD-8 for 117 days (●). About 60% of amino groups were modified with MPBI. The immobilized enzyme was stored in a cold room at 7°C. The beads were ground before the activity assay.
Figure 28. Changes in activity of immobilized chymotrypsin with time.

Chymotrypsin was immobilized on XAD-8 at three loading levels: 10 (O), 15 (●) and 200 (★) mg/g beads. Amino groups of chymotrypsin were modified with MPBI (60%). The beads were stored in a cold room at 7°C.
However, as the result given previously (Figure 25), the activities of immobilized trypsin appeared to decrease after immobilization. The time course of activity decrease at three different loading levels is shown in Figure 29. Unlike immobilized chymotrypsin, apparent activities of immobilized trypsin decrease significantly with time.

Trypsin is known to be more susceptible to autolysis than chymotrypsin. To determine whether autolysis accounts for the greater decline in the activity of immobilized trypsin, the beads on which trypsin was immobilized were crushed at various times and assayed for both activity against BAEE and for the number of active sites with pNPGB. The results obtained with phenylbutyramidinated trypsin and native trypsin adsorbed on Amberlite XAD-8 are compared in Figure 30. As shown in the figure the activity and the number of active sites changed very little with time, indicating very little autolysis in the beads. The activity of phenylbutyramidinated trypsin after immobilization on Amberlite XAD-8 was initially somewhat higher than that of unmodified trypsin but slowly decline to a comparable level without significant decrease in the number of active sites in the time period.
Figure 29. Changes in activity of immobilized trypsin with time.

Trypsin was immobilized on XAD-8 at three loading levels: 10 (○), 15 (●) and 200 (▲) mg/g beads. Amino groups of trypsin were modified with MPBI (60%). The beads were stored in a cold room at 7°C.
Figure 30. The changes of activity and the number active site of immobilized trypsin on ground beads with time.

Trypsin was immobilized on XAD-8 at 10 mg/g beads. Native trypsin (▲) and trypsin was modified with MPBI on its amino groups about 60% (●). A: active site titration; B: activity assay. The beads were stored in a cold room at 7°C.
Besides autolysis, another factor, the "diffusion" of trypsin from near the external surface of the beads to their interior, can cause the decrease of activities of immobilized trypsin. The activities of phenylbutyramidinated trypsin immobilized on Amberlite XAD-7 and XAD-8 are compared with that of unmodified trypsin adsorbed on the Amberlite XAD-7 and XAD-8 beads in Figure 31. The activities of phenylbutyramidinated trypsin was initially higher than those of unmodified trypsin but decreased with time, while the activities of adsorbed trypsin without modification was low but relatively stable. These results were consistent with a picture of beads with stained protein showing the unmodified trypsin diffusing faster than modified trypsin. As shown in figure 32, at the first day after immobilization, unmodified trypsin is already located throughout the Amberlite XAD-8 beads and after 3 days, was more or less uniformly distributed throughout the beads. On the other hand, phenylbutyramidinated trypsin appear to diffuse much more slowly and, as shown in Figure 33, was located initially only near external surface and was not far removed from the exterior even after 80 days.
Figure 31. The activity changes of trypsin immobilized under different conditions.

Trypsin was immobilized at loading level of 10 mg/g beads. Native trypsin adsorbed on XAD-7 (○) and XAD-8 (○) and trypsin with 60% of its amino groups modified with MPBI, and then immobilized on XAD-7 (■) and XAD-8 (●). The beads were stored in a cold room at 7°C.
Figure 32. The diffusion of adsorbed native trypsin from the exterior to the interior of beads.

Trypsin was adsorbed on Amberlite XAD-8 (100 mg/g beads) A: for 1 day; B: for 3 days. The beads were stored in a cold room at 7°C. The bead sizes ranged from 425-700 μm.
Figure 33. The diffusion of immobilized trypsin from the exterior to the interior of beads on XAD-8.

A: Trypsin was modified with MPBI (about 60%) then immobilized on Amberlite XAD-8 (100 mg/g beads) A: for 1 day; B: for 20 days and C: for 80 days. The beads were stored in a cold room at 7°C. The bead sizes ranged from 425-700 µm.
Another comparison was made between trypsin immobilized on Amberlite XAD-7 (average pore size 90 Å) and XAD-8, which has an average pore size 225 Å. Both beads have similar chemical compositions and are of similar particle size, but with different average pore sizes. As also shown in Figure 31, the activity of phenylbutyramidinated trypsin immobilized on Amberlite XAD-7 was initially higher than that on Amberlite XAD-8. This may be due to a faster diffusion of trypsin on Amberlite XAD-8, making a greater fraction of trypsin less accessible to substrate even at day 1. The difference in activities recovered on Amberlite XAD-7 and XAD-8 was greater in the beginning, and became smaller and smaller with time (Figure 31). The diffusion of trypsin immobilized on Amberlite XAD-7 as seen with stained protein (comparing figures 33 and 34), also appeared slower than that on Amberlite XAD-8.

The diffusion of chymotrypsin has also been observed as shown in Figure 35.

3.f The activity-loading curves on different beads

The most distinctive feature of the activity curves at different loading levels is that activities decrease with the increase of enzyme loading in certain ranges. The sharpness of the decrease of activity curve is related to the nature of the beads. α-Chymotrypsin was immobilized on
Figure 34. The diffusion of immobilized trypsin from the exterior to the interior of beads on XAD-7.

A: Trypsin was modified with MPBI (about 60%) then immobilized on Amberlite XAD-7 (100 mg/g beads) A: for 1 day; B: for 20 days and C: for 80 days. The beads were stored in a cold room at 7°C. The bead sizes ranged from 425-700 μm.
Figure 35. The diffusion of stained chymotrypsin from the exterior to the interior of beads.

A: α-chymotrypsin was immobilized on Amberlite XAD-8 for 1 day; B: for 450 days. About 60% of amino groups were modified with MPBI. The protein loading level in the beads is 100 mg/g beads. The beads were stored in a cold room at 7°C. The bead sizes ranged from 425-700 μm.
Amberlite XAD-7 and XAD-8 respectively. The activity-loading curves obtained at day 1 were given in Figure 36: Amberlite XAD-7 with a smaller micropore size displays a rapid drop in apparent activity as the enzyme loading increases. While the activity-loading curve of the chymotrypsin immobilized on Amberlite XAD-8 decrease less deeply and less sharply.

In both cases the activities change with time. Figure 37, for example, shows the activities on Amberlite XAD-7 and XAD-8 55 days after immobilization. The higher activity at about 20 mg/g at that time has decreased only slightly on Amberlite XAD-7 but is completely eliminated on Amberlite XAD-8.

3.g Immobilized chymotrypsin and co-immobilized chymotrypsinogen

To understand how loading levels and diffusion influence the activity, phenylbutyramidinated chymotrypsin was immobilized on Amberlite XAD-8 in various combinations with the phenylbutyramidinated derivative of its catalytically inert zymogen, chymotrypsinogen.

In Figure 38, phenylbutyramidinated chymotrypsinogen was immobilized on Amberlite XAD-8 at two different loading levels, 20 and 100 mg/g beads first. Phenylbutyramidinated chymotrypsin was then immobilized on those beads. The
Figure 36. Chymotrypsin immobilized on the different beads at day 1.

α-Chymotrypsin was immobilized on Amberlite XAD-7 (●) and XAD-8 (★). The amino groups of the enzyme were modified with MPBI about 60%.
Figure 37. Chymotrypsin immobilized on the beads with different pore sizes after 55 days.

α-Chymotrypsin was immobilized on Amberlite XAD-7 for 55 days (●), and XAD-8 for 55 days (▲). About 60% of amino groups were modified with MPBI. The immobilized enzyme was stored in a cold room at 7°C.
Figure 38. Activity of immobilized chymotrypsin with and without pre-immobilized chymotrypsinogen.

Chymotrypsin was immobilized on Amberlite XAD-8 at the loading levels shown in the figure (●). Chymotrypsin was immobilized on the beads pre-loaded with 20 mg/g (▲) and 100 mg/g of phenylbutyramidinated chymotrypsinogen (■). Amino groups of both chymotrypsin and chymotrypsinogen were modified with MPBI (60%).
activity-loading curves of chymotrypsin on Amberlite XAD-8 and on Amberlite XAD-8 pre-loaded with 20 mg/g and 100 mg/g of phenyl-butyramidinated chymotrypsinogen are shown in Figure 38. With 100 mg/g of pre-loaded chymotrypsinogen, the resulting activity profile differs markedly from that obtained without prior addition of phenylbutyramidinated chymotrypsinogen. The peak in activity at low levels of phenylbutyramidinated chymotrypsin appears to shift slightly when pre-loaded with 20 mg/g of phenylbutyramidinated chymotrypsinogen, whereas with 100 mg/g the activity has no such peak but rises smoothly, more like that observed on crushed beads (Figures 21 and 22). The possible explanation is that at this loading level, the beads become less porous. When loaded with phenylbutyramidinated protein at 100 mg/g, a noticeable shell of protein was formed on the outer surfaces of the beads, which was shown in the pictures of stained beads (Figures 33 and 34). Pictures of stained beads with different loading levels of enzyme are shown in Figure 39. When enzyme loading levels below 20 mg/g only a very faint shell of stained protein is seen at the beads surface. As the protein loading level increased, the protein shell became thicker and darker. Scanning electron microscope pictures, for example, appear to show some blockage of the surface of micropores at high loading levels (Figure 40).
Figure 39. The stained beads with different trypsin loading levels.

About 60% of amino groups of trypsin were modified with MPBI and immobilized on XAD-8 at day 1. The protein loading levels: A (10); B: 20; C: 50; D: 100; E: 300 and F: 500 mg/g. The bead sizes ranged from 425-700 μm.
Figure 40. Scanning electron microscopic picture of immobilized enzyme.

A: the external surface of Amberlite XAD-8; B: α-chymotrypsin immobilized on Amberlite XAD-8 for 2 days. About 60% of amino groups were modified with MPBI. The protein loading level in the beads is 300 mg/g beads.
The lower activities observed at high loading levels (above 50 mg/g beads) on intact beads thus appear to be related to the porous structure of the carrier, which is lost either by crushing or by pre-loading with large amounts of protein.

Phenylbutyramidinated chymotrypsinogen were also co-immobilized with phenylbutyramidinated chymotrypsin (1:1 mixture). At total protein loading levels above 50 mg/g, activity-loading curves appeared similar regardless pure chymotrypsin or chymotrypsin-chymotrypsinogen mixture loaded (Figure 41). However, at loading levels below 50 mg/g, mixture of chymotrypsinogen-chymotrypsin had lower activities, and activity peak was displaced to higher protein amount as compared with immobilized chymotrypsin. Also inert protein actually increases activity at higher levels.

The effect of phenylbutyramidinated chymotrypsinogen on previously immobilized phenylbutyramidinated chymotrypsin on Amberlite XAD-8 (10 mg/g), is shown in Figure 42. At low levels of phenylbutyramidinated chymotrypsinogen, from 5 to 20 mg/g beads, little effect was observed. But activity decreased steadily after more than 30 mg/g of phenylbutyramidinated chymotrypsinogen was immobilized.
Figure 41. Activities of immobilized chymotrypsin and immobilized mixture of chymotrypsin and chymotrypsinogen.

Chymotrypsin was immobilized on XAD-8 at the loading levels shown in the figure. Immobilized pure chymotrypsin (●); immobilized mixture (1:1) of chymotrypsin and chymotrypsinogen (▲). Amino groups of both chymotrypsin and chymotrypsinogen were modified with MPBI (60%).
Figure 42. Effect of added chymotrypsinogen on the activity of immobilized chymotrypsin.

Chymotrypsin was immobilized on Amberlite XAD-8 at 20 mg/g and its activities were determined with increasing amounts of chymotrypsinogen which was immobilized on the beads after immobilization of chymotrypsin (▲). Activities of immobilized chymotrypsin at same range of loading are shown for comparison (●).
4. DISCUSSION

4.a Activity-enzyme loading curves

Activity-loading curves for immobilized enzymes have only rarely been reported before (Traven, 1980). Those in this work which show a decline in activity when the amounts of immobilized enzyme increase are unusual. However, a similar decline activity with an increase in substrate concentration has been reported in the case of an immobilized enzyme by Engasser and Horvath (1974). No mechanism for the activity decline was discussed, but they did mentioned "substrate inhibition due to diffusion resistance" once.

As shown in Figures 21, 22 and 38, the decline with increasing enzyme levels was only observed for the enzyme immobilized on intact porous beads. When the beads were ground into finer particles before assay, or when the beads were pre-loaded with 100 mg/g of an inert protein, no decline in activity was observed. After the porous beads were crushed, a large portion of their original internal surface is exposed to the bulk solution as external surface. This suggests that the decline may be related to some limitation of internal surface, possibly a diffusional limitation on both adsorbed enzyme and its substrate. Pre-loading with inert protein may block some of those internal surfaces forcing a greater proportion of
subsequently adsorbed enzyme to reside at or near the external surface. In both cases, internal surface of beads are either eliminated or made less available. The high adsorption affinity and diffusional limitation of adsorbed enzyme in the porous beads seems to be the primary reasons for the unexpected decline observed in those activity-enzyme loading curves.

4.b The changes of activity-loading curve

The activities of immobilized enzymes changed significantly with time as shown in Figures 23, 24, 25, 28 and 29. With the ground beads, little change was displayed after immobilization (Figures 26, 27 and 30). This suggests that the intrinsic activity of the immobilized enzyme is relatively stable, and the changes are probably due to changes in protein distribution in the beads, presumably, as a result of diffusion. The diffusion of the immobilized enzymes from exterior surface into the inner surface with time can be seen by staining the beads (trypsin in Figure 33 and chymotrypsin in Figure 35). Immediately after adsorption, it is located at the periphery of the beads and with time appears to diffuse further into the beads, eventually achieving a relatively uniform distribution throughout the bead matrix.
4.4 Difference between trypsin and chymotrypsin

The changes in activity with time were different for immobilized chymotrypsin and trypsin. With high loading levels of immobilized chymotrypsin, the activity increased slightly with time (Figures 23 and 24). This may be due to an increase in surface area available to both the enzyme and the substrate as a result of enzyme diffusion. The activity increase after immobilization has also been reported as a result of the further diffusion of the substrate (Messing, 1974).

The activity of immobilized trypsin, however, always decreased with time at both high and low loading levels (Figures 25 and 29). From examinations of stained beads it was clear that the phenylbutyramidinated trypsin diffused into the beads as shown in Figure 33. The substrate employed in the case of trypsin, BAEE, however, is cationic and not adsorbed strongly by the beads (Figures 15 and 16). The absence of strong substrate-carrier adsorption, affects local concentrations of substrate, and may also influence the apparent activities of the immobilized enzymes and account for the described decrease in activity with time. This is the so-called partitioning effect in enzyme immobilization (Kennedy and Cabral, 1983). If the partition effect is unfavorable to the substrate, when the enzyme diffuses in the pores, the substrate can not easily
reach the enzyme. One possible explanation for the decrease of immobilized trypsin activities with time could be the consequence of the enzyme diffusion combined with the partition effect on substrate availability.

4.d The influence of different beads

The nature of the beads (ie. polystyrene vs. polyacrylate and different types of polyacrylates, Amberlite XAD-7 vs. XAD-8) influences the recovery of activity at low levels (see Figure 17) and at high levels (Figures 36 and 37), as well as the stability of those activities with time (Figure 37). Activities on Amberlite XAD-7 at low loading levels were initially somewhat higher than on Amberlite XAD-8. One possible explanation could be that Amberlite XAD-8, with larger micropores, has less diffusional limitation for the adsorbed enzyme which initially penetrates more deeply into the beads where it has less access to substrate.

The rate of change in the activity-loading curves is also affected by the types of polymer beads. About two months after immobilization, there still is a decline in the activity-loading curves between about 20 mg/g and 100 mg/g of chymotrypsin on Amberlite XAD-7 (Figure 37), similar to that observed immediately after immobilization (Figure 36). With Amberlite XAD-8, however, there is a
completely loss of the initial high activity at about 20 mg/g and a slightly increase at high levels, thus a smooth activity curve is observed (compare Figures 36 and 37). The activities of both types of beads after grinding, however, gave smooth gradual increases in activity which changed little over that time period (Figures 26 and 27). As shown in Figures 33 and 34, the adsorbed enzyme diffuses faster on Amberlite XAD-8 than XAD-7, so the changes with time may be a reflection of the diffusion factor. The faster diffusion on the other hand, probably results from larger pore size of Amberlite XAD-8, which offers less diffusional limitations to the adsorbed enzymes. Since the average micropores are no more than a few protein molecular diameters in width, the pore size is probably an important factor influencing the rate of enzyme diffusion in the beads.

4.e Effect of diffusional limitation

The high local concentrations of immobilized enzyme often achieved means that with normal substrate concentrations, only a fraction of the enzyme might be involved in the catalytic reaction (Trevan, 1980). With increasing amounts of enzyme, the activity would, however, presumably rise smoothly to some limiting value. The chemical and physical mechanisms responsible for the
observed decline in the activity-loading curves is not understood completely at this point. However, the following experimental results may lead to an explanation.

One possible mechanism might involve a decrease in the effective pore diameter due to partial blockage by the adsorbed enzyme as shown in Figures 39 and 40. Similar blockage might be responsible for the decline in activity-loading curves.

At low enzyme loading levels most phenyl-butyramidinated enzyme remains at the external surface of the beads, but some may diffuse deeply into the beads as shown in Figure 39.a.

At high loadings, large amount of protein was adsorbed on the outer surfaces of the beads. Evidences for the build-up of a blocking shell on external surfaces can be seen in Figures 33, 34 and 39. As shown in Figure 39, some faint staining appears to penetrates deeply into the beads. At 20 mg/g, a stained thin but darker protein shell would been seen the outer surface. At still higher loading levels the stained protein shell became thicker. This is the range in which activity declines (ie. about 20 to 50-70 mg/g). At 100 mg/g and above the stained shell is dark blue and increases mainly in its thickness. This is the range in which enzyme activities again begin to rise but only very slightly (see Figures 24, 38 and 41). As shown
in Figure 42 the addition of a catalytically inert protein, phenylbutyramidinated chymotrypsinogen decreased the activity of previously immobilized chymotrypsin to a similar or even greater extent. This suggests that the decline is due simply to the addition of protein and is not due to specifically the addition of chymotrypsin.

After a protein shell is built up on the outer surfaces of the beads, it could slowly "diffuse" deeper into the beads since it is not covalently immobilized on the surface. Upon so doing, the blocking of pore opening could be reversed. Therefore, initial activity-loading curves can be changed after immobilization (Figures 23, 24 and 25). If this is true, the rate of activity-loading curve change should be related to the pore size of the beads. The comparison of immobilized chymotrypsin on the beads with different pore sizes shows that adsorbed enzyme diffused faster on Amberlite XAD-8 than on Amberlite XAD-7 (Figures 33 and 34), and that the activity curves also changed faster on Amberlite XAD-8 than on Amberlite XAD-7 (Figure 37).
5. CONCLUSION

The porous carrier has several advantages: 1. it offers several more magnitudes of surface area; 2. the enzyme could be protected from physical abrasion and the turbulence of the bulk solution; and 3. larger particle can be employed, so it can be retained in fluidized bed or continuous-stirred reactors, and produces less pressure drop in a continuous packed-bed reactor. The major disadvantage is the pore diffusional limitation, which reduces the catalytic efficiency of immobilized enzymes, therefore the pore diameter should be optimized with respect to both the enzyme and the substrate to minimize the diffusional limitation.

The effect of loading levels on the activities of immobilized enzymes and activity changes with time have been investigated with porous Amberlite beads.

The most efficient concentration range is at low enzyme loading levels, for example, less than 20 mg/g beads. For the application of expensive enzyme, the concentration of the enzyme should be in this range for maximum utilization of the enzyme.
The feasibility of higher loading levels (above 20 mg/g) mainly depends on the utilization of internal surfaces of beads. For example, with immobilized chymotrypsin, it is possible to employ the relatively higher loading levels of enzyme in the beads to achieve a high volumetric productivity for large scale applications. But if enzymes with a substrate which can not be strongly adsorbed to the carriers, the enzyme immobilized on internal surfaces could not react with the substrate. So, these enzymes, like trypsin, should be kept on the outer surface of the beads, hence the nonporous carrier, or the carrier with extremely small pore diameter is more efficient. If the inner surface is not readily accessible to the substrate, co-factor, or activator, the diffusion of the adsorbed enzyme deeply into the beads should be prevented, thus less amount of enzyme should be employed.

Extremely high concentrations of enzyme should also be avoided in general applications, as the substrate will convert to product before it can diffuse very far in the beads to react with all the enzyme inside. Therefore, only a fraction of enzymes on the outer periphery of the beads are contributing the apparent reaction rate, whereas the some of enzyme are utilized little because the concentration of the substrate is negligibly low there.
APPENDIX A

STUDY ON ACTIVE SITE CONFORMATION
USING EPR WITH SPIN LABELING

1. Introduction

Electron spin resonance (ESR) or Electron paramagnetic resonance (EPR) has been an powerful technique in chemistry, biochemistry, and medicine for many years (Berliner and Reuben, 1988). It is one of the few methods that can be used to provide information about conformational changes in the active sites of enzymes immobilized on nontransparent solid phases (Berliner, et al., 1973). The technique is based on the detection of unpaired electrons, for instance, free radicals. However, due to the extremely short life-time of most free radicals in aqueous solution, EPR has not been extensively employed. It was with the discovery of nitroxide spin labels that the EPR applications stepped into a new era (Berliner, 1976). Because of stability of the nitroxides in aqueous solution and their sensitivity to motion as well as viscosity, they are especially useful in the field of biochemistry. As shown below, the nitroxides possess an unpaired electron located in a 2p orbital of the nitrogen bond.

\[\text{Appendix A}\]

\[\text{Study on active site conformation using EPR with spin labeling}\]

\[\text{1. Introduction}\]

Electron spin resonance (ESR) or Electron paramagnetic resonance (EPR) has been an powerful technique in chemistry, biochemistry, and medicine for many years (Berliner and Reuben, 1988). It is one of the few methods that can be used to provide information about conformational changes in the active sites of enzymes immobilized on nontransparent solid phases (Berliner, et al., 1973). The technique is based on the detection of unpaired electrons, for instance, free radicals. However, due to the extremely short life-time of most free radicals in aqueous solution, EPR has not been extensively employed. It was with the discovery of nitroxide spin labels that the EPR applications stepped into a new era (Berliner, 1976). Because of stability of the nitroxides in aqueous solution and their sensitivity to motion as well as viscosity, they are especially useful in the field of biochemistry. As shown below, the nitroxides possess an unpaired electron located in a 2p orbital of the nitrogen bond.

\[\text{Based on a draft of a publication by Y. Song, X. Wan, G. E. Means and L. J. Berliner.}\]

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$\overset{14}{N}^* - O$

Since naturally abundant $^{14}N$ possesses a nuclear spin of 1, the hyperfine splitting due to interaction of the nitrogen nucleus with the unpaired electron produces a three-line spectrum.

The site specific introduction of the nitroxide spin label onto an active site or a binding site, for example, can enable one to monitor subtle conformational variations. Hence, the spin labeling method has been widely used to study structure and functional relationships of macromolecules. Chymotrypsin is a well-studied case. A series of stable nitroxides including 4-(2,2,6,6-tetramethylpiperidine-1-oxyl)-m-fluorosulfonylbenzamide, m-IV (Berliner and Wong, 1974) have been synthesized for site specific covalent labeling of Serine 195 which is at the active site.

\[ m\-IV \]

m-IV is a active site directed reagent and labels essentially only catalytically active enzyme molecules (Clark and Bailey, 1984c). Studies have shown that m-IV labeled chymotrypsin gave a moderate restricted nitroxide motion spectrum in aqueous solution. The spectrum was
sensitive to the temperature, pH changes and also to certain ligands. In order to examine conformational changes in the active site of chymotrypsin during the immobilization processes, m-IV was employed in the site specific labeling.

2. Methods

2.a Synthesis of m-IV

The compound was synthesized according to Wong et al (1974). To a prechilled ether solution (150 ml) containing 1 g (5.6 mMoles) 4-amino-2,2,-6,6-tetramethyl-1-piperidinyloxy (4-amino-Tempo) and 0.8 ml (5.6 mMoles) of triethylamine, 1.3 g (5.6 mMoles) of 3-(Fluorosulfonyl) benzoyl chloride dissolved in 50 ml ether was added in small increment over one hour in an ice bath. The reaction was monitored by TLC. After standing at room temperature for three hours, the solid was collected on a filter and then dissolved in chloroform. The solution was filtered and passed through a silica gel column eluted with ethyl acetate. The yellow band was collected, and an orange solid was obtained after rotatory evaporation. The product was crystallized from benzene. A chemical ionization mass spectrum showed a single intensive peak at 359, and uncorrected M.P. is 177±1°.
2. b Spin labeling

α-chymotrypsin was labeled according to Wong (1974): 0.1 ml of m-IV stock solution (3.5 mg/ml acetonitrile) was gradually added during a period of 30 minutes to 20 mg of modified or unmodified α-chymotrypsin solution in 1.5 ml of 0.1 M potassium phosphate buffer, pH 7.75). The final organic solvent concentration was 12 % (v/v), which did not affect enzyme activity according to Wong (1974). The total amount of m-IV corresponded to about a 4-fold molar excess as compared to α-chymotrypsin. The labeling mixture was gently stirred at room temperature for 30 minutes and then subjected to exhaustive dialysis against diluted acetic acid (0.006 M), pH 4.0 at 4°C for several days. CaCl₂ (0.02 M) was also added into the dialysis solution according to Wong's procedure.

The reaction of chymotrypsin with methyl-4-phenylbutyrimidate (MPBI) was done prior to the spin labeling.

2. c EPR spectroscopy

EPR signals were examined at X-band (about 9.5 GHz) on a Varian E-4 spectrometer at room temperature. Both capillary quartz tubes (1 mm in diameter) and glass Pasteur pipettes (1-2 mm in diameter) were used as sample tubes. No differences in terms of spectral lineshape were detected. EPR spectra were digitized on a Varian E-935
data system interfaced to a Varian E-4 spectrometer.

Sucrose spectra were obtained by adding pure sucrose powder to m-IV labeled chymotrypsin in an appropriate buffer, pH 7.5, at room temperature. The solution was slowly stirred by using a small glass rod until the solution became saturated.

The thermal denaturation experiments were carried out by incubating both soluble and immobilized premodified and m-IV labeled enzyme preparations in a 50°C water bath for the desired time period before their EPR spectra were scanned. The temperature in the EPR cavity was adjusted to 50°C using a Varian temperature control unit.

2.d Decreasing and broadening of EPR signal

The m-IV compound labeled at the active site of chymotrypsin was reduced with ascorbate to decrease the EPR signal. Solutions of ascorbic acid, from 0.1 mM to 1.0 mM were adjusted to pH 7.5 by adding NaOH. The concentration of the solution varied from 0.1 mM to 1.0 mM. After recording the EPR spectrum in the absence of ascorbate, the buffer was drained from the sample tube, ascorbate solution was added to completely soak the polymer beads. The EPR spectra were then recorded at different times.
The EPR signals were broadened by K$_3$Fe(CN)$_6$ solution in a similar procedure. To the moisture cake of Amberlite XAD-8 beads with m-IV labeled immobilized chymotrypsin, 50-150 mM of K$_3$Fe(CN)$_6$ in an appropriate buffer was added at room temperature. After a 10 minute equilibration the EPR spectra were recorded at various time intervals.

3. Results and discussion
3.a Effect of modification on enzyme conformation

Spin labeling is a sensitive approach to detect alterations of the active site conformation that might result from the chemical modification of α-chymotrypsin. Figure 43 compares the EPR spectra of m-IV labeled α-chymotrypsin (II) and m-IV labeled, phenylbutyramidinated α-chymotrypsin (III) both in aqueous solution. A free m-IV spectrum (I) in aqueous solution is also included as a reference. The spectra of both m-IV labeled samples appeared to be moderately constrained reflecting a rather strong interaction between the nitroxide moiety and the active site environment. There is little difference between the spectrum of modified enzyme (III) and that of unmodified enzyme (II) under the experimental conditions based on calculations of their rotational correlation times, $\tau_R$, which correspond to the time required for the nitroxide moiety to rotate from one orientation to another.
Figure 43. Comparison of m-IV labeled chymotrypsin in aqueous solution with and without chemical modification

Free m-IV (I), m-IV labeled chymotrypsin (II), m-IV labeled chymotrypsin premodified with MPBI on about 60% of its NH$_2$ groups (III) in pH 4 water solution.

Instrumental conditions: Field set = 3395 G, Modulation level = 1.0 G, Microwave Power = 20 mW, Time Constant = 0.064 sec. Receiver Gain = 1.6 x 10$^4$, Scan Time = 2 minutes.
as follows:

\[ \tau (s) = 6.5 \times 10^{-10} \Delta H_0 \left( \frac{h(0)}{h(-1)} - 1 \right) \]  \text{(V-1)}

where \( \Delta H_0 \) is the line width of the center peak, \( h(0) \) and \( h(-1) \) are the peak heights of the middle peak and high field peak respectively (Knowles, et al., 1976) and \( \tau_R \) is inversely proportional to the mobility of the nitroxide moiety. Both m-IV labeled chymotrypsin and m-IV labeled phenylbutyramidinated chymotrypsin have similar \( \tau_R \): for spectrum II, \( \tau_R = 4.0 \) nsec. and for spectrum III \( \tau_R = 3.6 \) nsec. This indicates that the nitroxide mobilities were almost identical and the spin labels are moderately constrained in both cases. This is the evidence that the phenylbutyramidination with MPBI does not affect the active site conformation. This comparison can not, however, detect conformational changes beyond the active site.

The effect of immobilization on m-IV labeled chymotrypsin and m-IV labeled phenylbutyramidinated chymotrypsin were also examined. Figure 44 shows the EPR spectra of \( \alpha \)-chymotrypsin immobilized on Amberlite XAD-8 beads with (I) and without (II) prior modification by MPBI. Again, the two spectral peaks are perfectly superimposed. The two extrema in the figure, (I) and (II) indicated that \( 2T_{//} \), which is the distance between 2 extreme peaks, in both cases were identical within experimental error: \( 2T_{//} = \)
m-IV labeled chymotrypsin premodified with MPBI on about 60% of its NH₂ groups (I) and the chymotrypsin without phenylbutyramidination (II) immobilized on Amberlite XAD-8 beads (250-425 μm). Arrows indicate signals for a small subpopulation A of a slow tumbling and B of a fast tumbling component.

Instrumental conditions: Field set = 3395 G, Modulation level = 1.0 G, Microwave Power = 20 mW, Time Constant = 0.128 sec., Receiver Gain = 10 X 10³ (I) & 5 X 10⁴ (II), Scan Time = 2 minutes.
65.7±0.1 G. The much lower signal to noise ratio of spectrum (II) is due to the lower hydrophobicity of chymotrypsin prior to its modification, which results in less enzyme being bound on the same amount of beads.

3.b Effect of immobilization on enzyme conformation

The tumbling rate of the immobilized enzyme appears to be much slower than that of free enzyme in aqueous solution (comparing Spectrum III of Figure 43 to Spectrum II of Figure 44), as indicated by the shift of peaks to lower field. The nitroxide mobility exhibited in EPR spectra also includes a contribution from macromolecular rotation. The similarity of the spectra of spin labeled trypsin and immobilized, spin labeled trypsin in saturated sucrose (aqueous) solution has been reported before (Berliner, et al., 1973). Saturated sucrose slows down the tumbling rate of enzyme molecules. Figure 45 shows the saturated sucrose spectrum of m-IV labeled chymotrypsin (II) as compared to that of the free (I) and immobilized enzyme (III). The relatively constrained spectral component in (II) is essentially the same as that in (III). Therefore, the spectral difference between the soluble and the immobilized α-chymotrypsin shown in Figure 43 and Figure 44 is believed to be a consequence of the reduction of chymotrypsin molecular motion and not to a change in active site conformation.
Figure 45. Comparison of saturated sucrose spectrum with immobilization spectrum of m-IV labeled chymotrypsin.

m-IV labeled chymotrypsin in an appropriate buffer, pH 7.5, (I), in the same solution plus saturated sucrose (II), and immobilized on Amberlite XAD-8 beads (III) suspended in the same buffer at room temperature.

Instrumental conditions: Field set = 3395 G, Modulation level = 0.82 G, Microwave Power = 20 mW, Time Constant = 0.5 sec., Receiver Gain = 3.2 X 10^3 (I); 2.0 X 10^4 (II) & 6.3 X 10^3, Scan Time = 2 minutes.
3.c Heterogeneity of immobilized chymotrypsin

Immobilized enzymes are seldom homogeneous. Heterogeneous immobilized enzyme populations have been described in a few previous investigations. For example, two primary subpopulations of leucine aminopeptidase attached covalently on thiol-Sepharose 6B have been investigated by radiochemical and enzymological methods (Lasch and Koelsch, 1978). At least two different populations of covalently immobilized α-chymotrypsin with different numbers of surface attachments have been observed by several research groups (Lasch, et al., 1976; and Kawamura et al., 1981). Recently, EPR spectroscopy has been employed to study the heterogeneity of chymotrypsin immobilized on CNBr-Sepharose CL-4B (Clark and Bailey, 1984a) and on aminopropyl silic (Ulbrich, Schellenberger and Damerau, 1985). It has been suggested that different surface attachment points might result in two or more different enzyme conformations (Clark and Bailey 1984a).

The heterogeneity of immobilized α-chymotrypsin on the present polymer beads is also revealed by multi-component EPR spectra. For example, in Figure 44, each spectrum can be viewed as a composite of two individual spectra. "A" corresponds to the highly constrained spectral component, and "B" is the more weakly constrained component (likely to
be unfolded protein or/and weakly bound protein). Similar to Clark and Bailey's results (1984b), the weakly constrained subpopulation B has a spectrum very similar to that of soluble chymotrypsin (Figure 45). Here the term "constrained" describes the mobility of the nitroxide moiety in the active site pocket. The lineshapes and positions of the two components are very similar to the spectra reported by Clark and Bailey (1984b) and Berliner, et al., (1973). In contrast to other immobilized preparations, however, in the present case α-chymotrypsin had been immobilized on Amberlite XAD-8 beads by spontaneous adsorption of phenylbutyramidinated enzyme. The weakly constrained subpopulation B which to no more than 10% of the total in this case is also a smaller percentage as compared to other reports.

According to Clark and Bailey (1984b), the active site region of subpopulation B is more accessible to the surrounding solvent than that of subpopulation A. The specific activity of the B component was alleged to be 15 times greater than that of the A component (Clark and Bailey, 1984a); in other words, the more constricted form had very low activity. In our experiments, however, subpopulation B could be removed from the beads by extensive washing with 40% ethylene glycol in a buffered solution. Figure 46 shows the EPR spectra of immobilized
Figure 46. EPR spectra of chymotrypsin immobilized on Amberlite XAD-8 after washing different times.

Washed 3 times with 40% ethylene glycol (I), Washed 8 times (II), and Washed 15 times (III). Arrows indicate subpopulation A (slow tumbling component), B (fast tumbling component).

Instrumental conditions: Field set = 3395 G, Modulation level = 1.0 G, Microwave Power = 20 mW, Time Constant = 0.128 sec., Receiver Gain = 3.2 $\times$ 10$^3$, Scan Time = 2 min.
α-chymotrypsin on Amberlite XAD-8 with different washing times. Component B disappeared after washing 15 times with 2 to 3 ml of 40% ethylene glycol (for about 1 ml of beads), while subpopulation A remained the same. The activity of immobilized enzyme, on the other hand, did not decrease after washing, and no activity was detected in the wash. These results, in addition to the spectral similarity of component B to the soluble enzyme, suggest that component B is inactive and perhaps the fragment loosely bound near the surface of the beads or entrapped in the beads, whereas subpopulation A is the active enzyme.

3.d Denaturation on Amberlite XAD-2

One denaturation mechanism in enzyme immobilization is so called "carrier denaturation". It is believed that if the enzyme-carrier interaction is much stronger than essential intramolecular bonds, the protein molecule will undergo conformational perturbation or denaturation on the carrier surface (Trevan, 1980).

Amberlite XAD-2 is much more hydrophobic than Amberlite XAD-8. When enzymes were immobilized on Amberlite XAD-2, relatively low activities were observed as shown in Figure 17 and that activity decreased with time (Figure 19). To help determine why activities were so low, the EPR spin labeling method was employed to monitor active
site conformational perturbations on the Amberlite XAD-2 surface.

A distinct feature of α-chymotrypsin after immobilization on Amberlite XAD-2 was the existence of three spin labeled subpopulations: a strongly constrained population (A), a weakly constrained population (B) and a moderately constrained components population (C) (Figure 47). The characteristics of each subpopulation are discussed below.

The EPR spectra of component A on Amberlite XAD-2 and that on Amberlite XAD-8 are very similar. The value of 2T// differs slightly, about 2.1 G smaller on Amberlite XAD-2 than that on XAD-8. Since 2T// is directly proportional to the nitroxide mobility, this implies that component A on XAD-2 has a slightly weaker interaction with the Amberlite beads. The decrease in enzyme activity on Amberlite XAD-2 (Figure 48) with time and/or heating correlated with the decrease of subpopulation A on the beads and the increase in component C (Figure 49). Subpopulation A is, therefore probably the major active enzyme form on Amberlite XAD-2. However, as the number of washing increased, some component A appeared to be also converted into component C (Figure 47).
Figure 47. EPR spectra of chymotrypsin immobilized on Amberlite XAD-2 after washing different times.

Washed 3 times with 40% ethylene glycol (I), Washed 8 times (II), Washed 15 times (III). Chymotrypsin was modified with MPBI on its NH$_2$ about 60%, was stored 4 days after the immobilization. Arrows indicate subpopulation A (slow tumbling component), B (fast tumbling component) and C (moderate tumbling component).

Instrumental conditions: Field set = 3336 G, Modulation level = 1.0 G, Microwave Power = 20 mW, Time Constant = 0.128 sec. Receiver Gain = 2 X 10$^3$, Scan Time = 2 minutes.
Figure 48. Heat inactivation of chymotrypsin immobilized on Amberlite XAD-2.

Chymotrypsin was immobilized on Amberlite XAD-2 at 40 mg/g beads. The enzyme was modified with MEPI on about 60% its NH$_2$. The beads were incubated with 0.005 M triethanolamine/HCl buffer containing 0.01 M KCl and 0.02 M CaCl$_2$ (*) at 50°C.
Figure 49. EPR spectral change upon heat denaturation of chymotrypsin immobilized on Amberlite XAD-2.

Chymotrypsin modified with MPBI on about 60% its NH$_2$ was immobilized on Amberlite XAD-2 at 40 mg/g beads. The EPR spectra were recorded at 50°C at incubation times from 0 to 60 minutes. Component B was removed prior to the incubation by washing with 40% ethylene glycol.

a): individual spectral recordings for the indicated times and b): overlapped spectra. The arrows indicates the direction of the spectral change.

Instrumental conditions: Field set = 3350 G, Modulation level = .82 G, Microwave Power = 20 mW, Time Constant = 0.5 sec. Receiver Gain = 1.25 X 10$^4$, Scan Time = 2 minutes.
Components B resembles that of the same designation mentioned in the case of Amberlite 8. Component B was also removable by washing with 40% ethylene glycol as shown in Figure 47. Component B was completely eliminated after washing 15 times. The absence any change in hyperfine splitting (i.e. peak to peak) distance after the washing suggests this subpopulation is nonspecifically adsorbed, probably partially unfolded enzyme on the polystyrene surface similar to that described above on Amberlite XAD-8.

The major spectral difference observed between Amberlite XAD-2 and XAD-8 is the existence another subpopulation, component C. As shown in Figure 49, after washing with 40% ethylene glycol to remove all of the component B, heating brought about a gradual shift from a highly constrained ESR spectrum (subpopulation A like, $\tau$ is $> 3 \times 10^{-8}$ sec.) to a slightly less constrained spectrum (subpopulation C, where $\tau$ is about $10^{-8}$ sec.). This shift was also observed upon storage at 4 °C for more than 4 days (last spectrum in Figure 49a). This spectral shift was compared to a parallel experiment which showed a decrease in activity of modified chymotrypsin immobilized on Amberlite XAD-2 without spin label. The loss of enzyme activity during heating is shown in Figure 48 correlated with the spectral changes. The extra spectral component (C) observed on Amberlite XAD-2 appeared to reflect a
nitroxide moiety with a tumbling rate faster than that of immobilized component A. It was not possible to remove subpopulation C by washing the beads with either ethylene glycol or buffer. Hence, it is valid to assume that subpopulation C is the inactivated enzyme component. This subpopulation therefore appeared to lose its catalytic activity and became denatured on the carrier probably due to the direct contact with the surface of the Amberlite XAD-2 beads resulting from strong hydrophobic enzyme-carrier interaction.

3.e Accessibility of the active site after immobilization

Information about the accessibility of the immobilized enzyme may help to account for its lower efficiency after immobilization and may also provide information concerning the distribution of subpopulations in the beads. Two approaches were employed to obtain information on accessibility of subpopulations of spin labeled chymotrypsin on Amberlite XAD-8 beads as compared to that of soluble chymotrypsin.

One was an equilibration method with $K_3\text{Fe(CN)}_6$. Bailey and his colleagues have described the use of $K_3\text{Fe(CN)}_6$ as a broadening agent (Clark and Bailey 1984a). As a consequence of electron-electron dipole interactions between the broadening agent and the spin labeled molecule,
the EPR spectra should become wider and the amplitude of
the EPR signal should decrease. Using this approach, Clark
and Bailey (1984a) reported that the highly constrained
subpopulation "A" on CNBr-Sepharose CL-4B was less
accessible to the bulk solution than the weakly constrained
subpopulation "B", because the latter decreased much sooner
than the former as concentrations of K₃Fe(CN)₆ increased.
Similar results were observed in our studies. As shown in
Figure 50, component B decreases sooner than component A in
the presence of increasing concentrations of K₃Fe(CN)₆.
The plot of peak height versus K₃Fe(CN)₆ concentrations was
linear in the case of component B, while the plot of peak
height versus concentration for component A was not linear.
Like the results of Clark and Bailey (1984a and 1984b) from
spin labeled α-chymotrypsin-CNBr-Sepharose CL-4B, two
subpopulations were observed on Amberlite XAD-8. The more
constrained major component, "A", was less accessible to
the broadening reagent than the less constrained small
component, "B".

A kinetic approach: ascorbate reduction, was utilized
as an alternative approach to study the accessibility of
the spin label (Wan and Berliner, 1989). The quenching of
the ESR signal by ascorbate is due to electron transfer
from ascorbate to the nitroxide moiety converting the
latter into a diamagnetic (ESR silent) hydroxylamine
Figure 50. $K_3Fe(CN)_6$ broadening effect on signal of chymotrypsin immobilized on Amberlite XAD-8.

The changes of EPR spectra of m-IV labeled chymotrypsin immobilized on Amberlite XAD-8 in various concentrations of $K_3Fe(CN)_6$: 0 mM, 50 mM, 100 mM, and 150 mM. Arrows indicate the decrease in signal intensity and peak height plotted (A:*; B:●).

Instrumental conditions: Field set = 3350 G, Modulation level = .82 G, Microwave Power = 20 mW, Time Constant = 0.5 sec. Receiver Gain = $5 \times 10^3$, Scan Time = 2 minutes.
derivative: >N-OH. Figure 51a shows the rapid decay of the ESR signal of m-IV labeled phenylbutyramidinated chymotrypsin in the presence 0.1 mM ascorbate. A semi-log plot of the center peak height versus time was linear giving a pseudo first order rate constant of $k = 0.303 \text{ min}^{-1}$. Figure 52 shows the ESR spectral changes obtained with m-IV labeled phenylbutyramidinated chymotrypsin immobilized on Amberlite XAD-8 in 1.0 mM ascorbate solution (ie. 10 times higher than the concentration used for soluble enzyme). Both component A and B decreased rapidly at similar rates. Again the relative peak height was used to determine the rate constant assuming no change in line width during the measurements. A rate constant $k = 0.064 \pm 0.005 \text{ min}^{-1}$ was obtained by examining the low field extremum (A), whereas $k = 0.068 \pm 0.006 \text{ min}^{-1}$ was computed from the change of the second low field peak (B) and the middle peak. Considering the magnitude of the error bars, both constants are essentially identical.

The $K_3\text{Fe(CN)}_6$ broadening result suggested that weakly constrained component B decreased at a lower concentration of $K_3\text{Fe(CN)}_6$ than the strongly constrained component A as the concentration of $K_3\text{Fe(CN)}_6$ increased (Figure 50). However, it was clearly demonstrated with ascorbate reduction that both subpopulation A and B exhibited the same rate of signal decrease (Figure 52).
Figure 51. Reduction on the signal of soluble chymotrypsin by ascorbate.

The EPR spectral changes of m-IV labeled chymotrypsin in 0.1 mM ascorbate solution. The spectra were recorded every 2 min, and totally 20 min after the reduction. Instrumental conditions is similar as that in Figure 50, and receiver Gain = 8 X 10³.
Figure 52. Reduction on the signal of immobilized chymotrypsin by ascorbate.

The EPR spectral changes of m-IV labeled chymotrypsin immobilized on Amberlite XAD-8 in 1.0 mM ascorbate solution. The spectra were recorded every 5 min, and totally 30 min after the reduction. Component A (▲); component B (●) and middle peak (x). Instrumental condition is similar as that in Figure 50, and receiver Gain = 1.25 X 10^4.
The difference between $\text{K}_3\text{Fe(CN)}_6$ and ascorbate can result from the following reasons.

One of the main factors is the dependence of the broadening effect on the correlation time of participating molecules. As the broadening effect is most likely correlation time dependent, a broad spectral component may react to a broadening agent differently from a narrow component. Several groups have shown that line broadening ($\Delta H$) decreases as correlation time, $\tau_c$ increases (Anisimov, et al., 1971; Keith, et al., 1977; Berliner, 1979). It is also known that $H$ is actually reflects the sum of two kinds of broadening mechanisms: spin exchange and electron-electron dipole broadening. The latter has complex dependencies such as relaxation, translational and rotational diffusion of the nitroxide. For example, the fast tumbling of component B and rapid translational and rotational diffusion of the broadening agent tend to average out the net dipole field. However, the net dipole field might not be averaged away for subpopulation A molecules because of their slower tumbling. Therefore, component B may not even be able to sense the fluctuating dipole field resulting from nearby $\text{K}_3\text{Fe(CN)}_6$, while subpopulation A may experience a concentration dependent dipole field.
The ascorbate reduction method, in contrast, has no dependence on spin label correlation time. It is simply a pseudo-first order reaction (Figures 51 and 52). The calculation of the rate constant based on three well separated peaks clearly indicated that all spectral components diminished at the same rate. Besides the conclusion of equal accessibility for both subpopulations, one can also roughly estimate the diffusional limitation of ascorbate on Amberlite XAD-8 beads by comparing reduction rate constant of labeled soluble chymotrypsin to that of immobilized chymotrypsin. The ratio of these two rate constants, about 4.5, implies that diffusion hindrance on the bead slowed down the reduction rate approximately by about 4.5 time.

4 Conclusion:

The spin labeling technique has demonstrated that neither phenylbutyramidination or adsorption onto polymethacrylate beads (ie. Amberlite XAD-8) alters the active site conformation of α-chymotrypsin. The EPR spectra of immobilized phenylbutyramidinated chymotrypsin appeared heterogenous after adsorption to the beads. Complete elimination of a relatively minor, less constrained component "B" by washing with 40% ethylene
glycol suggested that this subgroup of chymotrypsin molecules was loosely bound on the surface of the beads. Subpopulation A which was the main spectral component was proportional to the activity of the immobilized enzyme on both Amberlite XAD-8 and Amberlite XAD-2 beads. The spectra of spin labeled phenylbutyramidinated chymotrypsin immobilized on Amberlite XAD-2 beads showed one more major component than those on Amberlite XAD-8. This component, "C", indicated a different active site conformation, and its increase correlated with the decrease in enzyme activity. Carrier denaturation in the case of Amberlite XAD-2 could result from denaturation of chymotrypsin by polystyrene beads and may account for the decrease in total enzyme activity on that support.
# APPENDIX B

## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>BAEE</td>
<td>Benzoyl-L-arginine ethyl ester</td>
</tr>
<tr>
<td>BTEE</td>
<td>Benzoyl-L-tyrosine ethyl ester</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical ionization</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>MPBI</td>
<td>Methyl phenylbutyrimidate hydrochloride</td>
</tr>
<tr>
<td>NPGB</td>
<td>p-nitrophenyl-m-guanidino benzoate</td>
</tr>
<tr>
<td>TNBS</td>
<td>2,4,6-trinitrobenzenesulfonic acid (TNBS)</td>
</tr>
</tbody>
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