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Studies on immobilization of horseradish peroxidase.

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University of Windsor

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STUDIES ON IMMOBILIZATION OF HORSERADISH PEROXIDASE

by

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ABSTRACT

The immobilization of horseradish peroxidase (HRPO) (Mw,40,000) a glycoenzyme, on long-arm polyacrylamide beads and nylon tubing is described. The long-arm polyacrylamide beaded material (≤400 mesh, 10-37 μm diameter, 1800 dalton exclusion limit) used for immobilization was derivatized (Bio-gel P-2) having .8 μeg/gm of dry beads or .1 μeg/gm of dry beads of amino group density and .3 μeg/gm of dry beads of hydrazide group density as determined by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method. Nylon tubing used for the immobilization (1 meter long, I.D.1.0 mm O.D.1.34 mm) had 0.66 nmoles amino groups/meter tubing.

Immobilization was carried out via a Schiff-base (imine) intermediate between amino groups of bead and oxidized HRPO. The HRPO was oxidized at pH 8.0, at 25°C, retaining 70% of its activity. In the case of long-arm polyacrylamide beads having high and low amino group density, stabilization of Schiff-base (imine) was further carried out by using different reducing agents. Sodium borohydride was added after the coupling was complete in order to avoid loss of the aldehyde functions necessary for the coupling reactions. By contrast, sodium cyanoborohydride was present throughout the coupling reaction to drive the reaction to completion by trapping the labile Schiff-base by reduction

at neutral pH.

In order to estimate the active immobilized HRPO on the beads, a sensitive chromogenic HRPO assay was developed. The assay was based on the rate of oxidative coupling of 2-hydroxy-3,5-dichlorobenzenesulfonic acid (HDCBS) and 4-aminoantipyrene (4-AAP). In the presence of hydrogen peroxide (H_2O_2), HDCBS and 4-AAP, peroxidase catalyses the formation of a deep pink colour which has an absorption maximum at 510 nm. The assay can detect levels of immobilized peroxidase on the beads in the picomolar range.

Different methods were used to quantitate peroxidase on the beads. An upper limit for the amount of bound enzyme could be calculated from the difference in protein content of the initial and coupling supernatants. This amounted to 28 nmol/g of beads, which would represent 52% surface saturation, for small beads (25 μm) having amino-terminated arms. Inactive material absorbing at 403 nm amounting to about 10% of the above estimate could be eluted from the beads with 10 mM aqueous hydrazine. However, from direct determination of enzyme activity on the beads it was evident that less than one percent of the above 28 nmol/g was immobilized in fully active form.

DEDICATION

to

my husband and family

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. K. E. Taylor, for his patient direction and encouragement during the course of this work.

I also thank Dr. N. F. Taylor, Dr. R. J. Thibert and Dr. B. Mutus for their helpful suggestions.

Many thanks to my friends, colleagues and students at the Department of Chemistry for their companionship.

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A	absorbance
Å	angstrom
HDCBS	2-hydroxy-3,5-dichloro-benzenesulfonic acid, sodium salt
4-AAP	4-aminoantipyrene
TNBS	2,4,6-trinitrobenzenesulfonic acid
FDNB	fluoro-2,4-dinitrobenzene
HRPO	horseradish peroxidase
ε	molar extinction coefficient

CHAPTER I

INTRODUCTION

The specificity of enzymes and their ability to catalyze reactions of substrate at low concentrations are of great use in chemical analysis. Enzyme catalyzed reactions have been used for analytical purposes for over a century for the determination of substrates, activators and inhibitors. However, disadvantages associated with the use of enzymes such as instability, unavailability and cost have seriously limited their usefulness. These limitations have made enzyme-catalyzed reactions unappealing for analytical chemists.

Attempts to circumvent (1,2) these problems led to a major breakthrough when a new technique known as enzyme immobilization was introduced in applied enzymology about 20 years ago. Klibanov (3) has defined immobilization of enzymes as the conversion of enzymes from a water soluble, mobile state to a water insoluble immobile state. According to Goldstein (4) immobilized enzyme can be defined as "enzymes physically confined or localized in a certain defined region or space with retention of their catalytic activities, which can be used repeatedly and

continuously."

Some remarkable successes have been reported on the utilization of immobilized enzymes in the food, pharmaceutical and chemical industries (2). Interest in the potential use of enzymes as catalysts in industry has increased during the last 10-15 years, as a result of the development of novel techniques for enzyme immobilization. The available techniques enable a high degree of flexibility in reactor design since they allow enzyme immobilization on polymeric supports of different geometric structures including powders, beads, tubes, fibres, filters and membranes. These provide the chemical engineer with heterogenous biocatalysts which can be utilized continuously in appropriate reactors. Katachalski-Katzir (2) has mentioned that the expected progress in the development and design of enzyme immobilization will provide biotechnologists with efficient pilot schemes for carrying out desired bioconversion reactions on an industrial scale.

The availability of enzyme columns, enzyme membranes and enzyme tubes has also facilitated more extensive use of enzymes for analytical purposes. Enzyme columns and tubes, when inserted into a suitable automatic analysers, permit the continuous assay of substrates such as glucose, lactose, amino acids, urea and uric acid (3,4).

Tosa, et al. (5) suggested that if active and stable

water insoluble enzymes are prepared by using immobilization techniques most of the aforementioned disadvantages can be eliminated and the following advantages are expected:

1. Enzymes can be recovered from a reaction mixture and can be reused.
2. Storage, thermal and operational stability of enzymes can be improved.
3. A catalyst can be tailor-made for specific use.
4. Continuous operation becomes practical.
5. Reaction requires less space, mild conditions of pressure and temperature.
6. Any detection technique can be employed using immobilized enzymes.
7. Characterization of products is possible due to ease of separation from enzymes.

The method used for immobilization were surveyed by Mosbach (6) in 1976. Also, a number of reviews have appeared which discuss various aspects of the field (7-10). Physical methods, adsorption (8,11) entrapment (10,12) and microencapsulation (13) were used frequently in industry due to simplicity. These methods suffer from the disadvantage of protein loss through leaching during application in industry and clinical analyzers. However, intermolecular crosslinking (14) and covalent methods (15) avoid these difficulties, but

sometimes result in a degree of conformational distortion which causes a substantial loss in enzyme activity after attachment. Even then, in practice, the method of covalent attachment is better suited to the requirement of product characterization.

The general strategy employed in enzyme immobilization is to incorporate chemically reactive and compatible groups into both support and enzyme in such a way that coupling can be accomplished in an efficient and chemically well-defined manner. Because a variety of organic functional groups are present in biological molecules and a vast array of modified supports are available, the chemical modification of both is rarely necessary. However, steric requirements usually demand that linkage to the support should be accomplished through a "spacer arm". Thus, the immobilization technique often consist of three steps:

1. Modification or derivatization of support.
2. Enzyme modification and coupling to support.
3. Estimation of bound and unbound enzyme activity on the support.

1. <u>Modification or Derivatization of Support</u>

Derivatization of the support is an important aspect of the immobilization process. In practice, derivatization of the support is undertaken to incorporate a spacer arm of appropriate length with a distal functional group that will

allow its specific covalent attachment to the enzyme. Cuatrecasas (16) reported that by attaching the same ligand to the same support through a flexible bridge or arm consisting of a chain of five or more atoms, a dramatic increase in binding may be possible. Previous work in this laboratory (17,18) has focused on the evaluation of the structural network of very long hydrophilic spacer-arm of the poly(oxyethylene) type shown in Figure 1.

Hydrophilicity was another desirable characteristic in selecting a proper support for the immobilization. Inman (19) showed that this property is very important for the preservation of enzymes in a highly active state after their immobilization. For this reason, immobilization on polysaccharide and polyacrylamide supports have found wide application in biochemistry (20). A few attempts to achieve both the hydrophilicity and binding capacity of these organic polymers involved crosslinking with polyethylene imine or maleic anhydride ethylene copolymer on the surface of the nonporous beads (20).

The degree of derivatization of the support is another important factor for better recovery of enzyme activity. The greater the substitution, the greater the possibility of multiple bond formation to the enzyme, possibly resulting in restriction of molecular orientation and a loss of activity. Therefore, it is required to control the sub-

Figure 1

Schiff-base Formation Involved in the Immobilization of Peroxidase to Long-arm Beads

Legend

HRPO was oxidized with sodium periodate and then coupled with long-arm-derivatized polyacrylamide beads.

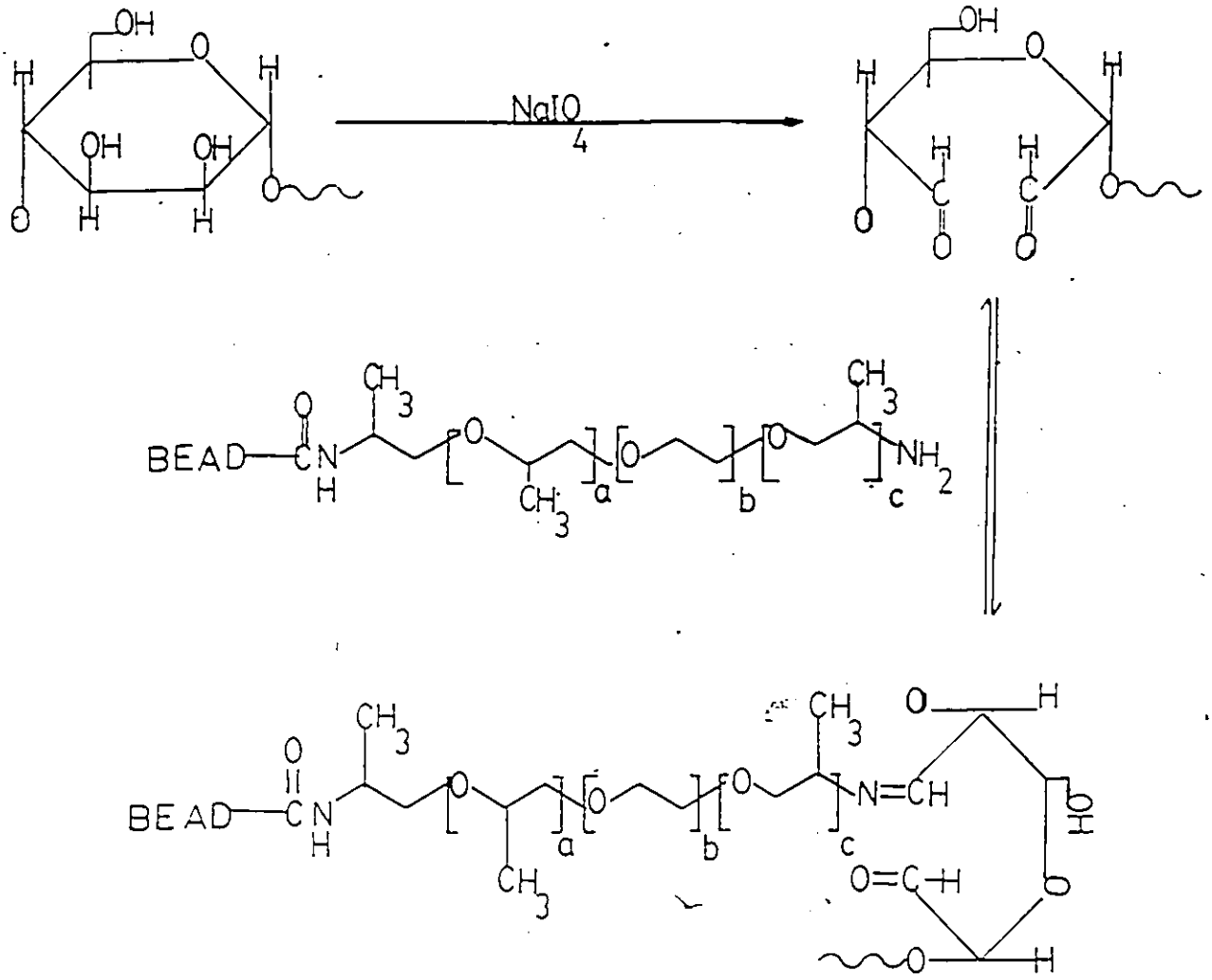

OH
O
H
OH
OH
O
H
H
NaIO
4
OH
O
H
H
C
O
C
O
O
BEAD
C
N
H
O
CH3
O
CH3
a
O
b
O
CH3
c
NH2
BEAD
C
N
H
O
CH3
O
CH3
a
O
b
O
CH3
c
N=CH
O
H
OH
O=C-H
O
O
H

Figure 1

stitution by varying the concentration of the activation reagents. Previous work in this laboratory (18) was also carried out by controlling substitution and derivatizing beads with different functional group density. Mosbach (6) suggested that immobilization of enzymes in such an artificial microenvironment is an important line of study for enzyme engineering because the well defined dimensions and chemistry of such matrices permit the study of parameters such as:

1. The surface area available for binding and the degree of its occupation.
2. The effect of hydrophilicity on the immobilized enzymes. Such data is especially useful for making tubular reactor or "tube-supported enzyme derivatives" in continuous flow analyzers (22).

The use of immobilized enzymes in the latter mode is extremely attractive when applied to automated analysis, since the tubular immobilized enzyme reactors can be readily incorporated into most standard analytical systems, without necessitating change in the existing technology. Nylon tube extruded from high molecular weight nylon contains relatively few sites for the attachment of protein and, therefore, in order to immobilize a reasonable quantity of an enzyme on the inside surface of the nylon tube, new active centres have to be introduced (21).

Many compounds could be used as spacers such as lysine, hexamethylene diamine and poly(oxyethylene)-α,ω-diamine. The use of a polyamino spacer is very attractive, since the attachment of the polyamino compound to one activated group in the nylon backbone yields a large number of active amino groups. The derivatization of nylon tubing used in our laboratory (work of S. Boss) does not involve cleavage of bonds, thus activating nylon without destroying the integrity of the polymeric structure.

Finally, in choosing the most advantageous immobilization technique, efforts are made to stabilize the immobilized enzyme in an artificial microenvironment and to avoid steric hindrance. Therefore, emphasis in this work has been placed on, (i) providing a hydrophilic spacer-arm for attaching enzyme without steric hindrance, (ii) selecting the chemical reaction and conditions which tend to minimize inactivation of enzyme.

2. Enzyme Modification and Coupling to Support

Enzyme selection for coupling was another important aspect of our immobilization study. Horseradish peroxidase (HRPO) was chosen as the enzyme for immobilization because it is readily available, stable on storage and its effect on hydrogen peroxide oxidation can be monitored spectrophotometrically (23). For the latter reason, peroxidase has been widely used for detection of micro amounts of

hydrogen peroxide and hydrogen-peroxide generating systems in analytical biochemistry (24).

HRPO is a glycoprotein having 21% carbohydrate residues (25) by weight. Figure 2 shows some information on its covalent structure. Welinder (26) speculated on the basis of compositional similarity that the carbohydrate chains of peroxidase (which have not been sequenced) may be similar to the carbohydrate moiety of another plant glycoprotein, pineapple stem bromelain. The HRPO glycans contain the two N-acetyl glucosamine, three mannose core found in all asparagine-linked glycans (25). These are further elaborated with one fucosyl and one xylosyl residue per glycan. Each of the bromelain N-glycans would be susceptible to periodate oxidation at seven to nine sites. All Asn-x-Ser/Thr sequences of peroxidase, except residues 286-288, have attached carbohydrate. The C-terminal half of glycoprotein carries six of the eight carbohydrate moieties.

Hsiao et al. (27) reported that carbohydrate residues of the glycoproteins have no apparent role in the catalytic activity of enzymes. Zaborsky et al. (28), Hsiao et al. (27) and Ugarova et al. (29) immobilized glycoprotein through carbohydrate residues of the enzymes. Therefore, it was considered more desirable to immobilize HRPO to polyacrylamide beads by these catalytically non-essential carbohydrate residues. The method of enzyme coupling consists

Figure 2

Structure of Horseradish Peroxidase C

Legend

Taken from Welinder (26)

Residues are represented by linear pieces of equal size. Bends are introduced to allow disulfide-bridged residues (11-91, 44-49, 97-301 and 177-209), and the proximal (residue 170) and the proposed distal histidine (residue 42)[6] to approach, and to give a compact structure (═══) indicates histidine, and (●) shows site of carbohydrate attachment. Line thickness of the polypeptide chain indicates its stability in apo-peroxidase to cold tryptic digestion: (━━━) very stable, (——) stable, and (-+-) completely cleaved. (----) Encircles domains of apo-peroxidase.

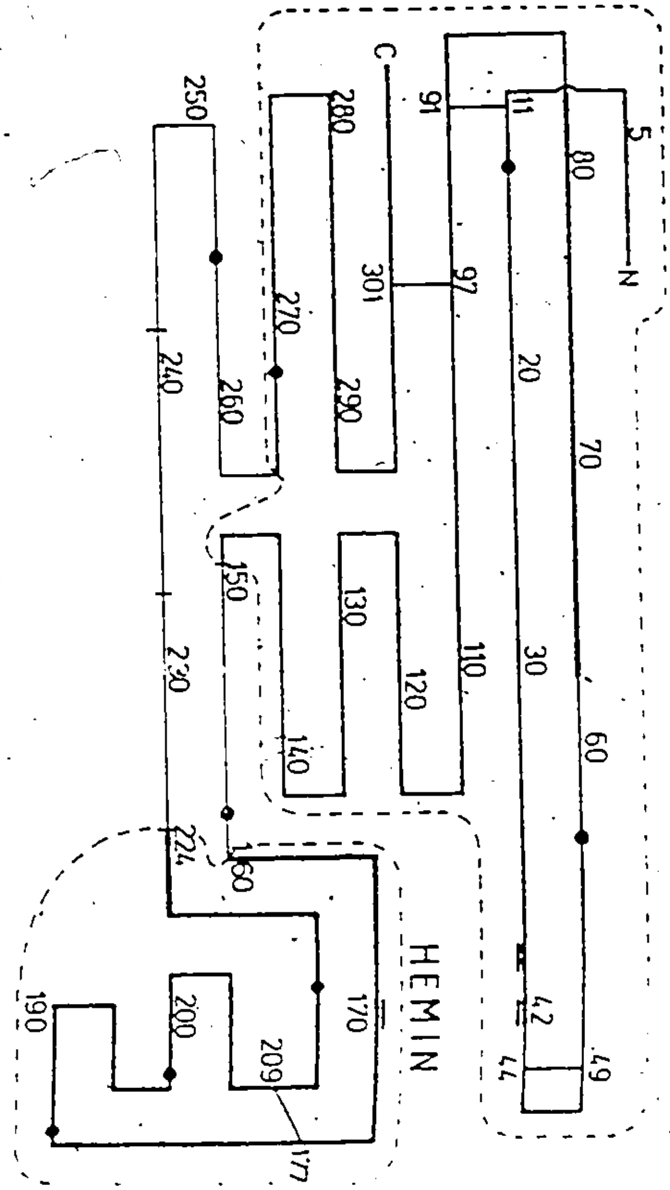

C
N
5
11
91
80
97
301
250
280
270
240
260
290
20
70
150
130
110
30
120
230
140
60
224
160
HEMIN
170
200
190
209
177
42
44
49

Figure 2

of two steps:

1. Activating HRPO via periodate oxidation.
2. Linking the modified aldehyde enzyme with amine groups attached on beads through spacer arms.

Periodate oxidation is a well established procedure for modifying carbohydrate residues in polysaccharides (30) and glycoproteins (31) to form active aldehydes which are available for Schiff-base formation with the amino group of any carrier. In glycoproteins, the carbohydrate moiety can be oxidized, with periodate or galactose oxidase with significant retention of activity (32). Capaldi (33) oxidized cell-surface membrane proteins with sodium meta-periodate or galactose oxidase to generate active aldehydes for stable Schiff-base formation. Heath et al. (34,35) used $NaIO_4$ oxidation to link HRPO to liposomes. Nakane et al. (32) used periodate oxidation to oxidize HRPO for the preparation of stable antibody conjugates. It is impossible to predict the efficiency with which these reactions will couple peroxidase to polyacrylamide beads since it depends upon many factors including the concentration of reactants, the number of reactive groups available, pH, and intrinsic reactivity of the specific types of aldehydes and nitrogen nucleophiles.

Schiff-base formation is the first stage in reaction of the oxidized glycoprotein and amino groups of the hydrophilic supports. The reactants may be irreversibly linked if II

in Figure 1 is reduced to an amine by $NaBH_3CN$ or $NaBH_4$. As a rule $NaBH_4$ reduction must be carried out at alkaline pH (36), while $NaBH_3CN$ coupling is achieved at neutral pH (37,38). Usually coupling can occur via a primary or secondary amine since either can form an imine.

3. Estimation of Bound and Unbound Enzyme Activity on the Support

The ultimate goal of this research is immobilization of HRPO on nylon tubes for a clinical analyzer and thus estimation of the amount of active peroxidase attached covalently to the support is important. Chan et al. (39) outlined the modifications required in enzyme assay techniques because of the particulate nature of insoluble enzyme derivatives. In contrast to catalysis in homogenous solution, some means of maintaining a steady diffusion of substrates and products must be found. This has been achieved either by continuous stirring of the mixture (6,39) or by continuous passage of substrate solution through a stationary column of the insoluble enzyme (6). The most used and often the easiest way of following both approaches is to use spectrophotometry, thereby studying changes in absorbance caused by the consumption of substrate or generation of product. In the first approach, the activity of the enzyme, immobilized on matrices that are not too optically dense can

be measured in a cuvette provided that the particles are kept in suspension by means of stirring during the assay procedure. In the second approach the enzyme-matrix is kept outside the light path. Reaction is carried on outside the photometer and the product containing solution is passed through a flow-cuvette for spectrophotometric analysis. In choosing any technique the following points should be considered:

1. The choice of assay procedure will be governed by the nature of product formed, e.g., carbon dioxide gas, protons, products visible by various kinds of spectroscopy.
2. Properties of the support, size of particle, particle size distribution, fragility.
3. Stirring and flow rate.
4. Control measurements of free enzyme in the assay mixture.
5. Ionic strength.

Study

This thesis focuses on the immobilization of HRPO on long arm-derivatized, surface-limited polyacrylamide beads having different group density, different functional groups and an analogously derivatized nylon tubing. In order to develop a successful immobilization procedure, different steps will be varied in terms of conditions. After the

selection of the best immobilization procedure the amount of total, active and inactive HRPO immobilized on beads will be estimated. Further, the established procedure and methods will be used to immobilize HRPO inside nylon tubing already loaded with amine functional groups.

CHAPTER II

EXPERIMENTAL

A. MATERIALS

Peroxidase (horseradish) [donor: hydrogen peroxide oxidoreductase; EC 1.11.1.7] Grade I, Grade II were purchased from Boehringer-Mannheim Canada, Ltd., Dorval, Quebec.

Sodium bicarbonate, ethylene glycol, cobalt nitrate, Tris (hydroxymethyl) aminomethane, hydrogen peroxide (30%), methanol, sodium acetate, sodium carbonate, sodium chloride and standard buffer solutions (pH 4.0, pH 7.0, and pH 10.0) were purchased from Fisher Scientific Company, Ltd., Don Mills, Ont., Canada.

Sodium periodate was purchased from BDH Chemicals, Toronto, Ontario.

YM-30 membranes were purchased from Amicon Corporation Scientific System Division, Danvers, MA.

Sodium cyanoborohydride and Hepes buffer were purchased from Sigma Chemical Company, St. Louis, MO.

4-Aminoantipyrene (4-AAP) was purchased from ICN Pharmaceuticals, Plainview, N.Y.

2-Hydroxy-3,5-dichlorobenzenesulfonic acid, sodium

salt and sodium borohydride ($NaBH_4$) were purchased from Aldrich Chemical Co., Milwaukee, WI.

Polyacrylamide gel P-2 (≤400 mesh) was purchased from Bio-Rad Laboratories, Richmond, C A.

Nylon tubing was obtained from Portex Ltd., Hythe-Kent, U.K.

Aqua-Sil was purchased from Pierce Chemical Company, Rockford, Ill.

B. EQUIPMENT

All automatic pipetting was done using Pipetman and Oxford pipetters from Mandel Scientific Company Ltd., Rockwood, Ontario, Canada, and the Fisher Scientific Company Ltd., Don Mills, Ontario, Canada, respectively.

The pH measurements were obtained on a Corning Digital III, general purpose pH meter from Canadian Laboratory Supplies Limited, Toronto, Ontario, equipped with a pencil combination electrode from the Fisher Scientific Company Ltd., Don Mills, Ontario.

For ultrafiltration, an Amicon cell (10 ml volume) with YM-30 membrane was used for pressure dialysis under nitrogen at 4 atmospheres pressure. The membranes were preconditioned with BSA (1mg/ml) before they were used and stored in 10% ethanol when not in use.

All spectrophotometric measurements and recordings were

made on the Beckman model 35 or Acta MVI spectrophotometer, as well as a Shimadzu 240 instrument. For activity measurements on bead suspensions, advantage was taken of Acta's built in magnetic stirrer.

C. REAGENTS

All aqueous solutions were prepared using deionized distilled water. The following buffers and solutions were used:

TNBS solution: 0.01 M

Glycine solution: 0.03 M

Phosphate buffer: 0.1 M, pH 7.4

Tris-HCl buffer: 0.1 M, pH 8.0

Hepes buffer: 0.1 M, pH 7.4

Borate buffer: 0.1 M, pH 9.5

Sodium carbonate buffer: 0.3 M, pH 9.5.

Sodium periodate stock solution: 40 mM. In distilled deionized water prepared immediately before use.

Sodium cyanoborahydride stock solution: 0.1 M in water prepared immediately before use.

Chromogen stock solution: HDCBS and 4-AAP were dissolved in 0.1 M Tris-HCl pH 8.0 to 18 mM and 4.8 mM, respectively. The solution was kept in the dark and used on the same day.

Stock hydrogen peroxide: 10 mM stock solution was prepared fresh in distilled deionized water immediately before the experiment.

Hydrazine solution: 10 mM hydrazine was prepared in 0.1 M phosphate buffer at pH 7.5.

D. METHODS

1. Washing the Glassware

In order to get rid of polyacrylamide gel particles which adhered to glass surface strongly, glassware was siliconized with Aqua Sil. Also, it was observed that the HRPO sticks to the surface of glassware even when washed with strong detergents. Therefore, glassware was usually soaked in chromic acid for 24 hours and always rinsed with 1N HCl after washing the apparatus with water. Finally, the glassware was rinsed with distilled deionized water.

2. Determination of Amino and Semi-Carbazido Group Density on Long-arm Polyacrylamide Beads Using the TNBS Test

The TNBS test was performed according to Antoni et al. (42). One or two ml of 50% bead suspension (pre-washed with 0.01 M borate buffer of pH 9.4) was made up to 10 ml with the same buffer. One milliliter 0.01 M TNBS was then added. A reference sample was prepared at the same time and composed of 10 ml of tetraborate, without the beads, to which 1 ml of .01 M TNBS was added. After 40 minutes at 37°C with stirring, the beads were centrifuged, 1 ml of

supernatant sample solution was diluted with 5 ml of 0.1 M tetraborate and 0.5 ml of 0.03 M glycine. For the blank, 0.5 ml of water was used in place of glycine. After 25 min at 25°C 10 ml of cold methanol was added and the absorbance of each sample was determined against its own blank at 340 nm using $\epsilon = 12400\ M^{-1}cm^{-1}$ (42). However, to distinguish amino and semi-carbazido groups the test was sometimes determined as above but in 0.1 M phosphate buffer pH 7.4; in these cases methanol was not added.

3. Performance of HPRO Assay

Rate method: The reaction was measured at 25°C in 0.1 M Tris-HCl buffer at pH 8.00. The sample and blank were always prepared fresh to contain 2.4 mM 4-AAP, 9 mM HDCBS and 0.3 mM H_2O_2 in 1 ml. Reaction was initiated by adding 50 µl of peroxidase solution to the sample cell and 50 µl of 0.1 M Tris-HCl buffer to the blank (reference cell). The change in absorbance at 510 nm was continuously monitored in 1 cm pathlength cuvettes.

4. Immobilization of HRPO on the Beads

Oxidation of HRPO: A fresh solution of HRPO (2 mg/ml) was made in .3 M $NaHCO_3$ buffer pH 8.00. Usually, the initial concentration of HRPO solution was calculated spectrophotometrically using an extinction coefficient of $9.1 \times 10^4 M^{-1}cm^{-1}$ at 403 nm (29). Oxidation was started by adding 1 ml of

HRPO to 1 ml of 12 mM $NaIO_4$ solution. The mixture was allowed to stir gently for one hour in a shaker at room temperature in the dark at 25°C. Excess of sodium periodate was destroyed by adding 1 ml of .32 M ethylene glycol to the above mixture. The mixture was again stirred gently for one hour using the same shaker and same speed. The absence of periodate was verified with starch iodide paper after one hour. Three ml of oxidized HRPO was dialyzed in the Amicon ultrafiltration cell at 0°C. The oxidized HRPO was removed from the dialysis cell using coupling buffer. Before coupling, the concentration of oxidized-HRPO was checked by spectrophotometric measurement at 403 nm. Immobilization of oxidized-HRPO to long-arm beads was carried out using three different procedures:

(a) Coupling without reduction

(b) First coupling then reduction

(c) Coupling and reduction together.

(a) Coupling without reduction: One ml of a 50% suspension (v/v) of beads was adjusted to coupling pH by centrifugation-resuspension with the coupling buffer three to four times. For coupling, 0.5 ml of the packed beads at pH 9.5 and 2 ml of oxidized HRPO at pH 9.5 in .1 M Na_2CO_3 buffer were stirred gently in a shaker for two hours at room temperature using siliconized test tubes.

(b) Coupling followed by reduction: One ml of 50% (v/v) suspension of beads at pH 9.5 in 0.1 M Na_2CO_3 buffer was incubated with 1 ml of oxidized HRPO at the same pH and in the same buffer for three hours at room temperature. After coupling, excess of HRPO was washed from the beads by washing beads with 10 ml of same buffer 4 times. Subsequently, the beads were reduced with 16 mM $NaBH_4$ at 0°C for three hours in a volume of 2 ml at pH 9.5.

(c) Coupling and reduction together: One ml of 50% suspension (v/v) of beads at pH 7.4 in 0.1 M Hepes buffer or 0.1 M phosphate buffer was incubated with 1 ml of oxidized HRPO at pH 7.4 in 0.1 M Hepes buffer or 0.1 M phosphate buffer for two hours at room temperature. An amount of $NaBH_3CN$ from 0.1 M solution was added into the coupling mixture to give a final concentration of 20 mM.

5. Estimation of Bound HRPO on the Beads

Three different methods were used:

(a) Differential determination method for the total bound HRPO on the beads

(b) Elution method for the determination of HRPO on the beads

(c) Assay method for the determination of active HRPO on the beads.

(a) Differential determination method for the total bound HRPO on the beads. Bound HRPO on the beads was estimated by making a differential determination of the amount of HRPO before and after the coupling reaction. Washing solution was also added to the unreacted HRPO after the coupling reaction. The difference in the protein content due to immobilization was made by measuring heme of HRPO spectrophotometrically at 403 nm and using $\varepsilon = 9.10 \times 10^4 M^{-1} cm^{-1}$ (29).

(b) Elution method for the determination of HRPO on the beads: For samples which had not been reduced after or during coupling, HRPO on the beadswas eluted by treating 0.5 ml of packed beads at pH 7.5 in 0.1 M phosphate buffer with 1 ml of 10 mM hydrazine prepared in 0.1 M phosphate buffer at pH 7.5. The beads were allowed to stir for 2 hours at room temperature. The amount of peroxidase was estimated at 403 nm using $\varepsilon = 9.1 \times 10^4 M^{-1} cm^{-1}$ (29).

(c) Assay method for determination of active HRPO on the beads: For the determination of active HRPO on the beads a method modeled after the procedure of Chan et al. (39) was used. Active HRPO immobilized on the beads was determined by stirring 2-5 mg beads in a cuvette, with teflon-coated stirring bead, containing 2 ml of peroxidase assay solution. In general, 1 ml of 50% suspension was diluted ten times so

that the run could be initiated by addition of the beads in a volume of 50 µl to 200 µl. A blank containing everything but peroxide, was stirred at the same time with same amount of beads. Another sample of the same bead dilution was taken for dry weight determination. Beads were continuously stirred in the cuvettes with teflon-coated stirrer for 20 min while absorbance at 510 nm was continuously monitored. A rate was calculated and the amount of HRPO immobilized on beads was estimated from the standard curve already developed for soluble peroxidase assay.

6. Immobilization of HRPO on Nylon Tubing

HRPO was immobilized on nylon tubing using the method (4a) mentioned earlier, without reduction. Coupling of HRPO to nylon tubing (derivatized by Kierkus) having amino groups, was carried out at pH 7.4 in 0.1 M phosphate buffer by filling nylon tubes with oxidized and ultrafiltred HRPO at 0°C. Nylon tubing was filled with 0.1 M phosphate buffer at pH 7.4 before coupling.

7. Estimation of HRPO on Nylon Tubing

The differential method mentioned earlier for beads, procedure (5a) was used to estimate the total amount of immobilized HRPO. The elution method already used for beads, procedure (5b), was used to estimate inactive HRPO on nylon tubes. For the estimation of active HRPO on nylon tubing

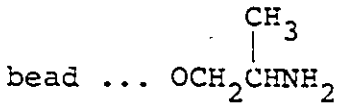
a different procedure from that used for beads was employed. The tubing was washed thoroughly with 0.1 M Tris-HCl buffer until no peroxidase activity was detected in the washes. The nylon tubing was then flushed with 10 ml of peroxidase assay solution for 1 minute at a rate of 1.8 ml/min. The assay solution was collected and absorbance was measured at 510 nm. The amount of HRPO activity on the nylon tubing was calculated using standard curve of soluble peroxidase.

CHAPTER III

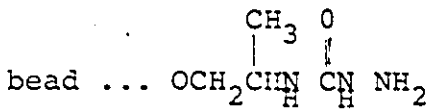
RESULTS AND DISCUSSION

Determination of Protein Coupling Group Density on Long-arm Polyacrylamide Beads

The beads used in this work had poly(oxyethylene) spacer arms ending either in a primary amine (identical to those used by Kristalovich (18)) or a semi-carbazide as follows:



or



Nylon tubing used here had spacer arms ending in a primary amine. Beads provided by Taylor (40) had amino groups like Kristalovich beads (18) and beads provided by Taylor and Kierkus had semi-carbazide groups. Nylon tubing provided by Kierkus had amino groups.

In all these cases initial characterization of amino groups and semi-carbazido groups was carried out by potentiometric titrations (41). In this laboratory, Chen and

Kristalovich (17,18) had used potentiometric titrations for the characterization of amino groups according to the method of Inman and Dintzis (41). However, the functional group densities desired in the present work (based on Kristalovich's results with high and low functional group density beads) are at the low end of the range of reliable estimation by this method.

After this project got started the paper by Antoni et al. (42) appeared which seemed to offer a more sensitive spectrophotometric means of characterizing amino group densities (reaction with excess TNBS, then back-titration of the TNBS remaining with glycine). It occurred to us that we might, with slight modification of the method (42), be able to determine and distinguish semi-carbazido and amino groups. The following model reactions were set up to test this possibility. In control experiments following Figure 3 it was observed that reaction between .55 mM ethanolamine and 10 mM TNBS was completed in 1 hour at pH 9.5 in 0.1 M borate buffer, shown in Figure 3A. In a parallel experiment, at lower pH of 7.5 in 0.1 M phosphate buffer it was also observed (shown in Figure 3B) that the reaction between .55 mM ethanolamine and 10 mM TNBS was not completed even in two hours. Therefore, the TNBS test for amino groups was found to require a higher pH 9.5 in 0.1 M borate buffer. Subsequently the detection of amino groups was carried out at higher pH.

Figure 3

Spectrophotometric Scans at Two Different pH's

Legend

Figure 3A. Shown are the spectrophotometric scans of a model reactions at pH 9.5 in 0.1 M borate buffer between 10 mM TNBS and .55 mM semicarbazide (———) or 10 mM TNBS and .55 mM ethanolamine (------) at different intervals of time (a) 0 min, (b) 10 min, (c) 20 min, (d) 30 min, (3) 40 min.

Figure 3B. Shown are the spectrophotometric scans of the same model reactions at pH 7.5 in 0.1 M phosphate buffer between 10 mM TNBS and .55 mM semicarbazide (———), 10 mM TNBS and .55 mM ethanolamine (------), at different intervals of time, (a) 0 min, (b) 10 min, (c) 20 min, (d) 30 min, (e) 40 min.

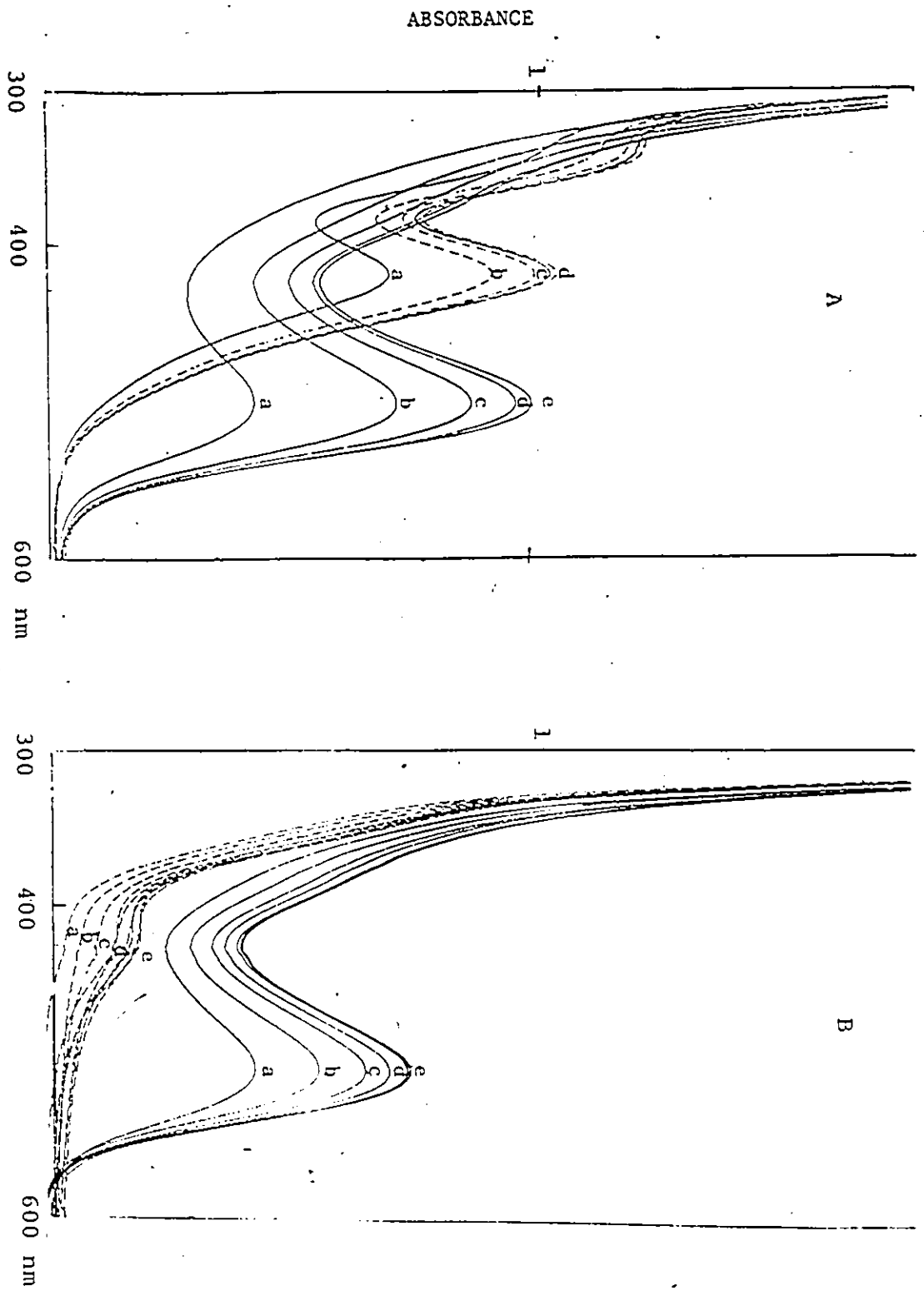


Figure 3

In another control experiment for semi-carbazido groups shown in Figures 3A and 3B, it was observed that reaction between .55 mM semi-carbazide and 10 mM TNBS was completed in 40 minutes both at pH 9.5 in 0.1 M borate buffer and at pH 7.4 in phosphate buffer. Therefore, the TNBS test for semi-carbazido group was carried out at pH 9.5 in 0.1 M borate buffer, and pH 7.4 in .1 M phosphate buffer.

The total concentration of protein coupling groups, amino and semi-carbazido, expressed as coupling groups per gram dry weight of the beads is summarized in Table I. Group density was calculated spectrophotometrically. As shown in Table I, final group density was a difference of total amino group density and amino group density due to native beads, where native beads consisted of washed but underivatized polyacrylamide beads (Bio-gel P-2). Unexpectedly, the native beads, despite extensive prior washing, showed substantial reaction with TNBS at both pH's. Amino group density on big beads (average diameter 225 μm) was determined using the same strategy (mentioned above) and procedure.

Amino group density, experiments 1 and 2, Table I, can be determined only at a higher pH 9.5 in 0.1 M borate buffer. Semi-carbazido group density, experiments 3 and 4, Table I can be determined at pH 9.5 in 0.1 M borate buffer and pH 7.5 in 0.1 M phosphate buffer. From these experiments, it was observed that semi-carbazido group density is less than amino group density. Also amino group density on

TABLE I

DETERMINATION OF FUNCTIONAL GROUP DENSITY ON LONG-ARM-DERIVATIZED POLYACRYLAMIDE BEADS

Size of the beads (Ave. diam.)	Functional Group	Native Beads[c] μeg/gm of dry beads	Functionalized Beads μeg/gm of dry beads	Net Functional Group Density μeg/gm of dry beads
25 μm	Amino[a]	2.4 ± .02	3.2 ± .03	0.8 ± .05
225 μm	Amino[a]	2.4 ± .02	2.5 ± .01	0.1 ± .03
25 μm	Semi-carbazido[a]	2.4 ± .02	2.7 ± .02	0.3 ± ,04
25 μm	Semi-carbazido[b]	2.4 ± .01	2.7 ± .02	0.3 ± .03

[a]Experiments 1-3 were carried out in duplicate at pH 9.5 in 0.1 M borate buffer according to TNBS procedure 2 in Methods. Functional group density was calculated spectrophotometrically using $\epsilon = 12400\ M^{-1}cm^{-1}$ (42) at 340 nm.

[b]Experiment 4 was carried out in duplicate at pH 7.4 in 0.1 M phosphate buffer according to TNBS procedure 2 in Methods.

[c]Native beads consisted of unmodified Bio-gel P-2 (polyacrylamide, exclusion limit 1800) which had been soaked in water overnight, degassed, washed 20 times with water, 0.1 M KCl and 0.1M borate buffer.

big beads is less than small beads by approximately the same factor as surface area per dry weight is diminished.

Peroxidase Assay

The assay was based on the oxidative coupling of HDCBS and 4-AAP in the presence of H_2O_2 and perodixase. This chromogen system has been used extensively in this Department for the determination of peroxide and peroxide-generating systems, but it was never used here to determine enzyme activity until the need arose in the present work. Initial studies showed that peroxidase is inhibited by its substrate hydrogen-peroxide as shown in Table II. In choosing the final concentration of hydrogen peroxide, 0.3 mM was considered as a suitable excess of substrate for making a continuous assay. Ugarova et al. (29) also used 0.3 mM H_2O_2 for the oxidation of o-dianisidine in presence of peroxidase and reported a K_m^{app} of 40 µM for hydrogen peroxide and K_m^{app} of 39 µM for the oxidized peroxidase immobilized on AH-sepharose 4B. Using 0.3 mM of H_2O_2, 9 mM HDCBS and 2.4 mM 4-AAP the rate of the reaction was found to be directly proportional to the concentration of the peroxidase. The assay results are shown in Table III, Figure 4 and Table IV, Figure 5. In constructing these standard curves two different methods were used to make dilutions. It is important to mention here, the method of making serial dilutions in different glassware (or outside the cuvette) has a limitation since

TABLE II

OPTIMIZATION OF HYDROGEN PEROXIDE CONCENTRATION[a]

$[H_2O_2]$ mM	ΔA_{510}/min
10	0.006 ± .001[b]
1	0.350 ± .002
.75	0.400 ± .02
.45	0.500 ± .01
.30	0.66 ± .01
.25	0.61 ± .01
.20	0.62 ± .02
.15	0.65 ± .01
.10	0.65 ± .01

[a]Rate of reaction was measured in the presence of 2.4 mM 4-AAP and 9 mM HDCBS with the cuvette varying concentrations of H_2O_2 shown above at 25°C, pH 8.00 in 0.1 M Tris-HCl buffer.

[b]Results in triplicate.

TABLE III

STANDARD CURVE DATA FOR PEROXIDASE

[HRPO] (pM)	$\Delta A_{510} \times 10^3/min$[b]
14.37	0.75 ± .50
28.37	1.20 ± .2
57.5	2.0 ± .5
115	5.8 ± 1.2
230	10.0 ± .8

[a] Peroxidase stock solution concentration was determined using a molar extinction coefficient 9.1×10^4 cm^{-1} at 403. Runs were formulated as in "Standard procedure for peroxidase" in triplicates. The amount of peroxidase was varied by making serial dilutions of the stock solution outside the cuvette.

[b] $\Delta A_{510}/min$ was change in absorbance at 510 nm per minute. Linear regression analysis of the HRPO versus the $\Delta A/min$ data yielded a Y-intercept -0.012 and a slope of .044, assuming 2 equivalents of H_2O_2 per mole of chromophore. The correlation coefficient was 0.993. Errors shown are simply mean uncertainties.

Figure 4

Standard Curve for Peroxidase

Legend

The assay was carried out in triplicates at pH 8.00 in 1 ml using a concentration of 4.8 mM of 4-AAP, 9 mM of HDCBS, 0.3 mM of H_2O_2. Concentration of peroxidase was varied. In this case dilution was made outside the cuvette. The line shown is the least square line (correlation coefficient 0.993, Y-intercept-0.012 and a slope 0.044). Error bars shown are simply mean uncertainties. ΔA_{510} is change in absorbance per minute at 510 nm.

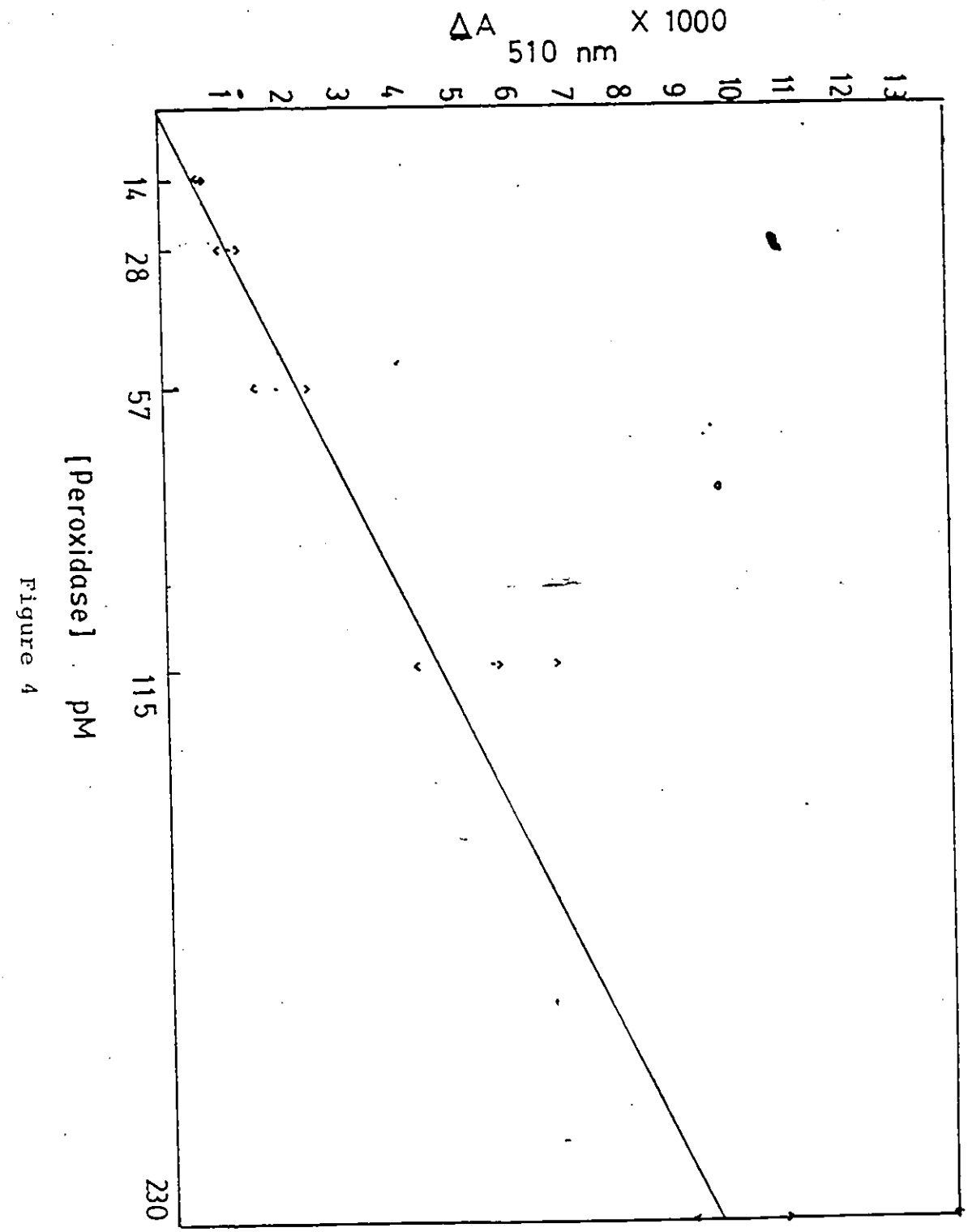

ΔA X 1000
510 nm
1
2
3
4
5
6
7
8
9
10
11
12
13
14
28
57
115
230
[Peroxidase] pM

Figure 4

TABLE IV

STANDARD CURVE DATA FOR PEROXIDASE[a]

[HRPO] (pM)	$\Delta A_{510} \times 10^3$/min
11.5	0.65 ± .05
23	1.30 ± .10
46	2.50 ± .10
92	5.1 ± 0.3
115	6.50 ± 0.5
184	9.80 ± 0.2
230	13.0 ± 0.1

[a] Runs were formulated as in the "standard procedure for peroxidase" in triplicates.

[b] The amount of peroxidase was varied in the cuvette as shown above from the same stock solution whose concentration was calculated on the basis of $\varepsilon_{403} = 9.1 \times 10^4 M^{-1} cm^{-1}$ (29). Linear regression analysis of the [HRPO] versus A_{510}/mm data yielded a Y-intercept of -0.006 and a slope of .055. The correlation coefficient was 0.9990. Errors shown are simply mean uncertainties.

Figure 5

Standard Curve for Peroxidase

Legend

The assays were carried out in triplicate at pH 8.00 in 1 ml using a concentration of 4.8 mM of 4-AAP, 9 mM of HDCBS and 0.3 mM of H_2O_2. Dilutions were made inside the cuvette. The line shown is the least-square line (correlation coefficient 0.9990, Y-intercept-0.006 and slope 0.055). Error bars shown are simply mean uncertainties ΔA_{510} is change in absorbance per minute at 510 nm.

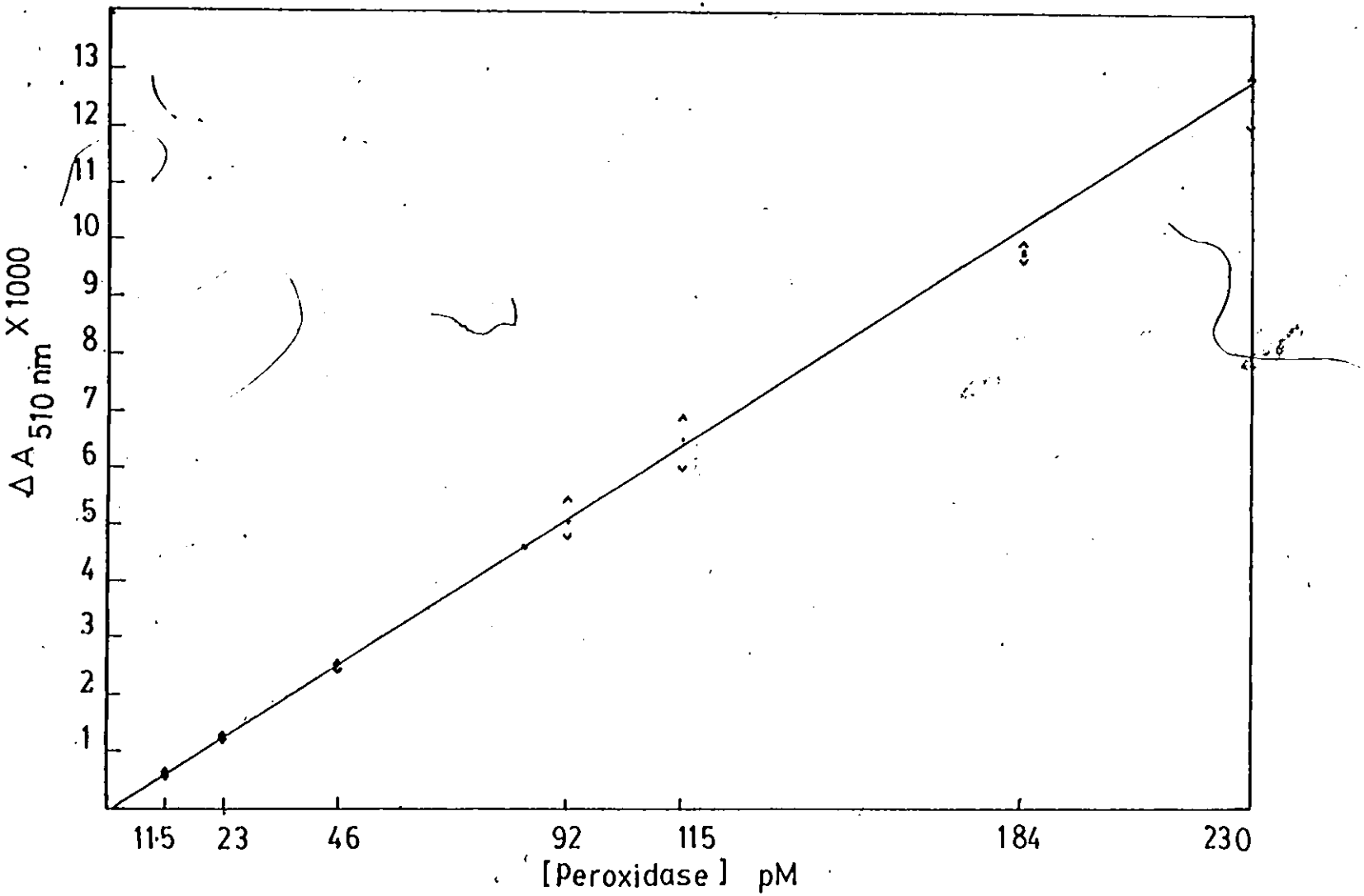

ΔA 510 nm X 1000
13
12
11
10
9
8
7
6
5
4
3
2
1
11·5
23
46
92
115
184
230
[Peroxidase] pM

Figure 5

peroxidase sticks to the glassware. As shown in Table III and Figure 4, when serial dilutions were made outside the cuvette, large deviations were observed, resulting in a poor correlation coefficient. On the other hand, the method of making dilution of peroxidase in the cuvette was found to be quite accurate and precise with a satisfactory correlation coefficient, Table IV and Figure 5. In fact, this assay procedure was adapted for the measurement of picomolar amount of insolubilized HRPO on the beads in the cuvette as described in the following discussion.

During these investigations, it was also found that the relationship between peroxidase concentration and absorbance was linear even in the nanomolar range, Table V and Figure 6. In fact, this was also required to measure perodixase activity on immobilized beads falling in the nanomolar range. In conclusion, a kinetic method has been developed to measure peroxidase activity in a wide range. Also, this procedure has the advantage over a two point procedure since formation of a product is continuously or intermittently measured at the start of the peroxidase reaction.

Estimation of HRPO Activity on the beads

For estimating active peroxidase immobilized on polyacrylamide beads the method of Weliky et al. (43) was used in which derivatized beads were stirred in peroxidase assay

TABLE V

STANDARD CURVE DATA FOR PEROXIDASE[a]

[HRPO] (nM)	ΔA_{510}/min
0.83	0.04 ± .005
1.67	0.072 ± .006
3.35	0.143 ± .007
6.7	0.29 ± .018
13	0.56 ± .036
26	1.11 ± .045
53	2.05 ± .015

[a]Runs were formulated as to the "standard procedure for peroxidase" in triplicates.

[b]The amount of peroxidase was varied in the cuvette shown above from the same stock solution whose concentration was calculated on the basis of $\varepsilon = 9.1 \times 10^4 M^{-1} cm^{-1}$ (29) at 403 nm. Linear regression analysis of [HRPO] versus A_{510}/min data yielded a Y-intercept of -0.021 and a slope of 0.038. The correlation coefficient was 0.9985.

Figure 6

Standard Curve for Peroxidase

Legend

The assays were carried out in triplicate at pH 8.00 in 1 ml using a concentration of 4.8 mM of 4-AAP, 9 mM of HDCBS, 0.3 mM of H_2O_2. Dilutions were made inside the cuvette. The line shown is the least square line (correlation coefficient 0.9985, Y-intercept 0.035 and slope 0.039. Error bars shown are simply mean uncertainties. ΔA_{510} is change in absorbance per minute at 510 nm.

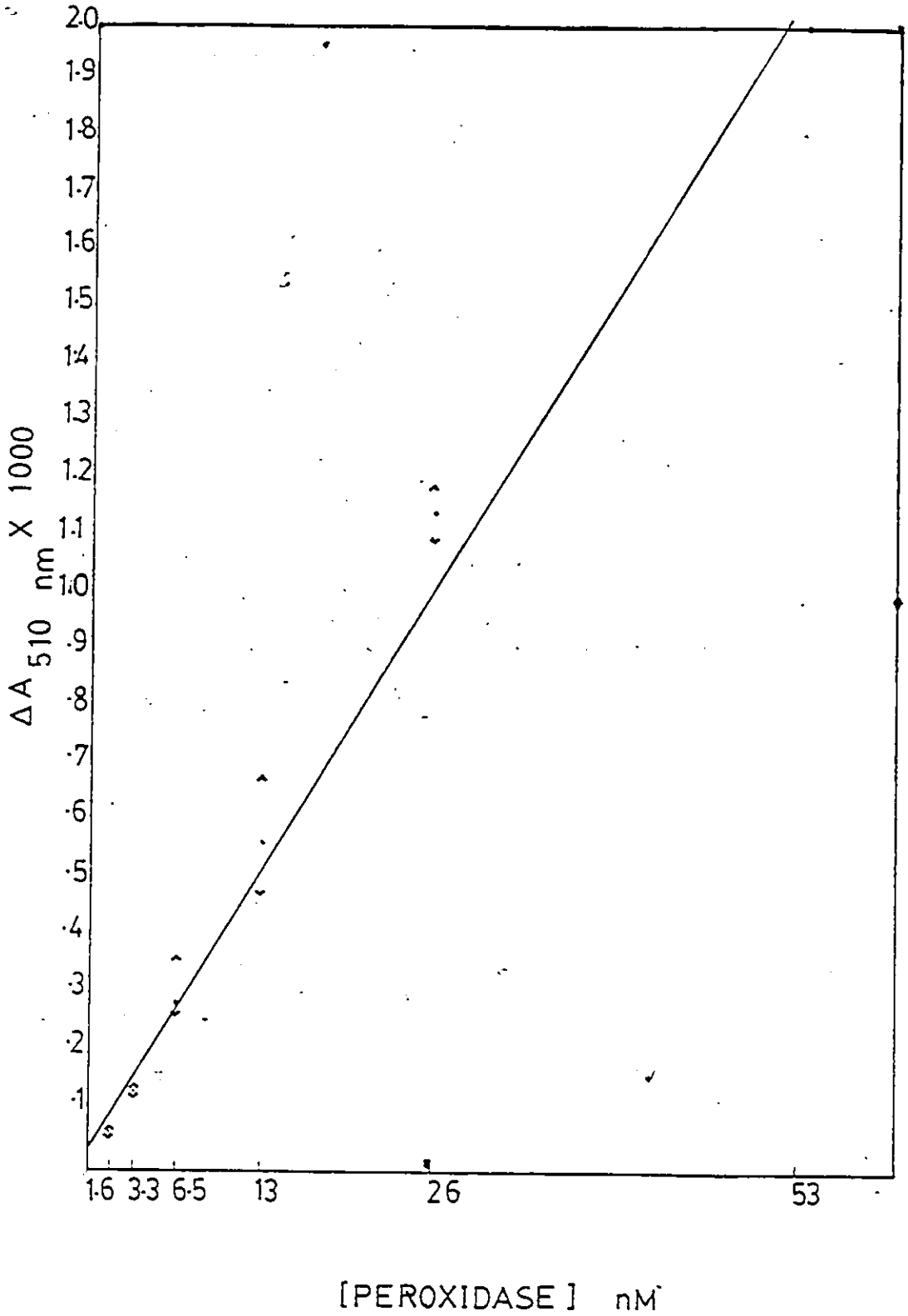

2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
·9
·8
·7
·6
·5
·4
·3
·2
·1
ΔA 510 nm X 1000
1·6
3·3
6·5
13
26
53
[PEROXIDASE] nM

Figure 6

solution in cuvettes under constant spectrophotometric monitoring. According to Mosbach and Mattiasson (44) the activity of enzyme immobilized on matrices that are not too optically dense can be measured in such a way provided the particles are kept in uniform suspension by means of stirring during the assay procedure. Chan et al. (39) had earlier demonstrated the feasibility of this approach. As shown in Figures 7a, and 7b a continuous linear reaction rate was observed as long as stirring was continued. When stirring ceased, the reaction rate dropped to zero, indicating that all the enzyme activity was associated with the matrix, which had sedimented, leaving no soluble enzyme in the supernatant. As observed resumption of stirring led to a linear increase in absorbance at the same rate as that observed during the previous period of stirring. It is apparent from the noise on the recorder trace that small particles in the cuvette cause small perturbations while these disturbances in the registered signal are more pronounced for bigger particles as shown in Figure 7b.

The slopes of recordings such as those shown in Figure 7 were used with standard curve (Figures 4-6) for soluble peroxidase activity to calculate enzyme activity on the beads. As shown in Table VI the method used was quite sensitive, as HRPO activity on 1.25 mg of beads can be

Figure 7

Spectrophotometric Scans for the Estimation of Active Immobilized HRPO on Beads

Legend

Figure 7a: Runs were formulated for small beads (25 μm average diameter) in triplicate as mentioned in procedure 5c "Assay method for determination of active HRPO on the beads."

Figure 7b: Runs were formulated for large beads (225 μm) average diameter) as mentioned above in duplicate.

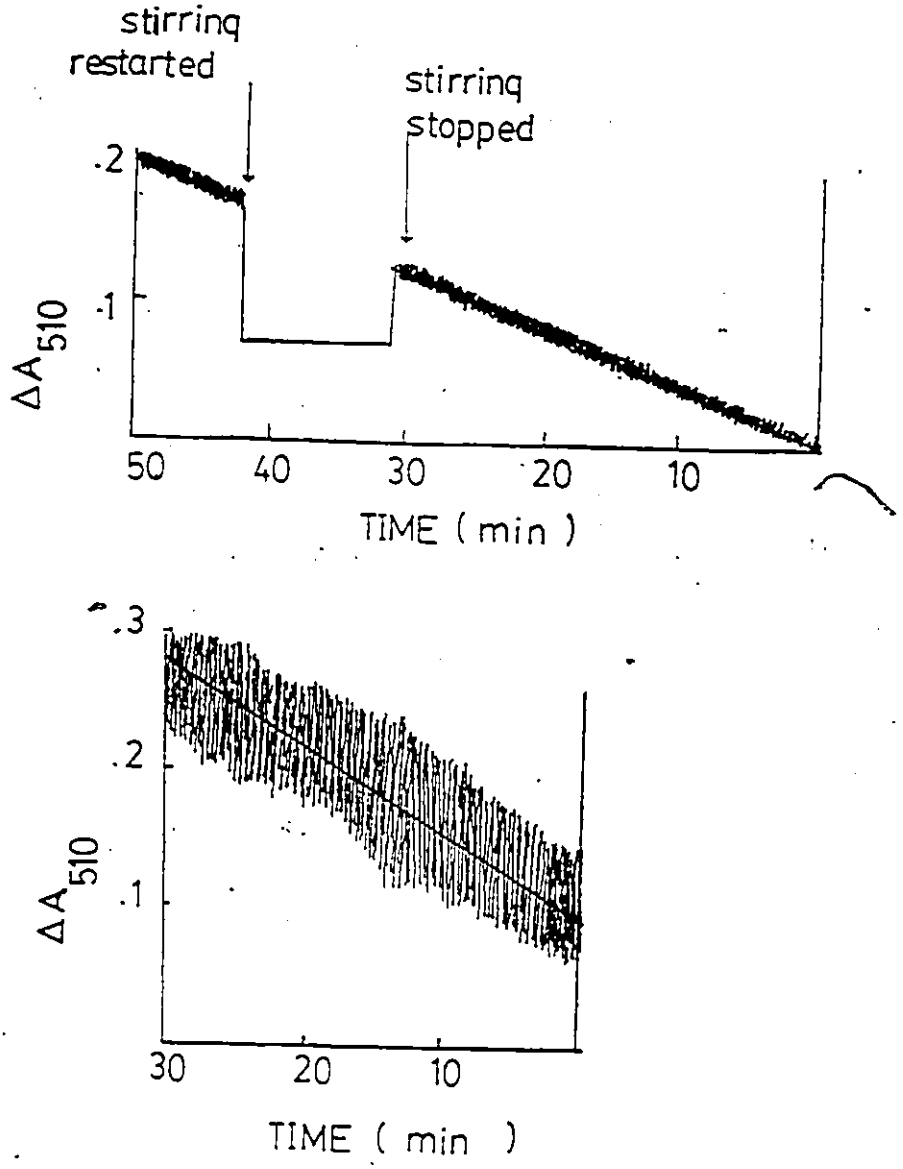

stirring
restarted
stirring
stopped
.2
.1
ΔA_{510}
50
40
30
20
10
TIME (min)
.3
.2
.1
ΔA_{510}
30
20
10
TIME (min)

Figure 7

TABLE VI

DETERMINATION OF HRPO ACTIVITY ON BEADS

Beads used[a] in Cuvette (ml)	Weight of Beads (mg)	ΔA_{510}/min	Activity on Beads	
			pM[c]	µg/gm of dry beads
0.100	1.25	.002	45	2.88
0.200	2.50	.004	90	2.88
0.250	3.12	.005	113	2.88
0.500	6.25	.010	225	2.88
10[d]	140	.044	1.25	2.87

[a]One ml of a 50% suspension of beads were diluted to 10 ml and this dilute suspension was shaken well before withdrawing the volumes shown.

[b]Weight of beads were determined according to dry weight determination (17) carried out on duplicate 0.5 ml sample of the dilute suspension.

[c]Activity on beads was estimated from the standard curve (Figure 5) formulated for soluble peroxidase.

[d]One ml of a 50% suspension of beads was diluted to 9 ml with 0.1 M Tris-HCl buffer. One ml of concentrated chromogen mixture was added to the diluted beads to achieve final H_2O_2, HDCBS and 4-AAP concentrations of 0.3, 9.00 and 2.4 mM, respectively. The beads were shaken well for 10 minutes in a shaker. After 10 minutes the absorbance of 1 ml was measured at 510 nm.

detected. In spite of the noise of the recorder, the slope of the line was measured satisfactorily. In this method only up to 6.2 mg of small beads having 25 μm average diameter can be detected. In the case of bigger beads (225 μm average diameter) the noise was more pronounced and therefore only up to 1.25 mg of beads could be used.

In conclusion this method for the estimation of peroxidase in the cuvette was found to be quite accurate. Its accuracy can be measured from the fact, that the total activity on one preparation (140 mg of dry beads) was a big additive number which is the sum of different numbers measured in a cuvette using different weights of beads.

Immobilization Procedure

Our initial immobilization procedure was based on the the method Nakane et al. used for forming HRPO-antibody conjugates (32). These workers attempted to limit self-coupling via amino group of HRPO (which were considered to be necessary for catalysis and substrate binding) by chemically blocking the amino groups of the enzyme with fluorodinitrobenzene (FDNB). However, self-coupling was not a limitation in the immobilization procedure according to our investigation as shown in Table VII, experiments 3 and 4. In fact, oxidized and FDNB-blocked HRPO was yellow which posed a number of problems in spectrophotometric determinations of HRPO concentration at 403 nm.

TABLE VII

IMMOBILIZATION PROCEDURE

Experiment No.	FDNB used	Ultrafiltration	µg of Activity/gm of dry beads
1	-	+	0.23 ± .02
2	-	+	0.19 ± .01
3	-	-	0.04 ± .01
4	-	-	0.06 ± .01
5	+	+	0.23 ± .02
6	+	+	0.19 ± .02

[a]In all these experiments 20 mM of $NaIO_4$ for 30 min at pH 8.00 in 0.3 M $NaHCO_3$ was used for oxidation at 25°C. Oxidation was terminated by addition of 0.32 M ethylene glycol. Ultrafiltration was done at 0°C in all experiments except 3 and 4 to remove small reagents and products. After coupling for three hours at pH 9.5 in 0.1 M carbonate buffer $NaBH_4$ was added to 20 mM and incubation was continued for 3 hours at 0°C.

Ultrafiltration of oxidized-HRPO to remove products of reagents already present in the oxidation mixture was checked with a control experiment. As shown in Table VII experiments 3 and 4, the amount of active HRPO on the beads was low, .04 µg/gm of dry beads. Also, the filtrate from the ultrafiltration cell was checked spectrophotometrically for the loss of HRPO during ultrafiltration. The amount of HRPO lost due to ultrafiltration was less than 1% of the total oxidized HRPO. From these observations ultrafiltration of oxidized HRPO before coupling was considered an important step in the immobilization procedure. The immobilization was carried out according to a two-step procedure (procedure 4 with or without reduction) in which Schiff-base formation occurred first at pH 9.5 followed by $NaBH_4$ reduction at the same pH.

A number of control experiments were performed to analyze different steps in the investigated methods. For example, in one control experiment, unoxidized HRPO was incubated with long-arm polyacrylamide beads, having high amino group density (0.8 µeg/gm of dry beads) according to the procedure mentioned in Methods (without reduction). After incubation, the beads were washed 14 times with 0.1 M phosphate buffer pH 7.4 and 0.1 M Tris-HCL buffer pH 8.00. HRPO activity, checked with the peroxidase assay was detected in first four washes, the remaining washes and

the beads themselves showed no activity. From this control experiment, it was clear that HRPO was not absorbed strongly on the carrier, perhaps due to hydrophobic or electrostatic attraction. The above observation suggests an area for future research into the use of organic hydrophilic supports which cannot absorb HRPO. This is mentioned because during this research it was observed that HRPO absorbed strongly on inorganic hydrophilic support like glass.

In another set of control experiments, Table VIII, oxidized-HRPO was incubated with native beads following the procedure mentioned in Methods (without reduction). Active HRPO was found on native beads even after 14 washes with 0.1 M phosphate buffer and 0.1 M Tris-HCl buffer. However, all wash solutions as shown in Table VIII showed a continuous leakage of HRPO activity from these beads. After washing these beads with buffer 500, mM NaCl solution was used to check retention of activity on the native beads. It was found that after this treatment, there was a complete loss of HRPO activity on the beads. In addition, the activity was found in the supernatant. The facts that unoxidized enzyme does not strongly associate with derivatized beads while oxidized enzyme does adhere strongly to native beads except in the presence of high salt concentrations suggests that the presence of amino groups on the beads might lead to electrostatic repulsion of

TABLE VIII

ADSORPTION OF HRPO ON NATIVE BEADS
(UNDERIVATIZED BEADS)[a]

No. of Washes	µg of Activity/gm of dry beads
14	4
10	1.14
10	0.6
10	0.1
10[b]	No activity

[a]Native beads consisted of undervatized (Bio gel-P-2 polyacrylamide beads exclusion limit 1800). These beads were soaked in water overnight and degassed, washed 20 times in water, 0.1 M KCl and 0.1M borate buffer before immobilization. Then 10 nanomoles of already oxidized HRPO (at pH 8.1 in 0.3 M $NaHCO_3$ at 25°C with 20 mM $NaIO_4$) was added at pH 7.5 M 0.1 M phosphate buffer for three hours. After three hours beads were separated from the added HRPO and washed thoroughly. For each wash, 10 ml of buffer solution (0.1 M phosphate buffer) was taken in the test tube and beads were shaken for 10 minutes vigorously.

[b]Before these washes the beads were washed with 500 mM of NaCl solution.

the enzyme at neutral pH.

However, in contrast long-arm polyacrylamide beads having amino groups for immobilization, mentioned in later discussion, never showed leakage of activity after six or seven washes and also a complete loss of activity after treatment with 500 mM NaCl solution. Thus it was concluded for permanent retention of enzyme activity on beads, there must be amino group density on the beads.

Variations in Oxidation Conditions

Initial studies on the oxidation of HRPO was done according to Nakane et al. (32) in which 20 mM $NaIO_4$ was used as oxidizing agent at pH 8.1 in 0.3 M $NaHCO_3$ buffer. Table IX shows that as the $NaIO_4$ concentration was increased there was a loss in HRPO absorbance at 403 nm and a loss in activity. These results were similar to the work of Nakane et al. (32).

The periodate reaction was chosen because of absence of side reactions, its high yield of aldehyde groups, and the fact that it can be carried out in aqueous solution at or near neutral pH. Nakane et al. (32) mentioned that other oxidizing agents such as lead tetraacetate which requires acidic media were to be avoided since such media can destroy HRPO activity. Tijssen and Kurstak (45) mentioned that the oxidation sensitivity of the carbo-

TABLE IX

EFFECT OF OXIDATION OF HRPO ON HEME ABSORBANCE

$[NaIO_4]$	No. of moles of HRPO after[a] treatment with $NaIO_4$	% of original number of moles.
20	20	95
40	15	71
80	14	66
160	12	57
320	13	62

[a]Concentration of HRPO was calculated by using $\varepsilon = 9.10 \times 10^4 M^{-1} cm^{-1}$ at 403 nm (29). In all these experiments 21 nmoles of HRPO were oxidized with different concentration of $NaIO_4$ shown above at pH 8.1 in 0.3 M $NaHCO_3$ buffer at room temperature in the dark for 30 minutes. $NaIO_4$, at the concentration noted, plus peroxidase were stirred for half an hour.

hydrate moiety is a central problem. In their opinion, HRPO is sensitive to impurities in water such as bacteria, bacteriostatic agents, polystyrene, etc. Yamasaki et al. (46) mentioned another possible side-effect of sodium periodate treatment. According to their work, even moderate concentrations of $NaIO_4$ leads to the oxidation of amino acid residues which may alter the conformation of protein molecules. In spite of the above mentioned problem, as mentioned earlier, $NaIO_4$ has been used frequently for the oxidation of carbohydrate residues of proteins (33,34,35).

Due to the foregoing uncertainties, a number of experiments were carried out at different $NaIO_4$ concentrations ranging from 2.5 mM to 20 mM for 30-120 minutes. The result of these experiments, shown in Tables X and XI suggested that a lower $NaIO_4$ concentration for a longer period of time resulted in better immobilization, both for beads having amino groups (Table X) and semi-carbazido groups (Table XI) available for coupling. The conditions selected, 6 mM for 90 minutes provided an efficient conjugation and prevented overoxidation. Almost the same observation and conclusion was mentioned by Tijssen and Kurstak (45). They suggested that too little oxidation prevents effective conjugation, whereas strong oxidation may result in the formation of carboxyl

TABLE X

EFFECT OF PERIODATE OXIDATION ON IMMOBILIZATION YIELDS USING AMINO BEADS

Time for Oxidation (min)	$NaIO_4$ (mM)	Activity on 1 gm of dry beads[a] (µgm/gm of dry beads)
30	2.5	2.2 ± .05
30	5	2.5 ± .07
90	6	9.2 ± .02
80	8	2.2 + .01
60	10	5.2 ± .05
30	20	3.2 ± .04

[a]Oxidation was carried out in 0.3 M $NaHCO_3$ buffer pH 8.1 at 25°C in the dark for different periods of time. In all these experiments ultrafiltration was done before coupling. Coupling and reduction were performed together in the presence of 20 mM $NaBH_3CN$ in 0.1 M Hepes buffer pH 7.5 for two hours. Uncertainties due to duplicate immobilization are given.

TABLE XI

EFFECTS OF PERIODATE OXIDATION ON IMMOBILIZATION YIELDS USING SEMI-CARBAZIDO BEADS[a]

Time for Oxidation (min)	$NaIO_4$ mM	Activity on 1 gm of dry beads[b] (µgm/gm of dry beads)
120	2.5	0.32 ± .02
90	5.0	0.58 ± .03
60	10.0	0.97 ± .01
30	20.0	1.00 ± .01

[a]Oxidation was carried out in 0.3 M $NaHCO_3$ buffer pH 8.1 at 25°C in the dark for different periods of time. In all these experiments ultrafiltration was done before coupling. Coupling and reduction were performed together in the absence of $NaBH_3CN$ in 0.1 M phosphate buffer pH 7.5 for two hours.

[b]Uncertainties due to duplicate immobilization are given.

groups instead of aldehyde groups.

Galactose oxidase, which is another mild oxidizing agent, was considered as an alternative possibility for HRPO oxidation as mentioned earlier. From previous work, in this laboratory (33), galactose oxidase appeared to generate aldehyde from HRPO in the presence of 4-AAP and HDCBS. After oxidation, the resultant coloured mixture was measured at 510 nm to allow a rough estimate of the number of aldehydes formed per µg of HRPO.

However, this method of oxidation as shown in Table XII did not lead to an efficient conjugation possibly because little oxidation of HRPO took place. This is not unreasonable in view of the fact that the carbohydrate composition reported by Welinder (26) for peroxidase C showed no galactose or N-acetylgalactosamine content, although earlier reports (47) had indicated the presence of these sugar residues.

Influence of Reducing Agent on Immobilization

The immobilization reaction proceeds via Schiff-base intermediates formed by the interaction of an aldehyde group of oxidized HRPO and an amino group of long arm beads as shown in Figure 1. The Schiff-bases are converted to amine derivatives by reduction with either $NaBH_4$ of $NaBH_3CN$ (32).

Initial studies were carried out without reducing

TABLE XII

EFFECT OF GALACTOSE OXIDASE CATALYZED OXIDATION ON IMMOBILIZATION YIELDS USING AMINO BEADS[a]

Experiment No.	Coupling pH	µgrams of Activity/gm of beads
1[b]	4.00	1.136
2[b]	4.00	0.71
3[b]	4.00	0.80
4[b]	7.5	0.51
5[c]	7.5	0.12
6[d]	7.5	0.16

[a]In all these experiments 20 nmoles of HRPO were oxidized with 10 units of GAO in phosphate buffer pH 7.5 for one hour at room temperature. Coupling was carried out according to method 4a (Without Reduction).

[b]Oxidation was performed in presence of 4-AAP and HDCBS.

[c]Oxidation was performed without 4-AAP and HDCBS.

[d]Oxidation was performed in a test tube containing beads, diazable compounds were not removed.

agents (procedure 4a) (without reduction). Peroxidase activity on the beads, shown in Table XIII, experiments 1 and 2 suggested that a Schiff-base is formed between oxidized HRPO and amino groups of the beads. However, the activity on these beads was not stable but, rather, diminished after continuous washing. Usually, the wash buffer showed a continuous leakage of HRPO activity. From these experiments, it was concluded that the Schiff-base is not stable.

In order to stabilize the Schiff base, some experiments were carried out according to Nakane et al. procedure (32) which was an adaptation of the Means and Feeney (36) procedure involving Schiff-base reduction by $NaBH_4$ (procedure 4b with reduction). These experiments, 3 and 4, Table XIII, showed that 10-20 mM $NaBH_4$ at 0°C for three hours was indeed capable of reducing the Schiff-base to give a stable immobilized preparation with no leakage of activity. The immobilized preparation was found to be stable for three months. After three months there was a dramatic loss of activity. In addition, wash supernatants never showed peroxidase activity. However, there are certain disadvantages inherent in the use of $NaBH_4$ reduction (37). The major drawback was its ability to reduce aldehyde groups directly thereby eliminating a possible coupling reaction. Furthermore, $NaBH_4$ is

TABLE XIII

ACTIVITY ON BEADS USING DIFFERENT REDUCING AGENTS

Experiment No.	Reducing Agents	μgram of HRPO g^{-1} of Dry Beads					
		Wash Number					
		1	2	3	4	5	6
1[b]	-	0.46 ± .02	0.31 ± .01	0.30 ± .01	0.25 ± .02	0.18 ± .01	0.09 ± .01
2[b]		0.41 ± .02	0.30 ± .01	0.20 ± .01	0.20 ± .01	0.15 ± .2	0.08 ± .01
3[c]	$NaBH_4$	1.70 ± .02	1.68 ± .01	1.65 ± .01	1.64 ± .01	1.64 ± .01	1.65 ± .01
4[c]	$NaBH_4$	1.29 ± .01	1.29 ± .01	1.28 ± .01	1.25 ± .01	1.24 ± .01	1.23 ± .01
5[d]	$NaBH_3CN$	5.0 ± .01	5.0 ± .01	4.8 ± .01	4.6 ± .01	4.8 ± .01	4.8 ± .01
6[d]	$NaBH_3CN$	8.0 ± .02	7.9 ± .01	7.9 ± .01	7.9 ± .01	7.8 ± .01	7.8 ± .01

[a]For all these experiments 20 mM $NaIO_4$ was used for oxidizing 21 nmoles of HRPO for 30 minutes at pH 8.1 in 0.3 M $NaHCO_3$ at 25°C. HRPO was ultrafiltered at 0°C to remove small molecule reagents and products. Beads with bound HRPO were stored in 0.1 M phosphate buffer at 0°C. Before activity measurement, supernatant was also checked for peroxidase activity using the peroxidase assay. Following this, the beads were washed 10 times with 0.1 M phosphate buffer and the activity on the beads was checked using same peroxidase assay.

[b]In experiment 1 and 2 coupling was done for two hours in Hepes buffer pH 7.5 at room temperature.

[c]In experiments 3 and 4 coupling was done for three hours in 0.1 M Na_2CO_3 buffer pH 9.5 at room temperature. Beads were washed with 10 ml of 0.1 M Na_2CO_3 buffer pH 9.5 five times washing was considered to be complete when the buffer showed no enzyme activity with the peroxidase assay. After complete washing, reduction was done with 16 mM $NaBH_4$ in Na_2CO_5 buffer pH 9.5 for three hours at 0°C.

[d]In experiment 5 and 6 coupling and reduction were performed simultaneously at pH 7.5 in 0.1 M Hepes buffer using 20 mM $NaBH_3CN$ at room temperature for two hours. Uncertainties in the activity determination are the result of triplicate determination on different bead preparations.

unstable at neutral pH, and is also capable of reducing disulfides (37) which are probably associated with stablizing the activity of this enzyme.

Nevertheless, the reductive amination method of coupling did result in stable conjugates, suggesting little alternation in physio-chemical properties of immobilized HRPO. However, the amount of immobilized HRPO was much less than could be calculated for surface saturation (17). The low activity of HRPO could be due to self-coupling or hydrolysis of $NaBH_4$ at coupling pH. To overcome the first problem a few experiments were carried out by blocking amino groups of HRPO with FDNB. As already discussed, Table VIII (immobilization procedure), there was no significant difference in the amount of HRPO immobilized compared to the first set of experiments.

The second problem was addressed by using a different reducing agent, sodium cyanoborohydride, which is a selective reagent for Schiff-bases at neutral pH. Borch, Bernstein and Durst (48) characterized its extra stability in acidic conditions and selectivity of action. Dottavio-Martin et al. (49) suggested $NaBH_3CN$ treatment is also less denaturing to proteins than $NaBH_4$ treatment because disulfide bonds are not reduced. Moreover, since $NaBH_3CN$ is a weaker reducing agent, it does not reduce aldehyde groups of neutral pH. Robyt (50) reported that

$NaBH_3CN$ reduces aldehydes at negligible rates under conditions where it reduces Schiff-bases rapidly. Therefore, it can be present throughout the immobilization to drive immobilization to completion by trapping labile Schiff-bases by reduction.

Although $NaBH_3CN$ has recently become widely used in coupling formaldehyde, carbohydrates and other aldehyde containing compounds to proteins there are only a few reports of its use in covalent immobilization of proteins. This may be due to side reactions of protein with cyanide. For example, Jentoft and Dearborn (51) found that in the reductive methylation of proteins, the $NaBH_3CH$ used either contained cyanide or generated it during reduction, resulting in the loss of formaldehyde due to cyanohydrin formation, thereby resulting in a lower degree of protein derivatization. They minimized cyanohydrin formation by using a low $NaBH_3CN$ (20 mM recrystallized) concentration or by adding divalent ions such as Co^{2+}, Ni^{2+} or Zn^{2+} to complex the cyanide (51).

Initial experiments of this work were conducted according to conditions reported by Jentoft and Dearborn (procedure 4C) (with reduction)[51]. Immobilization was carried out at neutral pH 7.5 using 20 mM $NaBH_3CN$ for two hours at room temperature. It was found that there was a considerable increase in the amount of immobilized HRPO

on the beads as shown by experiments 5 and 6, Table XIII. The effect of $NaBH_3CN$ on HRPO was checked before doing these experiments. It was found that native and oxidized HRPO reacted differently with 20 mM of $NaBH_3CN$. With native HRPO, 20 mM $NaBH_3CN$ showed a change in the absorbance at 403 nm, which could be reversed back using 6.66 mM cobalt nitrate. Also, oxidized HRPO does not lose activity on addition of 20 mM $NaBH_3CN$ which could not be reversed with addition of cobalt nitrate. Instead, addition of 6 mM of cobalt nitrate reduced HRPO activity. Therefore, it was concluded that further experiments will be carried out using the same procedure - which is an adaptation of Dearborn and Jentoft procedure (37) and cobalt nitrate would not be added in the immobilization procedure.

In the next set of experiments coupling conditions were varied. As shown in Tables XIV and XV variation in coupling time during immobilization, resulted in almost the same amount of active HRPO on beads. However, longer incubation time was harmful for HRPO activity as $NaBH_3CN$ was present in the coupling reaction mixture. In the next set of experiments, Table XVI, $NaBH_3CN$ concentration was varied. From these results it seems that less than 20 mM of $NaBH_3CN$ leads to decreased coupling yields.

TABLE XIV

EFFECT OF VARIATION IN COUPLING TIME ON IMMOBILIZATION TO BEADS HAVING AMINO GROUPS[a]

Experiments	Coupling time Hour	µgm HRPO/gm of dry beads
1	2	2.15
2	4	2.88
3	6	2.07
4	8	2.17
5[b]	18	2.07

[a] In all these experiments oxidation was carried out by using 6 mM $NaIO_4$ for two hours at pH 8.00, 0.3 M $NaHCO_3$ buffer at room temperature in dark. Oxidized-HRPO was ultrafiltered before coupling. Coupling was carried out at pH 7.4 in 0.1 M phosphate buffer in presence of 20 mM $NaBH_3CN$ for different periods of time at room temperature.

[b] Coupling was carried out as above but at 0°C.

TABLE XV

EFFECT OF VARIATION IN COUPLING TIME ON IMMOBILIZATION TO BEADS HAVING SEMI-CARBAZIDO GROUPS[a]

Experiment No.	Coupling Time Hour	µgm of Activity/gm of beads
1	0.5	0.16
2	1	0.29
3	2	1.25
4	4	1.26
5	8	0.89
6	24	0.34
7	48	0.20

[a]In all of these experiments oxidation was carried out by using 6 mM $NaIO_4$ for two hours at pH 8.00, 0.3 M $NaHCO_3$ buffer at room temperature in dark. Oxidized HRPO was ultrafiltered before coupling. Coupling was carried out at pH 7.4 in 0.1 M phosphate buffer in the absence of $NaBH_3CN$ for different periods of time.

TABLE XVI

EFFECT OF VARIATION IN SODIUM CYANO-BOROHYDRIDE CONCENTRATION ON IMMOBILIZATION YIELD[a]

Experiment No.	$NaBH_3CN$ mM	µgram HRPO on 1 gm of dry beads
1	8	3.22
2	12	3.12
3	20	5.3

[a] In all these experiments oxidation was carried out by using 6 mM $NaIO_4$ for two hours at pH 8.00, 0.3 M $NaHCO_3$ buffer at room temperature in the dark. Oxidized-HRPO was ultrafiltered before coupling. Coupling was carried out in 0.1 M phosphate buffer at pH 7.5 for two hours using different concentration of $NaBH_3CN$.

Coupling Yields and Surface Utilization

The work presented so far has focused on the enzyme activity immobilized and methods for its maximization. However, if a material balance is attempted for a typical coupling experiment it was observed that the amount of peroxidase lost during the coupling procedure is far greater than the active enzyme found on beads. In the coupling step, as shown in Table XVII there was a constant loss of 22-28 nanomoles/gm of beads (.8-1.12 mg/gm of beads) of HRPO from the coupling solution according to spectrophotometric determinations. However, it was found, Table XVII, the amount of active HRPO on these same beads was in the range of .15-.18 nanomoles/gm of beads (6-7 µg/ gm of beads). The lost peroxidase was a major point of investigation.

In order to estimate the amount of lost peroxidase from the immobilized beads, enzyme coupled to beads without reduction was subjected to exchange with another nucleophile, hydrazine at 10 mM (procedure 5b in methods). Two experiments, Table XVIII, showed that some of the lost HRPO was on the beads and was eluted upon treatment with hydrazine. Eluted peroxidase was measured spectophotometrically at 403 nm and the concentration was calculated using $\varepsilon = 9.1 \times 10^{4} M^{-1} cm^{-1}$.

After hydrazine treatment, the activity on beads was

TABLE XVII

INDIRECT ESTIMATION OF TOTAL HRPO IMMOBILIZED ON BEADS

Amt. of Peroxidase Taken for Coupling	Amt. of Peroxidase in Coupling Supernatant	Loss of HRPO During Coupling	Active HRPO During Coupling
Amount of Peroxidase/gm of Dry Beads			
69.5 nanomoles 2.78 mg	43.9 nanomoles 1.75 mg	25.6 nanomoles 1.03 mg	.15 nanomoles 6 µg
70 nanomoles 2.8 mg	40 nanomoles 1.6 mg	30 nanomoles 1.2 mg	.18 nanomoles 7.2 µg
67.45 nanomoles 2.6 mg	39.05 nanomoles 1.56 mg	28.4 nanomoles 1.13 mg	.17 nanomoles 6.8 µg

HRPO concentration was determined spectrophotometrically at 403 nm and using $\varepsilon = 9.1 \times 10^{4} M^{-1} cm^{-1}$. Coupling was done with 20 mM $NaBH_3CN$ in .1 M phosphate buffer at pH 7.4 for two hours at room temperature. For coupling 0.5 ml of packed beads which is 50% (v/v) suspension of 1 ml of beads (ca 140 mg of beads as determined by dry wt. determination (17) was used.

TABLE XVIII

ESTIMATION OF INACTIVE HRPO ON BEADS USING HYDRAZINE[a]

HRPO Lost in Coupling During Immobilization	HRPO Released by Treating with Hydrazine	Active HRPO Found on Beads
Amount of Peroxidase gm of Dry Beads		
20 nanomoles 852 µg	2.65 nanomoles 106 µg	.125 nanomoles 5 µg
28 nanomoles 1136 µg	3.46 nanomoles 137 µg	.15 nanomoles 6 µg

[a] one ml of 10 mM hydrazine at pH 7.5 in 0.1 M phosphate buffer was added to 0.5 ml of immobilized packed beads on which HRPO had been immobilized without reduction at pH 7.5 in 0.1 M phosphate buffer. The beads were allowed to stir for 1-2 hours at room temperature. The amount of peroxidase released was measured spectrophotometrically at 403 nm. The amount of peroxidase was estimated using $\epsilon = 9.10 \times 10^4\ M^{-1}cm^{-1}$. Beads were washed with 0.1 M phosphate buffer pH 7.5 10-12 times before hydrazine treatment.

checked with the peroxidase assay method (procedure 5c in methods). There was a complete loss of HRPO activity after this treatment. Unfortunately, activity in the hydrazine elute could not be checked since 10 mM hydrazine abolishes enzyme activity. Further re-immobilization of HRPO on these beads achieved the previous activity of about .125 nanomoles/gm of beads (5 ug/gm of beads). The cyclic process was repeated on beads six times. Each time, lost HRPO and eluted HRPO was similar to the above mentioned values. The amount of peroxidase obtained in the supernatant after treatment of immobilized beads with hydrazine is only 10% of lost HRPO, but is about 20 times the amount of active HRPO found on beads. According to Trevan et al. (52) the enzyme lost during coupling could be due to covalent immobilization. They mentioned that covalent bonding of an enzyme to the surface of a solid matrix could result in a multiple fixation of the enzyme on that surface. During coupling procedure this phenomenon leads to inactivation. In addition, they mentioned that uneven distribution of cross-linking "arms" on the surface could lead to heterogenity in the preparation because some enzyme molecules will be attached to the matrix surface by only one arm, others by two, etc. Moreover, uneven distribution of specific reactive groups on the enzyme surface could also lead to hetegeneity in

the preparation even when the cross-linking arms are evenly distributed.

For the alternative studies of immobilization of HRPO on long-arm polyacrylamide beads having amino functional groups and an average diameter of 25 μm (10-37 μm range, according to the manufacturer), two variations in this arrangement were investigated, variation in bead surface and variation in the types of Schiff-base forming nitrogen function at the end of the arm. Brief mention has been made of these variations earlier without much detail (data in Tables I, XI, XV).

From the results in Table XIX it may be seen that smaller diameter beads have more HRPO activity per unit weight than larger diameter beads of the same porosity (inclusion limit 1800 daltons). Thus, it may be concluded that coupling yield is surface area dependent. Sherrington et al. (53) found similar results in their study with glass beads and using a covalent method of immobilization. It is also observed from Table XIX that a 15-fold increase in activity was related to an approximate 10-fold decrease in diameter. Similarly Table I shows an 8-fold activity difference accompanying the same 10-fold difference in surface area. Given the large size ranges of the beads and lack of knowledge of the distribution within these ranges, these two factors agree well with expectation for a surface area related phenomenon.

TABLE XIX

EFFECT OF VARIATION IN SURFACE AREA[a] ON IMMOBILIZATION TO BEADS

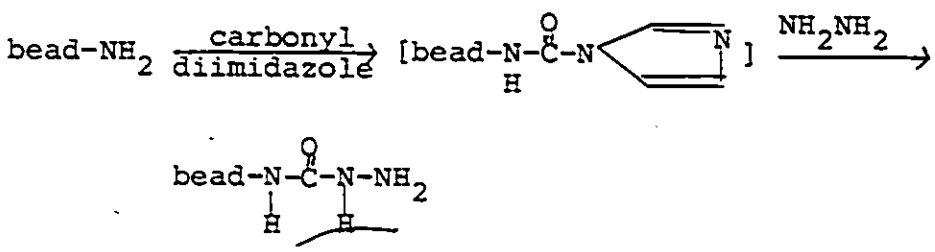
Functional Group	Particle Diameter μm	μg HRPO/gm of beads
Amino	225	0.595
Amino	225	0.560
Amino	25	5.0
Amino	25	6.0
Semicarbazido[b]	225	0.052
Semicarbazido[b]	225	0.056
Semicarbazido[b]	25	0.246
Semicarbazido[b]	25	0.257

[a]Particle diameters given are the averages of the respective ranges claimed by the manufacturer. Thus, 25 μm and 225 μm are the 10-37 μm (-400 mesh) and 150-300 μm (50-100 mesh), respectively. In all these experiments immobilization was done according to procedure #4 (with or without reduction) mentioned in methods. 21 nmol of HRPO was oxidized with 6 mM $NaIO_4$ for 90 min in 0.3 M $NaHCO_3$ buffer pH 8.00 at 25°C in the dark. Ultrafiltration was done before coupling. Oxidized HRPO was coupled with beads in 0.1 M phosphate buffer at pH 7.4 for two hours in the presence of $NaBH_3CN$ except as noted.

[b]$NaBH_3CN$ was not added in the coupling mixture.

[c]Active immobilized HRPO was determined according to procedure 5c mentioned in methods.

The second variation in bead construction represented in Table XIX concerns a different Schiff-base forming functional group which, it is assumed, resulted from a two-step treatment of the usual amino beads as follows:



However, the two methods used so far to characterise the transformation, potentiometric titration and TNBS reaction as a function of pH, have not been entirely satisfactory. Nevertheless, due to the availability of such provisionally-characterized beads from others in this laboratory, their evaluation in the immobilization method developed here was of interest.

According to Rando et al. (54) semi-carbazide functional groups are better for making stable Schiff-bases. From Table XIX it is evident that the final activity on beads was less than that on beads having amino functional group. However, the activity was apparently related to surface area as in the amino series. The lower activity on semi-carbazido beads is also evident in Tables I, XI and XV presented earlier.

Immobilization of HRPO on Nylon Tubing

The total amount of HRPO immobilized on nylon tubing was

calculated by difference spectrophotometrically at 403 nm, Table XX, to be 0.15 nmoles/metre (0.5 µg/metre). This upper limit estimate should be compared with that estimated from peroxidase assay activity measurement, which showed 0.02 ± 0.01 nmol of active enzyme per metre of tubing. Furthermore, the hydrazine elution method for the replacement of Schiff-bases showed 0.11 nmoles HRPO/metre. The amount of active enzyme estimated should be taken as a lower limit because the tubing was washed exhaustively with a concentrated 0.1 M Tris-HCl buffer, a procedure which could result in exchange of Schiff-bases. Nevertheless, it is evident from the results that the amount of HRPO immobilized on nylon tubes were almost the same as that replaced in the hydrazine exchange experiment. In addition, the amount of active peroxidase immobilized in nylon tubing was a much higher fraction compared to that in bead immobilization experiments. Also there was not any continuous leakage of peroxidase in the wash solution even though coupling had been carried out without any reducing agent. The activity on nylon tubes remained constant even after two weeks with storage in 0.1 M phosphate buffer at 0°C.

TABLE XX

ESTIMATION OF HRPO ON NYLON TUBING

Amt. of HRPO Taken for Coupling	Amt. of HRPO in Coupling Supernatant[a,b]	Loss of HRPO During Coupling	HRPO Released by Treating with Hydrazine	Active HRPO Found on nylon tubing	
nmoles/metre of nylon tubing	nmoles/metre of nylon tubing	nmoles/metre of nylon tubing	nmoles/metre of nylon tubing	nmoles/metre of nylon tubing	
10	9.84	.16	.11	First time	After 2 weeks
				.02 ±.01	.02 ±.01
10	9.82	.18	.12	.02 ±.01	.02 ±.01

[a] HRPO concentration was determined spectrophotometrically at 403 nm and using $\varepsilon = 9.1 \times 10^4 M^{-1} cm^{-1}$.

[b] Coupling was done without reducing agent in .1 M phosphate buffer at pH 7.4 for two hours at room temperature. Nylon tubing was filled with 5 mM hydrazine hydrate prepared in phosphate buffer at pH 7.5. The tubing (dimensions 1m x 1.00 mm 1.d.) was allowed to stand for two hours at room temperature. The amount of peroxidase released was measured spectrophotometrically at 403 nm.

[c] Ten ml of chromogen mixture having a final concentration of 9 mM HDCBS, 4.8 mM 4-AAP, 0.3 mM H_2O_2 was flushed through nylon tubing at a flow rate of 1.8 ml/min. Activity on tubes was estimated using standard curve formulated for peroxidase. Before checking activity, nylon tubes were washed thoroughly with .1 M Tris-HCl buffer until no peroxidase activity was detected in washes.

CHAPTER IV

SUMMARY

The major aim of our studies was to maximize immobilization of HRPO on long-arm derivatized polyacrylamide beads carrying distal amino or semi-carbazido functional group. Consideration of available surface area on these beads suggested an upper limit of 53 nmoles or 2.1 mg of peroxidase per dry gram of beads. Also, our work was an attempt to produce an analytical assay using the immobilized enzyme on beads, so that immobilized HRPO can be re-used continuously without losing its activity. Therefore, major emphasis in this work was given to:

quantitation of functional group density on the beads;

quantitation of active inactive and total HRPO on beads;

optimization studies to increase the amount of HRPO on beads

As mentioned earlier a TNBS test (42) was used in this work to quantitate amino and semi-carbazido group density on beads. This method was comparatively better than the titration method used earlier in this lab (17,18) although there is a fairly large background reaction of TNBS with un-derivatized beads for which correction must be made.

This method with little modification was used to distinguish amino functional groups from semi-carbazido groups and to determine functional group density as a function of bead size. In the future this method should be useful for estimating functional group density on different supports.

A spectrophotometric peroxidase assay, sensitive in the picomolar range, was developed and used to estimate the amount of active HRPO on beads and thus was the main criterion used in evaluating variations in coupling conditions.

Immobilization of periodate-treated peroxidase via Schiff-base formation with amino terminated spacer arms on the support was optimized with respect to conditions of periodate oxidation (reagent concentration, time of exposure and influence of excess reagents and products) and the conditions of coupling (coupling time, presence of reducing agent, type and concentration of reducing agent). Optimization of semi-carbazido group terminated arms on polyacrylamide was less systematically carried out in order to discern trends with respect to the amino-terminated arms. Brief examination was made of immobilization on analogously derivatized nylon tubing in the absence of reducing agent.

In the quantitative characterization of those con-

jugates it was apparent that the amount of enzyme which disappeared from the coupling solution was a substantial fraction of that predicted for surface saturation but enzyme activity expressed on these surfaces was far below that expected for the corresponding amount of soluble enzyme. Elution of unreduced conjugates with hydrazine showed amounts of heme protein release accounting for .7% and 12.5% of the discrepancies between amount coupled and fully active enzyme on the surface, for polyacrylamide and nylon conjugates, respectively. The less than quantitative elution by hydrazine cannot be explained at present but the fact that the elution furnished substantial amounts of heme protein suggests that the immobilization occurs with a large decrease in specific activity of the enzyme.

Despite the above uncertainties and apparent loss in specific activity it must be emphasized in closing that the solid phase enzymes produced in the present work still have a degree of activity and stability to be very useful analytically. To this end the nylon tubing version of peroxidase has already been successfully incorporated into Autoanalyzer procedures for peroxide and glucose (work of S. Boss). It can readily be imagined that, should the chemical problems in the present approach be solved in the future, expression of full enzyme activity in such tubular reactors

would provide analytical systems of exquisite sensitivity and specificity.

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