Long-arm thiolated polyacrylamide beads and their application in coupling experiments with papain.

Beverly Ann. Kristalovich

University of Windsor

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS RÉCU
LONG-ARM THIOLATED POLYACRYLAMIDE BEADS
AND THEIR APPLICATION IN COUPLING
EXPERIMENTS WITH PAPAIN

by
Beverly Ann Kristalovich

A Thesis
submitted to the Faculty of Graduate Studies through the Department of Chemistry in Partial Fulfillment of the requirements for the degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada
1983
LONG-ARM THIOLATED POLYACRYLAMIDE BEADS
AND THEIR APPLICATION IN COUPLING
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ABSTRACT

In this study, the synthesis, characterization, and
evaluation of a long-arm thiolated polyacrylamide bead
reagent is described. The bead reagent was prepared from
beaded polyacrylamide (±400 mesh, 10-37 μm diameter, 1800
dalton inclusion limit) using the long spacer arm Jeffamine
ED-600 via aminolysis, followed by reaction with dithiobis
(succinimidyl propionate) (DTSP) and reduction with
dithioerythritol (DTE). Beads of high and low functional
group densities were synthesized. The thiol (SH) titre of
the beads was determined spectrophotometrically using
5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and/or
2,2'-dithiodipyridine (2-Py-S-S-2-Py). However, upon
recycling of the low functional group density beads in
2-Py-S-S-2-Py the thiol titres increased, which gave
evidence that excess reagent was adhering non-specifically
to the beads. The beads were characterized in the activated
mixed disulfide form bead-S-S-2-Py by coupling experiments with the sulfhydryl protease papain.

In coupling experiments with the high functional group density beads (1.22 μmoles SH/g of dry beads), when protein was measured in the dialyzed cleavage supernatants, the coupling yields were 22 nmol/g of dry beads and 20 nmol/g of dry beads. Thiopyridone release exceeded coupled protein released by reductive cleavage by up to 4-fold.

In coupling experiments with the low functional group density beads (0.19 μmoles SH/g of dry beads), when protein was measured in the dialyzed cleavage supernatants, the coupling yields were 6 nmol/g of dry beads and 3 nmol/g of dry beads. Thiopyridone release was equal within experimental error to the coupled protein released by reductive cleavage. This result revealed that use of the low thiol titre beads was preferred over that of the high thiol titre beads, although coupling yields were not increased by the use of the low thiol titre beads. Assay using N-α-benzoyl-D,L-arginine-ß-nitroanilide (D,L-BAPA) predicted approximately the same amounts of protein in the undialyzed and dialyzed cleavage supernatants. Moreover, the specific activities obtained using D,L-BAPA assay demonstrated that even after immobilization on the beads and reductive cleavage the papain remained catalytically active.
DEDICATION

To

my parents, sister,
and Mittens
ACKNOWLEDGEMENTS

I thank Dr. K. E. Taylor, my research advisor, for his help and guidance in this project, and for the high functional group density bead reagent.

I also thank Drs. R. J. Thibert, P. B. Taylor, and B. Mutus for their helpful suggestions.

Thanks to Linda Chen for the use of her thesis.

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<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>Ac₂O</td>
<td>acetic anhydride</td>
</tr>
<tr>
<td>D-BAPA</td>
<td>N-α-benzoyl-D-arginine-p-nitroanilide</td>
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<tr>
<td>D,L-BAPA</td>
<td>N-α-benzoyl-D,L-arginine-p-nitroanilide</td>
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<td>bead-NH₂</td>
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<td>long-arm thiolated polyacrylamide beads</td>
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<td>bead-S-S-2-Py</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSA-SH</td>
<td>bovine serum mercaptalbumin</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>corr</td>
<td>corrected</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTE</td>
<td>dithioerythritol</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5′-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTSP</td>
<td>dithiobis (succinimidyl propionate)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Et₃N</td>
<td>triethylamine</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>inhibitor constant</td>
</tr>
<tr>
<td>PDP-CaM</td>
<td>[3-(2-pyridyldithio)-propionyl] calmodulin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td>PEG-DMP</td>
<td>polyethylene glycol di-(3-mercaptopropionate)</td>
</tr>
<tr>
<td>prep</td>
<td>preparation</td>
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<tr>
<td>SH</td>
<td>thiol group</td>
</tr>
<tr>
<td>SIGA</td>
<td>secretory immunoglobulin A</td>
</tr>
<tr>
<td>SPDP</td>
<td>N-succinimidyl 3-(2-pyridyldithio)-propionate</td>
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<td>2-Py-S-S-2-Py</td>
<td>Aldri thiol-2; 2,2'-dithiodipyridine</td>
</tr>
<tr>
<td>TNB</td>
<td>thionitrobenzoate ion</td>
</tr>
<tr>
<td>TNBS</td>
<td>2,4,6-trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
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<td>w/v</td>
<td>weight per volume</td>
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CHAPTER I

INTRODUCTION

Covalent Chromatography via Thiol-Disulfide Interchange in Soluble Systems

In 1973 Brocklehurst et al. (1) prepared fully active papain from both dried papaya latex and from commercial 2x crystallized partially active papain using a new type of affinity chromatography, covalent chromatography via thiol-disulfide interchange.

Affinity chromatography, which has been reviewed in our lab by Chen (2) in 1979, involves passing a solution containing the macromolecule to be purified through a column containing an insoluble support to which a specific competitive inhibitor or other ligand has been covalently attached (3). If the proteins do not exhibit any appreciable affinity for the ligand they will pass unretarded through the column, but those which recognize the inhibitor will be retarded in proportion to the affinity existing under the experimental conditions. Then in order to elute the specifically absorbed protein the composition of the solvent is altered so that dissociation of the protein
occurs. Affinity chromatography has many applications, as seen in Table 1 (4).

Covalent chromatography, which is a special type of affinity chromatography, relies upon formation and scission of covalent bonds (5). It involves the chemical reaction of the material of the chromatography column with only one of the components of the mixture. After the other components are removed from the column by washing, the covalently bound component is released by reaction with a suitable reagent. The reaction that releases the component of interest should preferably leave the column in a form that can be readily "reactivated" (5).

Brocklehurst et al. (1) developed a type of covalent chromatography for thiol-containing proteins, known as covalent chromatography via thiol-disulfide interchange. This type of covalent chromatography was developed using the following principles (5):

A protein containing one reactive thiol (SH) per mole would be expected to react with a polymer containing mixed disulfide residues (P-S-S-X) by thiol-disulfide interchange, as shown in Fig. 1 (5). The selection of P-S-S-X is done in a way to facilitate the isolation of E-SH from a mixture. The desirable conditions for the selection of P-S-S-X are such that: (i) the reaction of E-SH producing X-SH (Fig. 1a)
Table I

TYPES OF AFFINITY CHROMATOGRAPHY AND THEIR APPLICATIONS

<table>
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<th>Applications</th>
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<tr>
<td>Molecular affinity chromatography</td>
<td>Enzymes, antibodies, antigens, binding or receptor proteins, complementary proteins, repressor proteins, denatured and chemically modified proteins, nucleic acids and nucleotides, concentration of dilute solutions, storage of otherwise unstable proteins in immobilized form, investigation of kinetic sequences and mechanisms, purification of synthetic macromolecules.</td>
</tr>
<tr>
<td>Cellular affinity chromatography</td>
<td>Cells, cell organelles, cell membranes, phages and viruses.</td>
</tr>
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\(^a\) Adopted from the review of Sharma and Mahendra (4).
Figure 1

The Application of Thiol-Disulfide Reactions in Covalent Chromatography

Legend

Taken from the review of Brocklehurst et al. (5).
(a) \[ P-S-S-X + E-SH \iff P-S-S-E + X-SH \]

(b) \[ P-S-S-X + E-SH \iff P-SH + E-S-S-X \]

(c) \[ E-S-S-X + E-SH \iff E-S-S-E + X-SH \]

(d) \[ P-S-S-E \xrightarrow{\text{Excess} \; R-SH} P-SH + E-SH + R-S-S-R \]

Figure 1
predominates over that producing E-S-S-X (Fig. 1b); (ii) the reaction of Fig. 1a is effectively irreversible; (iii) the thiol X-SH is readily detected by physical measurement, for example, spectrophotometry. In this case, the attachment of the thiol-containing protein to the column may be followed by monitoring the release of X-SH produced in reaction a of Fig. 1. Then a solution of low molecular weight thiol (R-SH) would be used to elute the protein containing 1 mole of reactive thiol per mole of protein, the thiolated column (P-SH), and the disulfide derivative of the low molecular weight thiol (R-S-S-R) (Fig. 1d) (5).

Brookehurst and Little (6) chose X-SH to be 2-thiopyridone (Py-2-SH) in their study of papain, where the thiol group of the matrix reacted with 2,2'-dithiodipyridine (2-Py-S-S-2-Py) so as to produce a polymer containing alkyl-2-pyridyl residues with the release of 2-thiopyridone as seen in Fig. 2a (5). Brookehurst et al. (6) used the following considerations as the basis for choosing Py-2-SH as X-SH. (1) Very large equilibrium constants result from reactions of 2-Py-S-S-2-Py with low molecular weight thiols and with papain, stoichiometric with the thiol content of the reactants, even in solutions of low pH. Therefore, reaction of E-SH with P-S-S-X might be similarly irreversible.

(2) The electrophilicity of 2-Py-S-S-2-Py is in-
Figure 2

Covalent Chromatography via Thiol-Disulfide Interchange using Py-2-SH as the Released Thiol

Legend

Taken from Brocklehurst et al. (5).
(a) (thiolated Sepharose)  

(b) (thiol-containing protein)  

(c) SH+ESH+R-S-R  

Figure 2
creased by about $4 \times 10^3$ times when it becomes protonated (6,7).

(3) The release of Py-2-SH is readily detected by spectrophotometry.

(4) At pH values approximately 4, 2-Py-S-S-2-Py reacts specifically only with intact papain catalytic sites (6-8). The reaction of 2-Py-S-S-2-Py with papain was described by Brocklehurst et al. (5) as an intra-complex thiol-disulfide-interchange involving the nonionized thiol group of the cysteine-25-histidine-159-asparagine-175 hydrogen-bonded system of papain with 2-Py-S-S-2-Py hydrogen-bonded at one nitrogen atom to the carboxyl group of aspartic acid-158.

(5) The reaction of Fig. 2a would likely predominate over that in Fig. 2b, at least when the pyridyl moiety is protonated.

Covalent chromatography via thiol-disulfide interchange has been widely applied, many of the applications are summarized below.

In 1974 covalent chromatography via thiol-disulfide interchange reactions was employed to isolate bovine serum mercaptalbumin (BSA-SH), using 2-Py-S-S-2-Py covalently attached to thiol-Sepharose (9).

In 1975 Egorov et al. (10) used covalent chromatography via thiol-disulfide interchange to rapidly and
specifically isolate the thiol-containing peptides parvalbumin, bovine serum mercaptalbumin, and ceruloplasmin from large proteins.

In that same year, human $\alpha_1$-antitrypsin, as well as $\alpha_1$-antitrypsin from dog, monkey, and baboon were purified from plasma through thiol-disulfide interchange by conjugation of the monomeric X-chains with CNBr-activated Sepharose 4B (11). In order to separate plasma proteins, the compound thionitrobenzoate ion (TNB$^-$) was used to convert the C-terminal cysteine of the conjugated X-chain to a mixed disulfide. The disulfide link between $\alpha_1$-antitrypsin and X-chains was selectively cleaved in the presence of 5,5'-dithobis(2-nitrobenzoic acid) (DTNB) which accepted the sulphydryl group of $\alpha$-antitrypsin and mild reduction liberated TNB$^-$ (11). The method of Laurell et al. (11) increased the $\alpha_1$-antitrypsin content of the plasma fraction from 5% of the total protein to 95% within one day with a yield of about 50%. Glutathione-Sepharose was also used by Laurell et al. (11) to purify $\alpha_1$-antitrypsin, but the X-chain conjugate yielded $\alpha_1$-antitrypsin less contaminated with mercaptalbumin and prealbumin. Mercaptalbumin, as well as prealbumin, have affinity for interchange reactions with mixed disulfides, so they were present in the $\alpha_1$-antitrypsin load (11).

In the same year, mercaptohydroxypropyl ether agarose
was used to covalently immobilize α-amylase and chymotrypsin (12). Conversion of the mercaptohydroxypropyl ether agarose to the mixed disulfide was achieved with 2-py-S-S-2-py and methyl-3-mercaptopropioimidate was used to thiolate the enzymes. Then coupling of the thiolated enzymes to the mixed disulfide derivative of agarose gel was carried out. This immobilization technique could be performed so that most of the inherent activity of the enzymes was conserved, and the immobilized α-amylase was used in a packed-bed reactor for the continuous hydrolysis of starch.

In 1976 Norris and Brocklehurst (13) prepared highly active urease from Canavalia ensiformis using covalent chromatography by thiol-disulfide interchange.

In the same year Malthouse and Brocklehurst prepared fully active ficin containing 1 mole of thiol per mole of protein from the dried latex of Ficus galbrata by covalent chromatography on Sepharose-glutathione-2-pyridyl disulfide gel (14).

In 1977 Laurell et al. (15) used covalent chromatography via thiol-disulfide interchange in their comparison of the efficiencies and selectivities of a series of Sepharose-conjugated thiol compounds for linking plasma proteins.

In that same year, multiple forms of glutathione S-transferase, a family of proteins involved both with
bilirubin transport and with the detoxification of electrophiles, were purified using epoxy-activated Sepharose coupled with glutathione (16). Good yields of highly purified transferase from human liver were obtained in the study (16).

In the same year, Svenson et al. (17) isolated the cysteine peptides of bovine serum albumin and pancreatic ribonuclease by covalent chromatography on thiol agarose derivatives.

In 1978 the separation of plasma proteins by thiol-disulfide interchange chromatography was done by Laurell (18), using various aromatic disulfides as activators. The aromatic disulfides used were 4,4'-dithiodipyridine, 6,6'-dithiodinicotinic acid, 2-Py-S-S-2-Py, 2,2'-dithiobis (5-nitropyridine), and DTNB. The efficiency of various thiol compounds bound to the Sepharose matrix was also investigated in that study (18). When the efficiencies of Sepharose-linked K-chains, glutathione, and cysteine were compared using various aromatic disulfides as activators, it was found that both the activator and the matrix-bound thiol influenced the selectivity and the efficiency of the fractionation of plasma proteins. More importantly, it was found that the optimal combination of matrix-bound thiol and activator depended on the properties of the desired protein (18).
In 1980 the mechanism of inactivation and stabilization of luciferase from *Lucola mingrelica* immobilized on Sepharose 4B was investigated (19). Brovko and Ugarova (19) showed that the rate of the inactivation process for immobilized firefly luciferase was limited by the oxidation of the SH groups of the enzyme. The SH groups were far more reactive in immobilized luciferase than in the soluble enzyme, and the rate constant of interaction with DTNB was 200 times higher for immobilized luciferase than for the soluble enzyme. The process of inactivation and reactivation of immobilized luciferase was investigated in the presence of dithiothreitol. Dithiothreitol effectively protected immobilized luciferase from inactivation but had no effect on soluble luciferase, which was an indication of a different rate-limiting step of the inactivation of luciferase in the soluble state (19).

In that same year luciferase was immobilized on various CNBr-activated polysaccharide carriers to investigate changes in the pH dependence of enzyme activity and stability of the enzyme (20).

Hillson and Freedman (21) used covalent chromatography to resolve protein disulfide isomerase and glutathione-insulin transhydrogenase by immobilization on thiopropyl-Sepharose 6B activated with 2-Py-S-S-2-Py and ion exchange chromatography. In the new stepwise elution procedure,
protein disulfide-isomerase was displaced from the thiopropyl-Sepharose 6B in mild reducing conditions, whereas glutathione-insulin transhydrogenase was only displaced from the matrix by more extreme reducing conditions (21). The protein disulfide-isomerase was purified 560-fold more by the new procedure in comparison to bovine liver homogenate, and it only had small traces of glutathione-insulin transhydrogenase activity in it (21).

In 1981 the thiol peptides of ceruloplasmin, a copper protein of molecular weight 134,000, were isolated by immobilization on thiopropyl-Sepharose 6B which had been activated with 2-Py-S-S-2-Py (22). There was a covalent attachment of cysteine side chains to the activated matrix by means of thiol-disulfide exchange as shown in procedure A of Fig. 3. Digestion of the immobilized protein was carried out with either pepsin or trypsin followed by elution of the liberated non-thiol peptides together with the protease. Then after washing, reducing buffer was used to elute the immobilized thiol peptides, followed by desalting on the hydrophobic gel LH-20 and carboxymethylation. Purification of the peptides was accomplished in two steps, first gel filtration, then either column electrophoresis or ion-exchange chromatography. Ryden and Norder isolated two sets of peptides from four different regions in ceruloplasmin (22). The peptides
Figure 3
Im mobilization of Thiol Peptides by Covalent Chromatography

Legend
Adopted from Ryden and Norder (22).
Figure 3

Procedure A

1. Immobilization on activated gel

2. Digestion of immobilized protein

Procedure B

1. Digestion of activated protein with a protease

2. Immobilization on reduced gel
were 12-39 residues in length and together accounted for 152 residues.

In that same year, thiol-disulfide interchange chromatography was used to purify low molecular weight human antihemophilic factor essentially free of Von Willebrand factor (23). Partially pure antihemophilic factor was prepared from fresh plasma by treatment with dithiothreitol for limited reduction, followed by reaction with 2-Py-S-S-2-Py. Then thiol-disulfide interchange was carried out with the protein-2-pyridyl mixed disulfide on thiopropyl-Sepharose. This resulted in approximately 96% of the coagulant activity being adsorbed chromatographically, of which approximately 20% subsequently eluted by reduction with 1 mM dithiothreitol. Then the antihemophilic factor was S-alkylated with [1,14C]-iodoaceta-mide. The ratio of coagulant activity of the antihemophilic factor to the Von Willebrand factor-antigen activity was greater than 30000:1 (23).

In 1982 cytoplasmic aldehyde dehydrogenase was purified on reduced thiopropyl-Sepharose 6B under conditions in which mitochondrial aldehyde dehydrogenase did not bind (24). Cytoplasmic aldehyde dehydrogenase was modified with disulfiram so that the modified cytoplasmic aldehyde dehydrogenase would bind covalently to the thiol groups of thiopropyl-Sepharose whereas the mitochondrial enzyme
and other protein would not bind. After the resin was washed, elution of pure cytoplasmic aldehyde dehydrogenase was accomplished by reduction with dithiothreitol.

In that same year Seelig and Meister (25) used covalent chromatography via thiol-disulfide interchange to immobilize \( \gamma \)-glutamylcysteine synthetase, an enzyme previously known to be potently inhibited by cystamine, covalently to cystamine-Sepharose. Treatment of the enzyme with reducing agents reversed the inhibition so that a mixed disulfide was formed between the enzyme sulfhydryl group near the active site and cystamine-Sepharose. The enzyme was cleaved from the matrix by treatment with dithiothreitol.

In 1982 covalent chromatography via thiol-disulfide interchange was used to bind and dissociate the pyruvate dehydrogenase complex of \textit{Azotobaeter vinelandii} on thiol-Sepharose (26). After reductive acetylation of the lipoyl groups in the presence of pyruvate, the pyruvate dehydrogenase complex was immobilized on thiol-Sepharose. The complex was eluted with hydroxylamine. By this method, pure components of the complex were obtained in a high yield in a one step procedure. Previous attempts to dissociate the complex were unsuccessful. This was partly due to the relatively small molecular weight differences of the components and subcomplexes obtained, and partly
due to incomplete dissociation and inactivation during lengthy procedures. The lengthy procedures involved using classical methods such as high pH (27), high salt concentration (28), or urea (27,28) and subsequent purification of the components by hydroxylapatite chromatography (27,28) or gel filtration (29).

In that same year, sulfhydryl oxidase from bovine kidney cortex was separated from \(\gamma\)-glutamyltransferase on cysteiny1succinamidopropyl-glass (30). As a result, there was a 2500-fold purification of renal sulfhydryl oxidase and a 12000-fold increase in the ratio of oxidase to transferase activities over that present in the crude cortical homogenate (30). The nature of the thiol-containing ligand appeared to be important for the binding of renal sulfhydryl oxidase by covalent chromatography, because renal sulfhydryl oxidase activity was not bound by a sulfhydryl cellulose column (31). This was thought to reflect the specificity of bovine sulfhydryl oxidase for cysteine-containing substrates (30).

In 1983 covalent chromatography on cysteiny1succinamidopropyl-glass was used to purify bovine milk sulfhydryl oxidase from \(\gamma\)-glutamyltransferase (32). As with bovine kidney sulfhydryl oxidase discussed above (30), bovine milk sulfhydryl oxidase was immobilized on a cysteiny1succinamidopropyl-glass matrix through the formation of a
mixed disulfide between the enzyme and the substrate cysteinylsuccinamidopropyl-glass. The bound protein was eluted by reduction with small molecular weight thiols. By this procedure sulfhydryl oxidase was completely resolved from γ-glutamyltransferase. Separation between the two enzymes was confirmed when antibodies prepared against purified sulfhydryl oxidase quantitatively precipitated 95% of the sulfhydryl-oxidase-initiated reduced glutathione-oxidative activity from detergent-solubilized skim-milk membranes, whereas 100% of the transferase activity remained in the supernatant fraction (32).

In that same year, calmodulin-activated brain cyclic nucleotide phosphodiesterase was purified using 3-(2-pyridyldithio) propionyl-substituted calmodulin linked to thiol-Sepharose 4B (33). The disulfide-linked calmodulin-Sepharose was prepared as shown in Fig. 4. The capacity of the gel for binding phosphodiesterase was 4 times that of CNBr-coupled calmodulin-Sepharose (34). Quantitative recovery was accomplished by treatment with a reducing agent which desorbed both ligand and bound proteins, followed by gel filtration which separated the phosphodiesterase from the thiolated calmodulin derivative. The overall recovery of enzyme activity was greater than 70%, and after a second chromatography step on CNBr-coupled
Figure 4
Preparation of Disulfide-Linked Calmodulin-Sepharose

Legend
Reaction (a) is the reaction of N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) with calmodulin (CaM) (H₂N-R-CaM). Reaction (b) shows coupling of the calmodulin derivative, [3-(2-pyridyldithio)-propionyl] calmodulin (PDP-CaM) with thiol-Sepharose 4B. Taken from the study of Kincaid and Vaughan (33).
(a) \[
\text{Py-S-S-CH}_2\text{-CH}_2\text{-C=O-N=O} + H_2\text{N}-R\text{-CaM} \rightarrow \text{Py-S-S-CH}_2\text{-CH}_2\text{-C-HN-R-CaM} + \text{HO-N=O} \]

(b) \[
\text{SH} + \text{Py-S-S-CH}_2\text{-C-HN-R-CaM} \rightarrow \text{S-S-CH}_2\text{-C-HN-R-CaM} + \text{Py=S} \]

Disulfide-linked CaM Sepharose  \( \text{Py-2-SH} \)

*Figure 4*
calmodulin-Sepharose, preparations of homogeneous phosphodiesterase were obtained in good yield.

In the same year Ishiguro et al. (35) developed a specific and sensitive enzyme immunoassay system for the measurement of human secretory immunoglobulin A (SIgA) using anti-a-chain antibodies coupled to thiol-Sepharose, and anti-secretory component antibodies labelled with \( \beta \)-D-galactosidase from *Escherichia coli*. The system can measure secretory IgA in saliva, feces, urine, intestine, and serum without interferences by the abundant IgA in the same samples (35).

**Covalent Chromatography via Thiol-Disulfide Interchange for Cells**

The focus of this study is the development of bead reagents to be used in covalent chromatography of whole cells via thiol-disulfide interchange of the surface components. The long-range goal of this study is the use of bead reagents in the isolation of membrane proteins, as shown below (2):

\[
\begin{align*}
\text{Bead-S-S-X} & + \text{Cell-protein-SH} \\
& \xrightarrow{\text{coupling}} \\
& \text{Bead-S-S-protein cell} + X\text{-SH} \\
& \xrightarrow{\text{detergent, washing}} \\
& \text{Bead-S-S-protein} \\
& \xrightarrow{\text{reduction}} \\
& \text{Bead-SH} \quad \text{HS-protein}
\end{align*}
\]
Covalent chromatography of cell membrane proteins via thiol-disulfide interchange has already been reported (36, 37), but until the work of Chen (2) of our lab in 1979, the coupling of the membrane surface components to the matrix was done after the membrane components were solubilized in detergent. In the study of Kahlenberg and Walker (36), done in 1976, the band 3 proteins of the human erythrocyte were solubilized along with the other major membrane glycoproteins with Triton X-100 prior to purification of band 3 by covalent chromatography via thiol-disulfide interchange on activated thiol-Sepharose 4B. One year later, Shami et al. (37) separated the major glycoproteins (PAS 1, 2, and 3) of the human erythrocyte membrane rapidly and quantitatively by covalent chromatography via thiol-disulfide interchange with an organomercurial gel, following extraction of the glycoproteins by the non-denaturing detergent Triton X-100.

Affinity chromatography of whole cells has been attempted in a variety of systems (4). There are basic requirements for cellular affinity chromatography. These are given below (4):

1. When a ligand molecule is covalently coupled to an insoluble support, the ligand should react biospecifically with the binding site in the surface membrane.

2. The binding site should be available on the outer surface of the cell membrane.
(3) Recovery of cells should be carried out by the use of a compound competing for the affinity sites.

(4) The conditions of elution should be in accordance with the maintenance of cell integrity and expression of bioactivity.

The required properties of the matrix material, or insoluble support, for cellular affinity chromatography are that it should:

(a) be mechanically and chemically stable,
(b) be in a bead form,
(c) have good flow characteristics,
(d) allow biologically active molecules to covalently couple in an unaltered form,
(e) not physically entrap cells,
(f) not absorb cells non-specifically,
(g) affect minimally, if at all, the viability of the chromatographed cells.

Affinity chromatography of whole cells has been attempted in a variety of systems (4). Plastic, glass, polyacrylamide, nylon fibres, and agarose have been employed as a solid support with absorbed or covalently attached biospecific ligands such as lectins, antigens, antibodies or hormones (4). However, cells are readily adsorbed on to glass and other charged or hydrophobic gels, so the usefulness of such matrices is decreased by the
non-specific adhesion of a large number of cells (4). Fortunately, satisfactory cell separation has been achieved on antigen-coated polyacrylamide and agarose and it has been found that the use of polyacrylamide beads as the solid support results in considerably less non-specific retention of cells (4). Polyacrylamide beads have a polyethylene backbone, as shown below (38), which gives them physical and chemical stability and they are available commercially (as "Biogels") in spherical form and in various pregraded sizes and porosities (39):

A residue of a linear segment of polyacrylamide:

\[
\begin{align*}
\text{CONH}_2 & \quad \text{CONH}_2 \\
\end{align*}
\]

Polyacrylamide beads lack charged groups and they have a greater number of chemically modifiable groups (carboxyamide) that can be substituted than the number of groups than can be substituted on agarose gels by the cyanogen bromide procedure. Thus, a much higher degree of ligand substitution is possible, if desired, with polyacrylamide beads than with agarose beads (39).

Chen (2) of our lab used beaded polyacrylamide to synthesize her long-arm thiolated beads (bead-SH). She used polyacrylamide beads (≤400 mesh, 10-37 μm diameter, 1800 dalton exclusion limit) which had low porosity because
within-pore functional groups could be a detriment to coupling. Uncoupled membrane components liberated upon detergent solubilization could couple with the within-pore functional groups and thereby be classed with the surface-exposed macromolecules. Bead size was also taken into account, to increase the surface area available for cell coupling without using large quantities of beads (2). The beads that Chen synthesized had long-spacer arms extending into the bulk solvent between the matrix and ligand in order to allow the bead-cell reaction to be more like one between a surface reactant and a solution phase reactant, instead of a reaction with steric hindrance between two solids, one of which, the cell, is very "rough" on the molecular scale (2).

Chen's beads (2) were synthesized by aminoethylolation, then conversion to the iodoacetamidoethyl form via the N-hydroxysuccinimide ester, followed by introduction of the long spacer arm polyethylene glycol di(3-mercapto-propionate) (PEG-DMP) via thioether formation. An outline of the synthesis of Chen's beads is given in Fig. 5. Iodide was chosen as the leaving group following a survey of the corresponding chloro-, bromo-, and iodo-haloacetamidoethyl forms of the beads (2). Determination of the thiol densities of the final derivative of Chen's beads was carried out using spectrophotometric titration with 2-Py-S-S-2-Py and DTNB (40)
Figure 5

Preparation of Long-Arm Thiolated Polyacrylamide
Beads containing the Long-Arm PEG-DMP

Legend

Taken from the work of Chen (2) of our lab.
to form the mixed disulfide along with Py-2-SH or TNB- release. The thiol group densities achieved for the beads were in the range 3-7 μeq/g of dry beads.

Chen (2) characterized her long-arm thiolated beads in the mixed disulfide form (bead-S-S-2-Py) with coupling experiments using thiol-disulfide interchange reactions with 2-mercaptoethanol, PEG-DMP, papain, bovine serum mercaptalbumin, and the human erythrocyte (ghost).

Chen's coupling experiments with the sulfhydryl protease enzyme papain were modelled after those of Shipton and Brocklehurst for agarose-based gels (41), using bead-S-S-2-Py in large excess (40-50-fold) over protein thiol. Papain is a soluble protein from the latex or fruit of the papaya (42) and it has a molecular weight of 23,000 daltons (43). It is a single chain globular protein and it contains a single sulfhydryl group somewhat buried in the active site (2). In the bead-S-S-2-Py coupling experiments with papain thiopyridone release corresponding to ≥70% of protein thiol groups occurred rapidly. Thiopyridone release indicated that that the coupling yields of the two experiments were approximately 40-50 nmol/g of dry beads. When protein was measured in the dithioerythritol (DTE) cleavage supernatant, the coupling yields were 28 and 7 nmol/g of dry beads, respectively. Thiopyridone release exceeded the amounts of protein released by reductive cleavage by 2.6- and 9.6-fold, respectively, in the two experiments (2). However,
given the scale of the experiments, the discrepancies between Py-2-SH release and protein recovered in the cleavage supernatant were not thought to be large (2).

Chen next did coupling experiments with bovine serum mercaptalbumin, which is a polypeptide containing one equivalent sulfhydryl per 68,000 daltons (2). Bovine serum mercaptalbumin has been isolated from partially blocked bovine serum albumin (BSA) preparations using covalent chromatography (9,10) as mentioned above and Chen modelled her BSA-SH coupling experiments after those studies. In the BSA experiments bead-S-S-2-Py was in a 5-15-fold excess over protein thiol. Unfortunately, in the BSA experiments the protein in the cleavage supernatant was 20- to 55-fold lower than expected from thiopyridone release. Variation of the coupling times and/or the pH did not change the situation. However, when bead-SH was coupled with BSA-S-S-2-Py, reasonable amounts of protein were detected in the cleavage supernatant in two out of three cases. In those two cases greater than 30 nmol mercaptalbumin were detected per gram of dry beads.

Since the coupling experiments in the two different modes of coupling resulted in contrasting results (2), it was thought that the properties of BSA itself were the cause (2). Norris and Brocklehurst have shown that the reactivity of the BSA thiol group is very high, with a second-order rate constant approaching that of cysteine
and 10-fold higher than that of the sulfhydryl of the active site of papain (13). For this reason, the failure of Chen's BSA-SH plus bead-S-S-2-Py coupling experiments was thought to be due to the extreme accessibility of the sulfhydryl on the native polypeptide which allowed a manifold of subsequent thiol-disulfide interchanges (2).

Chen carried out cell coupling with bead-S-S-2-Py on the human erythrocyte ghost using the reagent 2-acetamido-4-mercaptobutyric acid hydrazide (thiol hydrazide) developed by Taylor and Wu (44) of our lab for introduction of the thiol into the carbohydrate of the glycoproteins (2). As shown in Fig. 6 (2), the ghosts were first reduced with DTE and alkylated with iodoacetamide to block a large proportion of the endogenous thiols and disulfides. Then after mild periodate oxidation the thiol hydrazide (44) was introduced into the cell surface oligosaccharides via hydrazone formation as shown in Fig. 6. When ghost hydrazone was incubated with bead-S-S-2-Py, Py-2-SH release into the coupling supernatant was 47 nmol per gram of dry beads which amounted to 7 nmol per mg of membrane protein. When the bead sample was subjected to DTE cleavage, the protein found in the cleavage supernatant was 0.29 mg, which was 6.1% of the protein in the experiment (2).

This study, like Chen's study (2), focuses on the preparation, characterization, and evaluation of a long-arm
Figure 6

Formation of Ghost-Hydrazone by Reaction of the Human Erythrocyte Ghost with Thiol Hydrazide

Legend

The ghosts were reduced with DTE and were alkylated with iodacetamide. Then after mild periodate oxidation the ghosts were reacted with thiol hydrazide. All reactions were carried out at room temperature and at pH 7.5. Taken from the thesis of Chen (2).
Figure 6
thiolated polyacrylamide bead reagent capable of thiol-disulfide interchange reactions. However, in this study beads of high and low thiol titres with different long-arms were synthesized, characterized, and evaluated in coupling experiments with papain. The low thiol titre beads were synthesized and evaluated in coupling experiments with papain because it was thought that the thiol groups of the high thiol titre beads interacted with each other due to their close proximity to one another so the coupling yields were not as high as they might be. It was thought that this problem of low coupling yields might be decreased by the use of low thiol titre beads in coupling experiments with papain.

In this study the cleavage supernatant was assayed for papain using N-α-benzoyl-D,L-arginine-β-nitroanilide (D,L-BAPA) (45) to see if the protein in the cleavage supernatants of the low thiol titre bead coupling experiments with papain was still catalytically active.
CHAPTER II

EXPERIMENTAL

A. MATERIALS

N-α-benzoyl-D,L-arginine-p-nitroanilide (D,L-BAPA), hydrochloride, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), dithioerythritol (DTE), and TRIS (Hydroxymethyl) aminomethane, (Trizma base) (TRIS) (reagent grade) were obtained from the Sigma Chemical Company, St. Louis, MO.

2,4,6-trinitrobenzene-sulfonic acid (TNBS) was bought from the Eastman Kodak Company, Rochester, N.Y.

Papain (Cryst.) was obtained as a suspension in 50 mM sodium acetate buffer, pH 4.5, from Boehringer Mannhein Canada, Dorval, Que., Canada.

Acetic acid, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), ethylenediaminethetraacetic acid (EDTA) disodium salt dihydrate, hydrochloric acid, methanol, phosphorus pentoxide, potassium chloride, potassium hydroxide, sodium acetate, sodium carbonate, sodium chloride, sodium phosphate dibasic, and standard buffer solutions pH 4.0, pH 6.0, pH 7.0 and pH 10.0 were purchased from the Fisher Scientific Company, Ltd., Don Mills, Ont., Canada.
Sodium phosphate monobasic was purchased from the Matheson Coleman and Bell Company, Norwood, OH.

Aldrithiol-2 (2,2'-dithiodipyridine) (2-Py-S-S-2-Py) and triethylamine (Et\textsubscript{3}N) were bought from the Aldrich Chemical Company, Milwaukee, WI.

Acetic anhydride (Ac\textsubscript{2}O) was obtained from British Drug House (BDH) Chemicals Ltd., Toronto, Ont., Canada.

Jeffamine ED-600 was donated by the Jefferson Chemical Company, Inc., Houston, TX.

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

Sephadex G-25 medium (140-200 mesh) was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Dithiobis (succinimidyl propionate) (DTSP) was kindly supplied by Taylor, and was synthesized using the procedure of Lomant and Fairbanks (46).

All aqueous solutions were prepared using deionized distilled water.

Nitrogen gas was supplied by Bull Welding, Windsor, Ont., a division of Liquid Carbonic, Toronto, Ont., Canada.

B. EQUIPMENT

All automatic pipetting was done using pipetters from Mandel Scientific Company Ltd., Rockwood, Ont., Canada, and the Fisher Scientific Company Ltd., Don Mills, Ont., Canada.
A 1.5 x 90 cm column K15 from Pharmacia Fine Chemicals AB, Uppsala, Sweden was used to pack the Sephadex G-25.

The pH measurements were done using a Corning Digital III General Purpose pH Meter from Canadian Laboratory Supplies Limited, Toronto, Ont., Canada, equipped with a Pencil Combination Fisher Electrode from the Fisher Scientific Company Ltd., Don Mills, Ont., Canada, and a Radiometer Model 26 instrument equipped with a Radiometer semi-microcombination electrode, number GK 2301-C, obtained from Bach-Simpson Ltd., London, Ont., Canada.

Activated papain solution was collected using an LKB 2070 Bromma Ultra Rac II fractionator equipped with an LKB 2138 Uvicord S and a chopper bar recorder 6520-7/8, which was purchased from Fisher Scientific Company Ltd., Don Mills, Ont., Canada.

All spectrophotometric measurements were made on a Beckman Model 35 spectrophotometer, or on a Beckman Acta MVI spectrophotometer, both of which were purchased from Beckman, Toronto, Ont., or on a Gilford Model 2000 absorbance recorder attached to a Beckman DU monochromator which was obtained from Gilford Instrument Laboratories Inc., Oberlin, OH.

C. METHODS

1. Analytical Methods

(a) TNBS Test

A 3% (w/v) solution of TNBS was made up in water. Four ml of samples were mixed with 2 ml of saturated
sodium borate solution, followed by addition of 3-4 drops of TNBS solution. Following mixing, the samples were compared with a saline control after 5 minutes. When TNBS (47) reacts with primary amines an orange color, ranging from yellow to orange-red depending on the concentration of primary amines in the sample, appears after 5 minutes.

(b) Amino Group Determination

In order to determine the aliphatic amino group density of the beads, the beads were first washed with 0.2 M KCl and were suspended 50% (v/v) in 0.2 M KCl. Then 1 M KOH was used to adjust the pH of the suspension to 11, followed by addition of standard HCl (v, ml) of normality N in order to bring the pH to 7. The volume of the suspension (V, ml) was measured. The amino group content in the beads was determined by measuring the milliequivalents of HCl added (vN) minus millequivalents of free OH\textsuperscript{−} titrated [0.001(v−v)] as given by the equation below (47):

\[
\text{meq NH}_2 = vN - (V-v) \times 0.001
\]

(c) Dry Weight Determination of the Amino Derivative of the Beads (Bead-NH\textsubscript{2})

The sample of beads was placed in a preweighed Gooch crucible and was converted into the uncharged form by 2 washings with 0.2 M sodium carbonate (for amino groups) in order to minimize subsequent swelling (38). Subsequently
the beads were washed with deionized distilled water to remove electrolyte, then by 60% methanol in water, and finally with methanol. The beads were dried over P₂O₅ in a vacuum desiccator overnight using a vacuum pump equipped with a dry-ice-cooled vapor trap. On removal from the desiccator the beads were weighed immediately.

(d) **Dry Weight Determination of Thiolated Beads (Bead-SH)**

The beads were placed in a preweighed Gooch crucible or sintered glass funnel. Then the beads were washed twice with 0.2 M KCl, several times with 0.1 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA), several times with deionized distilled water to remove electrolyte, and twice with methanol. The beads were dried overnight over P₂O₅ on house vacuum. On removal from the desiccator, beads were weighed immediately.

(e) **DTE Back Titration of High Functional Group Density Bead-S-S-2-Py**

A 1 ml sample of 50% suspension (v/v) of bead-S-S-2-Py in 0.2 M KCl was washed several times with 0.1 M sodium phosphate buffer pH 7.6 (containing 0.3 M KCl and 1 mM EDTA). Then 5 ml of 10 mM DTE (in the same buffer) were added with stirring to the moist cake of the beads. After 15 minutes, beads were filtered to a moist cake and another 5 ml of 10 mM DTE were added with stirring as wash. After
15 minutes beads were filtered and the final volume of filtrate was recorded. The amount of 2-thiopyridone (Py-2-SH) released in the filtrate was determined using $\varepsilon_{343} = 8.080 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ (48). Dry weight determination of the thiolated beads was carried out as described above.

(f) **DTE Back Titration of Low Functional Group**

**Density Bead-S-S-2-Py**

A 7 ml sample of 50% suspension (v/v) of bead-S-S-2-Py in 0.2 M KCl was washed several times with 0.10 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA). Then 3.5 ml of 10 mM DTE (in the same buffer) were added with stirring to the moist cake of beads. After 15 min the beads were filtered to a moist cake and another 3.5 ml of 10 mM DTE were added as wash. After 15 min the beads were filtered and the final volume of the filtrate was recorded. The filtrate was measured at 343 nm for Py-2-SH release. The Py-2-SH release was calculated as described above and the dry weight determination of the thiolated beads was done as described above.

(g) **DTNB Titration of High Functional Group**

**Density Beads**

A 1 ml aliquot of 50% (v/v) suspension of bead-S-S-2-Py in 0.2 M KCl was removed and a DTE back titration was done on it as described above. Then the beads were washed several
times with 0.10 M sodium phosphate buffer, pH 7.0 (containing 0.3 M KCl and 1 mM EDTA). Then 10 ml of 1 mM DTNB (in the same buffer) were added with stirring to the beads. After 30 min the beads were filtered and the thionitrobenzoate ion (TNB\(^-\)) content of the filtrate was determined using \( \epsilon_{410} = 13.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1} \) (40). Then beads were washed with 0.10 M sodium phosphate buffer, pH 7.0 (containing 0.3 M KCl and 1 mM EDTA) several times and a DTE back titration was done on the beads as discussed above, except that the 10 mM DTE was made up in 0.10 M sodium phosphate buffer, pH 7.0 (containing 0.3 M KCl and 1 mM EDTA). The amount of TNB\(^-\) group was determined at 410 nm as described above and the dry weight of the beads was determined (described above).

(h) **DTNB Titration of Low Functional Group Density Beads**

A 7 ml aliquot of 50% (v/v) suspension of bead-S-S-2-Py in 0.2 M KCl was removed and a DTE back titration was done on it as described above. The beads were washed several times with 0.10 M sodium phosphate buffer, pH 7.0 (containing 0.3 M KCl and 1 mM EDTA) and then the DTNB procedure used for the high functional group density beads (described above) was used for the low functional group density beads. This was followed by DTE back titration and dry weight determination of the beads (described above).
2. **Synthesis of the High Functional Group Density Long-arm Thiolated Polyacrylamide Beads**

(a) **Preparation of the Aliphatic Amino Derivative of Polyacrylamide Beads**

Taylor (49) prepared the aliphatic amino derivative. In a fume hood 45 g Jeffamine ED-600 were pumped under vacuum for several hours at room temperature, then were pre-heated to 90°C in vacuo. Then 15 g dry beaded polyacrylamide (<400 mesh, 10-37 μm diameter, 1800 dalton inclusion limit) were added to the Jeffamine ED-600 at 90°C with stirring for 3 hours. After 3 hr the suspension was mixed with an equal volume of crushed ice until the temperature cooled to room temperature. Immediately the beads were filtered in a sintered glass funnel and washed with 1 mM HCl twice, then with 0.2 M NaCl by batch washes until supernatants were negative to the TNBS test (described above). Then the beads were washed twice with 0.2 M KCl and suspended in 0.2 M KCl. The amino group content of the beads was determined using the titration and dry weight determination described above.

(b) **Thiolation of the Amino Derivative of Polyacrylamide Beads to Form Bead-SH**

Synthesis of the long-arm thiolated beads was carried out by Taylor (49). The reaction was carried out by adding
506 mg DTSP (1.25 mmol) and 50 μL TE₃N to a 25 ml DMF solution containing 15.05 g of amino derivative of the beads. The reaction mixture was stirred for 4 hr at room temperature. Then the mixture was diluted with 40 ml water and 4 ml of 1 M sodium phosphate buffer, pH 7.4 and was filtered. The beads were washed several times with 0.2 M KCl and stored in same.

In this study Taylor's beads (above) were washed several times with 50 mM sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA). Then the beads were treated with 10 mM DTE (in the same buffer) for 30 min to reduce the disulfide to bead-SH.

3. Conversion of Bead-SH to the Mixed Disulfide Form Bead-S-S-2-Py

A stock solution of 2-Py-S-S-2-Py was prepared using the procedure of Brocklehurst and Little (6) by adding 0.120 g 2-Py-S-S-2-Py (0.5 mmol) to 150 ml of 0.10 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA) for 5 hr at room temperature. Filtration was used to remove insoluble material. The concentration of the solution was determined to be in the range of 0.9 - 1.7 mM using $\varepsilon_{281} = 9.7 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$ (50).

Thiolated beads were filtered to a moist cake and were washed several times with 0.2 M KCl. The beads then were suspended in 150 ml of 1 mM 2-Py-S-S-2-Py (in 0.10 M sodium
phosphate buffer, pH 7.6, containing 0.3 M KCl and 1 mM EDTA) for 60 min followed by filtration and washing with 50 mM sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA) several times. Next, the beads were washed with 0.2 M KCl several times with degassing and then were transferred to a graduate cylinder and were stored at 0-4°C. Dithioerythritol back titrations (described above) were done on the beads to determine the thiol content of the beads by Py-2-SH release.

4. **DTE Titration of 2-Py-S-S-2-Py Wash Samples**

After the beads were converted to the activated mixed disulfide form they were washed with MeOH:H₂O (1:1) twice and with 0.10 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA) several times. The wash was checked for 2-Py-S-S-2-Py present by a DTE titration. A 0.9 ml sample of the wash was mixed with 0.1 ml of 100 mM DTE (in 0.10 M sodium phosphate buffer, pH 7.6, containing 0.3 M KCl and 1 mM EDTA) for 15 min. Then the sample was read at 343 nm for Py-2-SH release, against a blank containing 0.9 ml of 0.10 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA) and 0.1 ml of 100 mM DTE (in the same buffer).

5. **Recycling of Bead-SH in 0.1 mM 2-Py-S-S-2-Py**

The DTE-titrated bead-SH samples of bead reagent were taken and combined with the remaining bead-S-S-2-Py in
0.2 M KCl in a sintered glass funnel. The beads were filtered and washed several times with 0.10 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA) with degassing. Following this, the moist cake of beads was treated with 10 mM DTE (in the same buffer) for 15 min. Then the beads were filtered to a moist cake and more 10 mM DTE was added as wash. After this the beads were filtered, washed with 0.10 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA), and were degassed. Then the beads were washed with 50 mM sodium acetate buffer, pH 4.5 (containing 0.15 M NaCl and 1 mM EDTA). The preparation was then transferred to a stirring solution of 0.1 mM 2-Py-S-S-2-Py (in the same buffer). After 25 min the beads were filtered, washed with MeOH:H₂O (1:1) twice and exhaustively washed with 0.10 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA). Py-S-S-Py and Py-2-SH contents in the wash were indicated by appropriate titration and absorbance measurements. Then the beads were washed twice with 0.2 M KCl and were stored in 0.2 M KCl at 0-4°C in the activated form. The thiol content of the beads was determined by DTE back titration and DTNB titration as described above. Recycling of the beads in 2-Py-S-S-2-Py was carried out as described above.
6. **Synthesis of Low Functional Group Density**

**Long-Arm Thiolated Polyacrylamide Beads**

Preparation of the amino derivative of the beads was done using the procedure of Taylor (49) (above) with some modifications to lower the amino group density on the beads. In a fume hood, 5 g of dried polyacrylamide beads were heated in 50 ml Jeffamine ED-600 for various times and temperatures as noted. Quenching, washing, and amino group titration were carried out as described above.

Synthesis of low thiol titre bead-SH (0.2 ± 0.1 µmoles SH/g of dry beads) was carried out using the procedure of Taylor (49) with some modifications. The reaction was carried out by adding limiting amounts of DTSP (e.g., 4.95 or 0.74 µmoles) and 10 µl Et$_3$N to 13 ml of DMF containing 4.62 g of bead-NH$_2$. The reaction mixture was stirred for 4 h at room temperature. Then 50 µl of Ac$_2$O were added to reaction mixture to acylate any unreacted amino groups. The reaction mixture was stirred for 1 h then another 50 µl Ac$_2$O were added. After 1 h the mixture was diluted with 0.10 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA):H$_2$O (1:1). Then the mixture was filtered, washed 3 more times with 0.10 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA):H$_2$O (1:1), twice with 0.2 M KCl, and then was treated with 10 mM DTE (in the same buffer). After 15 min the beads were filtered, and the moist cake was washed with 10 mM DTE, then with 0.2 M KCl twice.
The beads were then converted to the mixed disulfide form bead-S-S-2-Py.

7. **Large Scale Preparation of Low Functional Group Density Long-Arm Thiolated Polyacrylamide Beads**

The amino derivative of the beads, bead-NH₂, was prepared using the procedure of Taylor (49) with some modifications. Eleven g beaded polyacrylamide (≤400 mesh, 10-37 μm diameter, 1800 dalton inclusion limit) were added to 100 ml of preheated Jeffamine ED-600 at 60°C for 20 min. The amino group content and dry weight determination of the beads were done as described above.

Thiolation of the 10.51 g of bead-NH₂ was done with 1.24 umol DTSP using the procedure of Taylor (49) with some modifications, as described above for the smaller scale syntheses of low functional group density thiolated beads, except that the amounts of Et₃N and Ac₂O were doubled. Conversion of bead-SH to bead-S-S-2-Py and recycling of beads were done as described above.

8. **Preparation of Activated Papain Solution**

A 2 ml aliquot of papain suspension containing approximately 20 mg in 50 mM sodium acetate buffer, pH 4.5 was removed from stock papain suspension and 2 ml water were added to immerse the pH electrode. The pH was adjusted to 8.0 with 1 M TRIS and then water was added until the papain dissolved (fluid volume approximately 8 ml). Activation
of the papain (8) was accomplished by treatment with 0.4 ml 100 mM DTE (in 0.10 M sodium phosphate buffer, pH 7.6, containing 0.3 M KCl and 1 mM EDTA) (final concentration of DTE in the papain solution was 5 mM) for 15 min at room temperature. The papain solution was passed through a Sephadex G-25 column using N₂-saturated 50 mM sodium acetate buffer, pH 4.5 (containing 0.15 M NaCl and 1 mM EDTA). The A₂₈₀ chart recorder which was attached to the fractionator served as a guide to pooling the desired papain fractions. The amount of papain in the pooled fractions was determined using $E_{278}^{1\%}$ for papain as 25 (51) and the molecular weight of 23,000 daltons (43) for papain. Titration of activated papain samples with 2-Py-S-S-2-Py (in 50 mM sodium acetate buffer, pH 4.5, containing 0.15 M NaCl and 1 mM EDTA) was done as follows: 0.9 ml of papain solution and 0.1 ml of 1 mM 2-Py-S-S-2-Py were mixed and after 25 min the A₃₄₃ was measured for Py-2-SH release.

9. Coupling of High Functional Group Density Bead-S-S-2-Py with Activated Papain

The moist cake from 4 ml 50% (v/v) suspension of bead-S-S-2-Py was washed several times with N₂-saturated 50 mM acetate buffer, pH 4.5 (containing 0.15 M NaCl and 1 mM EDTA). Then 10 ml of activated papain from the Sephadex G-25 column were added with stirring to the moist cake of bead-S-S-2-Py for 30 min at room temperature. The
beads then were filtered to a moist cake, washed with acetate buffer, and the wash was added to the filtrate. The volume of the combined filtrate and its A$_{343}$ and A$_{278}$ values were measured for Py-2-SH release and protein content. A 2-Py-S-S-2-Py titration and a DTE titration were done on the coupling supernatant to determine the 2-Py-S-S-2-Py contents and thiol contents of the coupling supernatant, respectively. The DTE titration of the coupling supernatant was carried out by mixing 0.1 ml of 150 mM DTE (in 0.10 M sodium phosphate buffer, pH 7.6, containing 0.3 M KCl and 1 mM EDTA) and 0.9 ml of coupling supernatant for 30 min. Then the A$_{343}$ and A$_{278}$ were measured.

The beads were washed with N$_2$-saturated 50 mM sodium acetate buffer, pH 4.5 (containing 0.15 M NaCl and 1 mM EDTA), with 0.10 M sodium phosphate buffer, pH 7.6 (containing 0.3 M KCl and 1 mM EDTA), and then were treated with 5 ml of 15 mM DTE (in 0.10 M sodium phosphate buffer, pH 7.6, containing 0.3 M KCl and 1 mM EDTA) for 15 min. After 15 min the beads were filtered to a moist cake and another 5 ml of 15 mM DTE were added as wash. After 15 min the beads were filtered. The volume of the filtrate (DTE cleavage supernatant) was recorded and the A$_{343}$ and A$_{278}$ were measured. The filtrate was dialyzed over 2 nights against several changes of N$_2$-saturated 50 mM sodium acetate buffer, pH 4.5 (containing 0.15 M NaCl and 1 mM
EDTA). After dialysis, the cleavage supernatant was measured at 278 nm and 343 nm, and a 2-Py-S-S-2-Py titration was done at pH 4.5.

10. **Determination of the Specific Activity of Activated Papain using D,L-BAPA**

The assay was carried out using the procedure described by Arnon (45) with some modifications. Activated papain from the Sephadex G-25 column was diluted 2-fold in N₂-saturated 50 mM sodium acetate buffer, pH 4.5 (containing 0.15 M NaCl and 1 mM EDTA) so that the concentration of the papain was approximately 0.1 mg/ml as determined using $E_{278}^{1%} = 25$ (51). The substrate solution was prepared by dissolving 22 mg D,L-BAPA HCl (50.6 μmol) in 1.69 ml DMSO. For the assay procedure, enzyme solution, prepared above, was placed in test tubes in volumes from 0.1 ml to 0.8 ml and the volumes were made up to 0.8 ml with acetate buffer. Then 25 μl of 0.8 M TRIS were added to each tube with mixing to bring pH of reaction mixture to 6.0. At 1 min intervals 50 μl of 0.10 M sodium phosphate buffer, pH 6.0 (containing 37.5 mM DTE and 2.95 mM EDTA) and 25 μl of substrate solution were added to each tube with mixing. After 20 min at room temperature the reaction was terminated by addition of 0.1 ml 30% acetic acid solution. The quantity of p-nitroaniline was estimated spectrophotometrically at 410 nm using $ε_{410} = 8.80 \times 10^3 \ M^{-1} cm^{-1}$ (45). One unit of D,L-BAPA activity is the
amount of enzyme which will hydrolyze 1 μmole of substrate per minute (52). The specific activity is expressed as the number of units per milligram protein (52). A standard curve of $A_{410}$ versus amount of papain (μg) was plotted.

For comparison of the specific activity of the papain that had been activated with DTE and passed through the Sephadex G-25 column (described above), an assay was done of papain that had not been activated with DTE until the assay. The assay was carried out as described above except that the enzyme solution was prepared as follows: 0.25 ml of stock papain suspension was dissolved in 25 ml of $N_2$-saturated 50 mM sodium acetate buffer, pH 4.5 (containing 0.15 M NaCl and 1 mM EDTA).

11. Coupling of Low Functional Group Density Bead-S-S-2-Py with Papain

Coupling of the low functional group density bead reagent with papain was done using the same procedure as for coupling of the high functional group density bead reagent with papain, above, with some modifications. The bead samples used for coupling were 14 ml of 50% (v/v) suspension of bead-S-S-2-Py, and the buffer used for the DTE cleavage reaction was 0.10 M sodium phosphate buffer, pH 6.0 (containing 0.3 M KCl and 1 mM EDTA) because the papain was assayed in the coupling experiments at pH 6.0, using the D,L-BAPA assay above. When papain samples in
0.10 M sodium phosphate buffer, pH 6.0 (containing 0.3 M KCl and 1 mM EDTA) were assayed, 25 µl of 0.10 M sodium phosphate buffer, pH 6.0 were added instead of 0.8 M TRIS so the reaction mixture would have a pH of 6.0.
CHAPTER III

RESULTS

The polyacrylamide beads used in this study were Biogel P-2 (~400 mesh). The beads had a particle diameter of 10-37 μm and an inclusion limit (molecular weight cutoff) of ~1800 daltons.

The spacer molecule used in this study was Jeffamine ED-600 (polyoxyalkylene diamine) and this spacer molecule has a length of 70-100 Å. Jeffamine ED-600 is an aliphatic primary diamine structurally derived from propylene oxide-capped polyethylene glycol. It has an average molecular weight of 600 and its structure is shown below.

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{H}_2\text{NCHCH}_2 & \quad (\text{OCHCH}_2)_a \quad (\text{OCH}_2\text{CH}_2)_b \quad (\text{OCH}_2\text{CH})_c \quad \text{NH}_2
\end{align*}
\]

According to the manufacturer, the values of \(a+c\) and \(b\) in the diagram above are approximately 3.5 and 13.5, respectively.

**Synthesis of High Functional Group Density Long-Arm Thiolated Polyacrylamide Beads**

High functional group density beads (preparation I) (prep. I) were prepared by Taylor (49) using the proce-
dure shown in Fig. 7. The amino derivative of the beads, 9.6 μeq NH₂/g, was reacted with a large excess of DTSP which would correspond theoretically to 166 μmoles SH/g of beads to saturate all the amino groups with thiol groups followed by cleavage with DTE.

In this study Taylor's beads were added to a stirring solution of 1 mM 2-Py-S-S-2-Py and DTE back titrations were done on samples. Table II gives the results of the DTE back titrations, along with the resulting thiol titres after recycling the beads in 0.1 mM 2-Py-S-S-2-Py solution. Table III gives the results of treatment of the recycled beads with DTNB (40), along with the results of DTE titration of the DTNB-treated beads.

**Synthesis of Low Functional Group Density Beads**

(a) **The Aminolysis Reaction**

In order to lower the thiol titre of the beads, instead of carrying out the aminolysis reaction (shown in Fig. 7) for 3 h at 90°C (49), the aminolysis reaction was carried out at 90°C for 20 min. The amino group titre, which was determined as described by Inman (47), was found to be 7.2-13.6 μeq NH₂/g of beads. In an attempt to lower the amino group titre further, the aminolysis reaction was carried out at 60°C for 20 min. The resulting amino group titre was 7.3 μeq NH₂/g of beads. Since the amino group
Figure 7

Preparation of High Functional Group Density Long-arm Thiolated Polyacrylamide Beads

Legend

Taylor (49) prepared the amino derivative via aminolysis at 90°C for 3 hr using Jeffamine ED-600 as the spacer. Thiolation of the amino derivative was done by reaction with DTSP for 4 hr at room temperature followed by reduction with DTE.
Bead - C - NH$_2$  Polyacrylamide

\[ \begin{align*}
\text{NH}_2 & \quad \text{Jeffamine ED-600} \\
\text{CH}_3 & \quad \text{DTSP}
\end{align*} \]

(1) Bead - C - NH$_2$  Polyacrylamide

(2) Bead - C - NH$_2$

Long-Arm Thiolated Beads (Bead-SH)

Figure 7
<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>μmoles SH g⁻¹ of beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ᵃ</td>
<td>1.45</td>
</tr>
<tr>
<td>2ᵃ</td>
<td>1.46</td>
</tr>
<tr>
<td>3ᵃ</td>
<td>1.01</td>
</tr>
<tr>
<td>4ᵃ</td>
<td>1.23</td>
</tr>
<tr>
<td>5ᵇ</td>
<td>0.96</td>
</tr>
<tr>
<td>6ᵇ</td>
<td>0.97</td>
</tr>
<tr>
<td>7ᶜ</td>
<td>1.27</td>
</tr>
<tr>
<td>8ᶜ</td>
<td>1.26</td>
</tr>
</tbody>
</table>

ᵃExperiments 1-4 were carried out by treating the moist cake of 1 ml 50% suspension of synthesized bead-S-S-2-Py (prep. I) with 10 mM DTE (in 0.10 M sodium phosphate buffer, pH 7.6, containing 0.3 M KCl and 1 mM EDTA) for 30 min at room temperature.

ᵇExperiments 5 and 6 were carried out by treating the moist cake of bead-S-S-2-Py (prep. I) (recycled in 0.1 mM 2-Py-S-S-2-Py) with 10 mM DTE (in 0.10 M sodium phosphate buffer, pH 8.0, containing 0.3 M KCl and 1 mM EDTA).

ᶜExperiments 7 and 8 were carried out by back titrating the moist cake of bead-S-S-2-Py (prep. I) (recycled in 0.1 mM Py-S-S-2-Py) with 10 mM DTE (in 0.10 M sodium phosphate buffer, pH 7.0, containing 0.3 M KCl and 1 mM EDTA).
TABLE III
DETERMINATION OF THE THIOL CONTENT OF BEADS (PREP. I) BY DTNB TREATMENT

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>μmoles SH from DTNB forward titration&lt;sup&gt;a&lt;/sup&gt; (g⁻¹ beads)</th>
<th>μmoles SH from TNB⁻ release by DTE&lt;sup&gt;b&lt;/sup&gt; (g⁻¹ beads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.43</td>
<td>1.54</td>
</tr>
<tr>
<td>2</td>
<td>1.84</td>
<td>1.50</td>
</tr>
</tbody>
</table>

<sup>a</sup>Experiments were carried out by treating the moist cake of 1 mL 50% (v/v) suspension of recycled bead-S-S-2-Py (prep. I) with 10 ml 10 mM DTNB (in 0.10 M sodium phosphate buffer, pH 7.0, containing 0.3 M KCl and 1 mM EDTA).

<sup>b</sup>These experiments were carried out by treating the moist cake of DTNB treated beads (above) with 10 mM DTE (in 0.10 M sodium phosphate buffer, pH 7.0, containing 0.3 M KCl and 1 mM EDTA).
titre was not decreased sufficiently, it was decided that
ttempts to lower the thiol functional group density would
be attempted by lowering the concentration of DTSP (see
Fig. 7), and as for the aminolysis reaction it would be done
at 90°C for 20 min.

(b) Thiolation of the Amino Derivative of
Polyacrylamide Beads

The synthesis of low thiol titre beads containing
0.2 ± 0.1 μmoles SH/g of dry beads, using limiting DTSP
was first attempted with beads of 7.2-13.6 μeq. NH₂/g
using 2.5 mg DTSP (4.95 μmoles) for 4 hours, followed by
acylation of the unreacted amino groups with Ac₂O and then
reduction with DTE as shown in Fig. 8. The resulting thiol
titre of the beads (prep. II) by 2-Py-S-S-2-Py treatment
followed by DTE back titration was 1.41-1.54 μmoles SH/g
of dry beads. Since the thiol content of the beads was too
high the DTSP reaction was carried out using 0.74 μmoles of
DTSP with 4.58 g of the amino derivative of the beads which
would correspond to a theoretical thiol titre of 0.32 μmoles
SH/g of beads. The resulting thiol content of the beads
(prep. III), following treatment with 2-Py-S-S-2-Py as
monitored by Py-2-SH release, is given in Table IV, along
with the data found after recycling the beads in 2-Py-S-S-2-Py
solution. Since with increasing recycles there was more
Py-2-SH released than was theoretically possible (see Table IV),
Figure 8

Preparation of Low Functional Group Density Long-arm Thiolated Polyacrylamide Beads

Legend

The amino derivative of the beads was prepared via aminolysis for 2 min at 90°C using Jeffamine ED-600 as the spacer. Thiolation of the amino derivative was accomplished by reaction with DTSP for 4 hr at room temperature, unreacted amino groups were acylated with $\text{Ac}_2\text{O}$. Beads were reduced with DTE.
Bead—C—NH₂ Polyacrylamide

\[ \text{Jeffamine ED-600} \]

Bead—C—NH\_2

\[ \text{DTSP} \]

1

\[ \text{Ac}_2\text{O} \]

2

\[ \text{DTE} \]

3

Bead—C—NH\_2 Long-Arm Thiolated Beads (Bead—SH)

\[ \text{Figure 8} \]
<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Recycle no.</th>
<th>Concentration Py-S-S-Py used (mM)</th>
<th>μmoles SH g⁻¹ beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Formation of bead-S-S-2-Py</td>
<td>1</td>
<td>0.24</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0.35</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0.46</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1</td>
<td>1.08</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>0.1</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Experiments were carried out by treating the moist cake of bead-S-S-2-Py (prep. III) with 7 ml of 10 mM DTE (in 0.10 M sodium phosphate buffer, pH 7.6, containing 0.3 M KCl and 1 mM EDTA).*
the beads were recycled in 0.1 mM 2-Py-S-S-2-Py instead of 1 mM 2-Py-S-S-2-Py solution. The Py-2-SH released was 0.25 μmoles Py-2-SH per gram of dry beads (shown in Table IV), which was remarkably less than the last recycle of the beads in 1 mM 2-Py-S-S-2-Py which resulted in a thiol titre of 1.08 μmoles Py-2-SH/g of dry beads.

The large scale synthesis of low thiol functional group density beads was done using 1.24 μmoles DTSP for 10.51 g of amino derivative (see Fig. 8) (prep. IV) which corresponded to a theoretical thiol titre of 0.24 μmoles SH/g of dry beads. When the beads (prep. IV) were treated with 0.1 mM 2-Py-S-S-2-Py followed by DTE back titration, the resulting thiol titre was determined, and it is given in Table V. Table V also gives the thiol content of the beads after recycling them in 0.1 mM 2-Py-S-S-2-Py solution. Although the thiol content of the beads, exhibited by Py-2-SH release, increased even with recycles in 0.1 mM 2-Py-S-S-2-Py, coupling experiments with papain were carried out with the beads after the first recycle, in which the Py-2-SH release corresponded closely to the theoretical SH content of the beads (0.20 μmoles SH/g of dry beads). The thiol content of the low functional group density beads was determined also by DTNB (40) treatment after its second recycle in 0.1 mM 2-Py-S-S-2-Py. The resulting thiol titre is given in Table VI. Then the bead
TABLE V

THIOL CONTENT OF BEADS (PREP. IV) AFTER
0.1 mM 2-Py-S-S-2-Py TREATMENT FOLLOWED
BY DTE BACK TITRATIONa

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Recycle no.</th>
<th>μmoles SH g(^{-1}) beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Formation of bead-S-S-2-Py</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.20</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.40</td>
</tr>
</tbody>
</table>

aExperiments were carried out by treating 7 ml of 50% (v/v) suspension of bead-S-S-2-Py (prep. IV) with 7 ml of 10 mM DTE (in 0.10 M sodium phosphate buffer, pH 7.6, containing 0.3 M KCl and 1 mM EDTA) at room temperature for 30 min.
### TABLE VI

DTNB DETERMINATION OF THE THIOL CONTENT OF LOW FUNCTIONAL GROUP DENSITY BEADS

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>μmoles SH (g⁻¹ beads) from DTNB forward titration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>μmoles SH (g⁻¹ beads) from TNB released by DTE&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.53</td>
<td>0.54</td>
</tr>
<tr>
<td>2</td>
<td>0.46</td>
<td>0.57</td>
</tr>
</tbody>
</table>

<sup>a</sup> Experiments were carried out by treating the moist cake of 7 ml 50% (v/v) suspension of bead-S-S-2-Py (prep. IV) with 10 ml of 10 mM DTNB (in 0.10 M sodium phosphate buffer, pH 7.0, containing 0.3 M KCl and 1 mM EDTA).

<sup>b</sup> Experiments were carried out by treating the moist cake of DTNB-treated beads (above) with 10 ml of 10 mM DTE (in 0.10 M sodium phosphate buffer pH 7.0, containing 0.3 M KCl and 1 mM EDTA).
samples were washed with 0.10 M sodium phosphate buffer, pH 7.0 (containing 0.3 M KCl and 1 mM EDTA) and treated with 10 mM DTE (in 0.10 M sodium phosphate buffer, pH 7.0, containing 0.3 M KCl and 1 mM EDTA). The TNB⁻ release was measured at 412 nm and then the thiol content of the beads was determined. The results of the thiol content of the beads as determined by DTNB forward titration as well as by the TNB⁻ released by DTE titration were averaged together. The resulting thiol titre was 0.53 μmoles SH/g of dry beads.

**Coupling of High Thiol Titre Bead-S-S-2-Py with:**

Papain-SH

Coupling of bead-S-S-2-Py (prep. I) containing 1.22 μmoles-S-S-2-Py/g of dry beads (determined by 2-Py-S-S-2-Py titration followed by DTE back titration) with papain-SH was done at pH 4.5 using the procedure of Chen (2) with some modifications. Papain was activated with DTE and passed through a Sephadex G-25 column. titration of activated samples with 2-Py-S-S-2-Py solution yielded samples containing 0.66 moles thiol per mole of papain. Samples of activated papain in the literature (8) had thiol titres in the range of 0.4–0.6 moles thiol per mole of protein. In the coupling experiments of bead-S-S-2-Py with papain-SH, there was a 8.5 and a 9.1-fold excess of bead-S-S-2-Py over papain-SH. When coupling of bead-S-S-2-Py with papain
was carried out Py-2-SH release of experiment 1 indicated 53% coupling, but as seen in Table VII, the decrease in protein suggested 18% coupling. The protein in the DTE cleavage supernatant was determined after measuring the \( A_{278} \) and the \( A_{343} \) and correcting the \( A_{278} \) as described in Table VII for the Py-2-SH present. The DTE cleavage supernatant of experiment 1 showed only approximately one twelfth the protein monitored as Py-2-SH released. However, the correction of the \( A_{278} \) values due to Py-2-SH present was a large proportion of the absolute magnitude of the absorbances of the DTE cleavage supernatants of the 2 coupling experiments, which placed great uncertainty in the measurements. Therefore, the DTE cleavage supernatants were dialyzed over 2 nights, until the \( A_{343} \) values dropped to zero (or near zero) and then the \( A_{278} \)'s were remeasured and corrected if necessary. The amount of protein found in the dialyzed cleavage supernatant of experiment 1 (see Table VII) was 78% of the amount of protein not present in the coupling supernatant and this corresponded to 22 nmol/g of dry beads (0.51 mg/g of dry beads). The 0.25 mg of protein in the dialyzed DTE cleavage supernatant was 27% of the degree of protein coupling indicated by Py-2-SH release. The percentage coupling in experiment 1 was 14%.

In coupling experiment 2 (see Table VII) Py-2-SH release indicated 14% coupling but the decrease in protein
**TABLE VII**

**COUPLING OF HIGH FUNCTIONAL GROUP DENSITY BEAD-S-S-2-Py WITH PAPAIN-SH**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Bead-S-S-2-Py</th>
<th>Sample of papain applied</th>
<th>Coupling supernatant before dialysis</th>
<th>DTE cleavage supernatant before dialysis</th>
<th>Cleavage supernatant after dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td>0.65</td>
<td>0.076</td>
<td>0.04</td>
<td>0.54</td>
<td>0.09</td>
</tr>
</tbody>
</table>
| Py-2-SH available or released (μmoles)

| Protein that is papain-SH (mg)
| Protein (mg) |
|----------------|-----|
| 1.75 | 2.69 |
| 2.37 | 0.08 |
| 0.25 | 

| **Experiment 2** | 0.65 | 0.071 | 0.01 | 0.61 | 0.09 |
| Py-2-SH available or released (μmoles)

| Protein that is papain-SH (mg)
| Protein (mg) |
|----------------|-----|
| 1.63 | 2.44 |
| 0.24 | 1.89 |
| 0.23 | 

---

*a Coupling experiments were carried out by reacting samples of activated papain from Sephadex G-25 column with moist cake of 4 ml 50% (v/v) suspension of bead S-S-2-Py (prep. 1) at pH 4.5 for 30 min at room temperature as described in EXPERIMENTAL.*

*b The amount of Py-2-SH was calculated from the $A_{343}$ measurement.

*c Protein that was papain-SH was calculated from the number of μmoles Py-2-SH x molecular weight of papain (23,000 daltons) (43).*

*d Protein was determined from $A_{278}$ measurement. When Py-2-SH was present $A_{278}$, corr was calculated from $A_{278}$, corr = $A_{278} - 1.25 \times A_{343}$ (50).*

*e Amount of Py-2-SH was estimated by DTE back titration.*
suggested 34% coupling. When the DTE cleavage supernatant was checked for protein the $A_{278}^\text{corr}$ showed that only one twenty-fourth of the protein coupled during Py-2-SH release was recovered upon DTE cleavage. After the DTE cleavage supernatant was dialyzed over 2 nights the amount of protein was determined. The amount of protein in the DTE cleavage supernatant was 42% of the corresponding amount of protein not present in the coupling supernatant and corresponded to 20 nmol/g of dry beads (0.46 mg/g of dry beads). The 0.23 mg of protein recovered after dialysis was 96% of the degree of protein coupling indicated by Py-2-SH release, and the percentage coupling was 14% as in experiment 1.

**Coupling of Low Thiol Titre Bead-S-S-2-Py with Papain-SH**

Coupling experiments of bead-S-S-2-Py (prep. IV) containing 0.19 μmoles-S-S-2-Py/g of dry beads with activated papain were carried out at pH 4.5, as described in the EXPERIMENTAL. Titrination of activated papain samples with 2-Py-S-S-2-Py yielded a thiol titre of 0.85 moles thiol per mole of papain. The specific activity of the activated papain was determined using the substrate D,L-BAPA by the procedure of Arnon (45) with some modifications. The specific activity of the activated papain was determined to be 0.065 U·mg⁻¹ and a standard curve (Fig. 9) was prepared. Linear regression analysis was done on the data.
Figure 9

Standard Curve of Activated Papain from Sephadex G-25 Column using D,L-BAPA

Legend

The assay was done at pH 6.0 using a concentration of 0.83 mM D,L-BAPA in the reaction mixture. Samples were done in duplicate. The line shown is the least-squares line (correlation coefficient 0.9996): $A_{410} = (0.0109 \pm 0.0002)(\mu g \ papain) + (0.0233 \pm 0.0008)$. *Point not included in linear regression analysis.
Figure 9
The uncertainties in the slope and y-intercept were calculated as described in Appendix 2 (53). A standard curve, Fig. 10, was prepared for papain that was not activated with DTE and passed through a Sephadex G-25 column, although DTE was present in the assay mixture, for comparison to the papain from the Sephadex G-25 column (Fig. 9). Linear regression analysis was done on the data. The uncertainties in the slope and y-intercept were calculated as described in Appendix 2. The specific activity of the papain was determined to be 0.083 U·mg⁻¹.

In coupling experiment 1 (see Table VIII), there was a 4.9-fold excess of bead-S-S-2-Py over papain-SH. Coupling of bead-S-S-2-Py with papain-SH resulted in Py-2-SH release that indicated 15% coupling, whereas determination of the decrease in protein content suggested 24% coupling. When a 2-Py-S-S-2-Py titration was done on the coupling supernatant, the amount of protein in the coupling supernatant that was calculated to be papain-SH was 1.48 mg. The specific activity of the coupling supernatant was determined using the D,L-BAPA assay described in the experimental, the resulting specific activity was 0.058 U·mg⁻¹. The amount of catalytically active papain in the coupling supernatant was calculated using the A₄₁₀ value from the D,L-BAPA assay and the standard curve of activated papain (Fig. 9) after correcting for the difference in activity between the papain
Figure 10

Standard Curve of Papain using D,L-PAPA

Legend

The enzyme solution for the assay was prepared by dissolving 0.25 ml of stock papain suspension in 25 ml of 50 mM acetate buffer, pH 4.5 (containing 0.15 M NaCl and 1 mM EDTA). The assay was performed at room temperature as described in the EXPERIMENTAL using 0.83 mM D,L-BAPA in the reaction mixture. Samples were done in duplicate. The line shown is the least squares line (correlation coefficient 0.999): $A_{410} = (0.0147 \pm 0.0001)(\mu g \text{ papain}) - (0.0103 \pm 0.0005)$. *point not included in linear regression analysis.*
Figure 10
<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Bead-S-S-2-Py</th>
<th>Sample of papain applied</th>
<th>Coupling supernatant</th>
<th>DTE cleavage supernatant before dialysis</th>
<th>Cleavage supernatant after dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Py-2-SH available or released (µmoles)</td>
<td>0.37</td>
<td>0.076</td>
<td>0.011</td>
<td>0.30</td>
<td>0</td>
</tr>
<tr>
<td>Protein that is papain-SH (mg)</td>
<td>1.75</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (mg)</td>
<td>2.04</td>
<td>1.62</td>
<td>0.07</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Specific activity (U mg⁻¹)</td>
<td>0.065</td>
<td>0.058</td>
<td>0.022</td>
<td>0.004</td>
<td></td>
</tr>
</tbody>
</table>

| Experiment 2 | |
|-------------|--------------------------|----------------------|----------------------------------------|----------------------------------|
| Py-2-SH available or released (µmoles) | 0.37 | 0.076 | 0.006 | 0.31 | 0 |
| Protein that is papain-SH (mg) | 1.75 | 0.15 |
| Protein (mg) | 2.04 | 1.65 | 0.00 | 0.14 |
| Specific activity (U mg⁻¹) | 0.065 | 0.062 | 0.027 | 0.005 |

*a Coupling experiments were carried out by reacting samples of activated papain from Sephadex G-25 column with moist cake of 14 ml 50% (v/v) suspension of bead-S-S-2-Py (prep. IV) at pH 4.5 for 30 min at room temperature as described in the EXPERIMENTAL.

*b The amount of Py-2-SH was calculated from the A₃43 measurement.

*c Protein that is papain-SH was calculated from the number of µmoles Py-2-SH x molecular weight of papain (23,000 daltons) (43).

*d Protein was determined from A₂78 measurement. When Py-2-SH was present A₂78 corr was calculated from A₂78 corr = A₂78 - 1.25 x A₃43 (50).

*e Specific activities were determined using the D,L-RAPA assay as described in the EXPERIMENTAL and the A₂78 measurements of protein. The specific activity of the protein in the DTE cleavage supernatant prior to dialysis was calculated using the amount of protein determined at 278 nm after dialysis.

*f Amount of Py-2-SH was estimated by DTE back titration of beads.
in the coupling supernatant (0.058 U·mg⁻¹) and the activated papain from the Sephadex G-25 column (0.065 U·mg⁻¹).

The amount of catalytically active protein in the coupling supernatant was calculated to be 1.57 mg (Table IX). When the supernatant of the DTE cleavage reaction was checked at 278 nm and corrected for the Py-2-SH present, only one quarter of the protein coupled during Py-2-SH release was recovered upon DTE cleavage, as shown in Table VIII.

There was uncertainty in the corrected \(A_{278}\) results of the DTE cleavage supernatant due to the large excess of Py-2-SH present, which was also true in the two coupling experiments of the high thiol titre bead reagent with papain, described previously. Therefore, the DTE cleavage supernatant was dialyzed against \(N_2\)-saturated 50 mM acetate buffer, pH 4.5 (containing 0.15\(\mu\)M NaCl and 1 mM EDTA) until the \(A_{343}\) value dropped to zero, and then the \(A_{278}\) was remeasured. The protein content of the dialyzed DTE cleavage supernatant is given in Table VIII. The amount of protein in the dialyzed cleavage supernatant was 55% of the amount of protein not present in the coupling supernatant. The 0.23 mg protein in the dialyzed cleavage supernatant corresponded to 6 nmol/g of dry beads (0.13 mg/g of dry beads) and represented a percentage coupling of 13%. This same amount of protein was 92% of the degree of coupling indicated by Py-2-SH release.
TABLE IX

DETERMINATION OF THE CATALytICALLY ACTIVE PAPAIN IN COUPLING EXPERIMENTS OF BEAD-S-S-2-Py (PREP. IV) WITH PAPAIN-SH USING THE STANDARD CURVE OF ACTIVATED PAPAIN FROM THE SEPHADEX G-25 COLUMN

<table>
<thead>
<tr>
<th></th>
<th>Coupling supernatant</th>
<th>DTE cleavage supernatant before dialysis</th>
<th>Cleavage supernatant after dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (mg)(^a)</td>
<td>1.57</td>
<td>0.23</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (mg)(^a)</td>
<td>1.64</td>
<td>0.09</td>
<td>0.11</td>
</tr>
</tbody>
</table>

\(^a\)The amount of protein was calculated from D,L-BAPA assay using Figure 9 after the differences in activities of the coupling supernatants and the DTE cleavage supernatants (before and after dialysis) in comparison to the activated papain from the Sephadex G-25 column were corrected for.
The specific activity of the papain in the undialyzed DTE cleavage supernatant was determined at pH 6.0 using the D,L-BAPA assay described in the EXPERIMENTAL (see Table VIII). The resulting specific activity of the papain in the undialyzed cleavage supernatant was 0.022 U·mg⁻¹.

The amount of catalytically active protein in the undialyzed DTE cleavage supernatant was calculated using the A₄₁₀ value from D,L-BAPA assay and the standard curve of activated papain from the Sephadex G-25 column after correcting for the difference in activity of the papain after the DTE cleavage reaction (0.022 U·mg⁻¹) as compared to the activated papain started with (0.065 U·mg⁻¹). The amount of protein in the DTE cleavage supernatant (see Table IX) that was catalytically active was calculated to be 0.23 mg which was the same result as the A₂₇₈ value of the dialyzed DTE cleavage supernatant. The 0.23 mg of catalytically active protein in the undialyzed DTE cleavage supernatant of experiment 1 corresponded to an active protein coupling yield of 0.13 mg/g of dry beads. The active protein coupling yield corresponded to 0.0029 U/g of dry beads.

The specific activity of the protein in the dialyzed cleavage supernatant was 0.004 U·mg⁻¹ (Table VIII). The amount of catalytically active protein in the dialyzed cleavage supernatant was calculated using the A₄₁₀ value of the dialyzed DTE cleavage supernatant from its D,L-BAPA assay and the standard curve of activated papain (Fig. 9).
after correcting for the difference in specific activity between the papain in the dialyzed DTE cleavage supernatant and that of the activated papain from the Sephadex G-25 column (Table VIII). The amount of papain in the dialyzed DTE cleavage supernatant that was calculated to be catalytically active was 0.20 mg (Table IX).

In experiment 2, as shown in Table VIII, there was 4.9-fold excess of bead-S-S-2-Py over papain-SH. Coupling of bead-S-S-2-Py with papain-SH resulted in Py-2-SH release that indicated 8% coupling, whereas, determination of the decrease in protein content suggested 22% coupling. When a 2-Py-S-S-2-Py titration was done on the coupling supernatant, the amount of protein in the coupling supernatant that was calculated to be papain-SH was 1.48 mg. The specific activity of papain in the coupling supernatant was determined in the same manner as for experiment 1 above. The resulting specific activity was 0.062 U·mg⁻¹ (see Table VIII). Then the amount of catalytically active protein in the coupling supernatant was calculated using its \( \lambda_{410} \) value from D,L-BAPA assay and the standard curve of activated papain (Fig. 9), as in experiment 1 above. The amount of papain that was catalytically active was 1.64 mg (Table IX). When the supernatant of the DTE cleavage reaction was checked at 278 nm and corrected for the Py-2-SH present (see Table VIII), none of the protein coupled during Py-2-SH release was indicated
to have been recovered upon DTE cleavage. As in experiment 1, the DTE cleavage supernatant was dialyzed until the $A_{343}$ value dropped to zero, and then the $A_{278}$ was remeasured, which resulted in 0.14-mg protein recovered (see Table VIII). The amount of protein in the dialyzed cleavage supernatant corresponded to 3 nmol/g of dry beads (0.07 mg/g of dry beads) and indicated 8% coupling. This same amount of protein was 36% of the amount of protein not present in the coupling supernatant, and was 93% of the degree of coupling indicated by Py-2-SH release (see Table VIII).

The specific activity of the undialyzed cleavage supernatant was determined to be 0.027 U/mg$^{-1}$ by assay using D,L-BAPA as in experiment 1. The amount of catalytically active protein in the undialyzed DTE cleavage supernatant was calculated from D,L-BAPA assay as in experiment 1 above, and the calculation revealed that 0.09 mg papain was catalytically active (Table IX). The 0.09 mg of catalytically active protein in the undialyzed DTE cleavage supernatant of experiment 1 corresponded to an active protein coupling yield of 0.04 mg/g of dry beads. The active protein coupling yield corresponded to 0.0014 U/g of dry beads.

The specific activity of the protein in the dialyzed DTE cleavage supernatant, as determined by the D,L-BAPA
assay described in the EXPERIMENTAL, was 0.005 U·mg⁻¹. The amount of catalytically active papain in the dialyzed cleavage supernatant was calculated as for experiment 1 above and the amount of catalytically active papain was determined to be 0.11 mg (Table IX).

As shown in Table VIII, the specific activities of the protein in the DTE cleavage supernatants were much smaller than the specific activity of the papain started with in the coupling experiments and in the coupling supernatants. One possible cause of this was the fact that a high concentration of DTE (15 mM) was used in the cleavage reaction. Sluyterman and Wijdenes (54) have shown that DTE causes inactivation of papain owing to autolysis of papain. It was decided that an assay would be done of papain using D,L-BAPA after papain was incubated with 15 mM DTE at pH 6.0 for 40 min to simulate the conditions of the DTE cleavage reaction of the coupling experiments. A standard curve (Fig. 11) was prepared for the assay of papain which had been incubated for 40 min with 15 mM DTE in 0.10 M sodium phosphate buffer, pH 6.0 (containing 1 mM EDTA and 0.3 M KCl). The specific activity of the papain incubated in 15 mM DTE for 40 min prior to being assayed was 0.033 U·mg⁻¹. To compare the activity to that of the assay done of papain which had been incubated with 15 mM DTE for 40 min prior to being assayed, another assay was done using D,L-BAPA for papain in 0.10 M sodium phosphate buffer, pH 6.0 (containing 0.3 M KCl and 1 mM EDTA). A standard curve was
The enzyme solution for the assay was prepared by incubating 0.25 ml of stock papain suspension in 25 ml of 0.10 M sodium phosphate buffer, pH 6.0 (containing 15 mM DTE, 0.3 M KCl and 1 mM EDTA). Then assay was performed as described in EXPERIMENTAL using 0.83 mM D,L-BAPA in the reaction mixture. The assay was carried out at room temperature. Samples were done in duplicate. The line shown is the least-squares line (correlation coefficient 0.9994): \[ A_{410} = (0.0056 \pm 0.0001) (\mu g \text{ papain}) - (0.0011 \pm 0.0005). \] The uncertainties in the slope and y-intercept were calculated as described in Appendix 2.
Figure 11

PAPAIN (μg)

ABSORBANCE
drawn (Fig. 12) and linear regression analysis was done on the data. The specific activity of the papain was calculated to be 0.063 U·mg⁻¹.
Figure 12

Standard Curve of Papain in 0.1 M Sodium Phosphate Buffer, pH 6.0, using D,L-BAPA.

Legend

The enzyme solution for the assay was prepared by dissolving 0.25 ml of stock papain suspension in 25 ml of 0.10 M sodium phosphate buffer, pH 6.0 (containing 0.3 M KCl and 1 mM EDTA). The assay was carried out as described in the EXPERIMENTAL using 0.83 mM D,L-BAPA in the reaction mixture. Samples were done in duplicate. The line shown is the least-squares line (correlation coefficient 0.9996): \( \lambda_{410} = (0.0103 \pm 0.0001)(\mu g \text{ papain}) + (0.0217 \pm 0.0009) \). The uncertainties in the slope and in the y-intercept were calculated as described in Appendix 2.
CHAPTER IV

DISCUSSION

Preparation of High Thiol Titre Long-Arm Polyacrylamide Beads

High thiol titre long-arm polyacrylamide beads

(prepar. I) were prepared by Taylor (49) using the procedure given in Fig. 7. In this study Taylor's beads were titrated with 2-Py-S-S-2-Py followed by DTE back titration. The thiol titre of the beads was 1.01-1.45 \( \mu \)moles SH/g of dry beads, as seen in Table II. When the beads were recycled in 0.1 mM 2-Py-S-S-2-Py followed by DTE back titration, the thiol titre of the beads was 0.96-1.27 \( \mu \)moles SH/g of dry beads, as shown in Table II. When the beads were treated with DTNB the thiol titre of the beads was 1.43-1.84 \( \mu \)moles SH/g of dry beads, and when the DTNB-treated beads were reduced with DTE the thiol titre of the beads was 1.50-1.54 \( \mu \)moles SH/g of dry beads (Table III). These differences in the thiol titres of the beads are thought to be due to excess reagent adhering non-specifically to the beads, even in the pores of the beads. Non-specific adsorption to the beads was thought to have occurred in the work of Chen (2) also. Spectrophotometric
titration of Chen's beads revealed thiol densities in the range 3-7 μeq/g of dry beads (2). However, when Chen (2) carried out radiochemical titration on her bead samples using excess [1-14C]-iodoacetamide of known specific activity, the incorporation of [1-14C]-iodoacetamide was 40-43% of that determined by titration with 2-Py-S-S-2-Py. The differences in the thiol titre results of Chen's beads were thought to be a reflection of the accessibility of the two reagents or, more likely, non-specific adsorption of 2-Py-S-S-2-Py to the polyacrylamide beads, as mentioned above.

Preparation of Low Thiol Titre Long-Arm Polyacrylamide Beads

The synthesis of low thiol titre beads (0.2 ± 0.1 μmoles SH/g of dry beads) was attempted first by decreasing the aminolysis reaction (see Fig. 8) from 3 h (49) to 20 min at 90°C in order to decrease the amount of amino groups. This modification furnished preparations with amino group titres in the range 7.2-13.6 μeq NH₂/g of beads, which was close to the amino titre of Taylor's beads (49), 9.6 μeq NH₂/g of beads. The aminolysis reaction was also done at 60°C for 20 min to attempt to lower the amino titre of the beads, but the resulting amino titre was 7.3 μeq NH₂/g of beads. Since lowering the temperature and time of the aminolysis reaction did not result in sufficient reduction in the amino group titre, it was decided that, instead,
lower amounts of DTSP (see Fig. 8) would be used in the synthesis of the low thiol titre bead reagent. The 4.62 g beads (prep. II), which were synthesized using the aminolysis reaction done at 90°C for 20 min, and which had an amino group titre of 7.2-13.6 μeq NH₂/g of beads, were added to a DMF solution containing more than sufficient DTSP (for a theoretical thiol titre of 2.14 μmoles SH/g of beads) for 4 h and then Ac₂O was added to block the unreacted amino groups. After DTE reduction and 2-Py-S-S-2-Py titration, the thiol titre of the beads was determined to be 1.41-1.54 μmoles SH/g of beads. Since the thiol titre of the beads was too high the amount of DTSP used was lowered to correspond to a theoretical thiol titre of 0.32 μmoles SH/g of beads. The resulting thiol titre of the beads (bead prep. III) was 0.24 μmoles SH/g of dry beads as seen in Table IV. However, with subsequent recycles of the beads in 1 mM 2-Py-S-S-2-Py solution, the thiol titre of the beads increased right up to 1.08 μmoles SH/g of beads, as shown in Table IV. For the amount of DTSP taken, the thiol titres of 0.35, 0.46 and 1.08 μmoles SH/g of beads (Table IV) were impossibly high. The high thiol titres were possibly due to excess 2-Py-S-S-2-Py entering the pores of the beads and adhering non-specifically to the beads, as discussed above, until DTE release. As a precaution, after 2-Py-S-S-2-Py titration the beads were washed exhaustively
and then the wash was checked at 281 nm for 2-Py-S-S-2-Py to make sure the beads were washed properly. Then the beads were recycled in 0.1 mM 2-Py-S-S-2-Py. The thiol titre dropped to 0.25 μmoles SH/g of beads, which was comparable to the theoretical maximum thiol titre of 0.32 μmoles SH/g of beads.

A large scale synthesis of low functional group density bead reagent (prep. IV) was prepared using sufficient DTSP to correspond to 0.24 μmoles SH/g of beads. As seen from Table V, upon recycling the beads in 0.1 mM 2-Py-S-S-2-Py the thiol titre of the beads was 0.20 μmoles SH/g of dry beads, which was close to the maximum theoretical titre. Coupling experiments with papain (which will be discussed later) were done with the beads which contained 0.20 μmoles SH/g of dry beads. Then the beads were recycled in 0.1 mM 2-Py-S-S-2-Py. When the beads were back titrated with DTE, the thiol titre that resulted was 0.40 μmoles SH/g of dry beads. The reason for the thiol titre being so high with subsequent recyclines in 0.1 mM 2-Py-S-S-2-Py is possibly non-specific adsorption of 2-Py-S-S-2-Py to the beads, as discussed above. Titration of the beads with DTNB followed by DTE back titration (see Table VI) gave even higher thiol titres, which supported the theory that excess reagent was adhering to the beads in the pores.
Coupling of High Thiol Titre Bead-S-S-2-Py with Papain-SH

Having been characterized, the high thiol titre bead reagent (see Table II), containing 1.22 μmoles SH/g of dry beads, was used in the mixed disulfide form, bead-S-S-2-Py, in coupling experiments with activated papain-SH. Activated papain for the coupling experiments had a thiol titre of 0.66 moles thiol per mole papain. Coupling experiments of bead-S-S-2-Py with papain, modelled after those of Chen (2) of our lab, showed Py-2-SH which corresponded to 53% and 34% of protein thiol groups in the two experiments (see Table VII). Determination of the decrease in protein content in the coupling supernatant indicated 18% and 34% coupling. When protein was measured in the dialyzed DTE cleavage supernatants the coupling yields were 22 nmol/g of beads and 20 nmol/g of beads, respectively, and the percentage coupling for both experiments was 14%. The 22 nmol/g and 20 nmol/g coupling yields were in the range of coupling results of Chen (2). The coupling yields of Chen's bead coupling experiments with papain were 28 nmol/g and 7 nmol/g, respectively (2). However, the coupling yields of both experiments of this study, and those of Chen (2), were lower than the 10% of theoretical or 200-400 nmol/g coupling yield capacity of macroporous agarose-glutathionyl-S-S-2-Py beads with papain (1). The capacity of agarose-glutathionyl-S-S-2-Py beads for papain (substantially less than 100%) was thought
to have arisen from the inaccessibility of some of the mixed disulfide residues to the papain thiol group due to their location in hindered regions of the gel or, more likely, by steric shielding of some glutathione-2-pyridyl disulfide residues by glutathione-papain disulfide residues (1). The coupling yields of protein in the dialyzed DTE cleavage supernatants were a reduction of up to 4-fold in comparison to the Py-2-SH released in the coupling supernatant (see Table VII). In the work of Chen (2), the coupling yields based on protein present in the DTE cleavage supernatant were also smaller than the coupling yields indicated by Py-2-SH release. In Chen's coupling experiments with activated papain, beads of 2 μeq SH/g of beads were used (2). Chen (2) suggested that functional group densities of 2 μeq/g were excessive with the beads she used. She did a calculation which indicated that if the surface of a 25 μm diameter bead were completely hidden by 50 Å diameter protein molecules only 2.6% of the sites on beads of 2 μeq/g functional group density would be utilized. In this study, similar calculations were done for the beads of 1.22 μeq/g functional group density (see Appendix 1) which were used in coupling experiments with papain-SH. Calculations were carried out for non-porous beads of 25 μm in diameter and for beads of 10 μm in diameter, assuming 74% space occupancy for close packing to determine the "patches" of surface
available per functional group. The number of beads per dry gram was estimated using the manufacturer's water regain data. For beads of 25 μm diameter with a functional group density of 1.22 μeq/g, a "patch" of surface 9.2 × 9.2 Å would be available per functional group. If the surface of a 25 μm diameter bead were completely hidden by 50 Å diameter spherical protein molecules only 4.3% of the sites of 1.22 μeq/g functional group density would be utilized. In the two coupling experiments of this study, using beads containing 1.22 μeq SH/g of dry beads, there was 1.8% and 1.6% utilization of total bead sites. On the basis of the hard-sphere type calculations presented earlier these amounts of site utilization would correspond to 42 and 36% masking of the available surface area of 25 μm diameter beads by 50 Å diameter proteins. For beads of 10 μm diameter with a functional group density of 1.22 μeq/g of beads, a "patch" of 14.6 × 14.6 Å would be available per functional group. If the surface of a 10 μm diameter bead were completely hidden by 50 Å diameter protein molecules just 10.8% of the sites of 1.22 μeq/g functional group density would be utilized. However, for beads of 0.19 μeq SH/g of beads similar calculations (see Appendix I) indicate that for 25 μm diameter beads, a "patch" of surface 23.8 × 23.8 Å would be available per functional group. If the surface of a 25 μm diameter bead were completely hidden by 50 Å
diameter spherical protein molecules 28.8% of the sites of 0.19 μeq/g functional group density would be utilized, which is a much greater proportion of sites utilized than when 1.22 μeq/g functional group density beads were used. For beads of 10 μm diameter, a "patch" of surface 37.4 x 37.4 Å would be available per functional group. If the surface of a 10 μm diameter bead were completely hidden by 50 Å diameter spherical protein molecules, 71.1% of the sites of 0.19 μeq/g functional group density would be utilized. The results for the 10 μm diameter beads containing 0.19 μeq SH/g of beads, above, give even more evidence that low functional group density beads would be preferable for use in coupling experiments to obtain higher coupling yields, because a higher percentage of sites on the beads would be utilized. Whereas, for the high functional group density bead reagent, since there are more functional groups per gram of beads, there is a higher chance that the thiol groups will interact with each other through thiol-disulfide interchange reactions to form bead-S-S-bead with the result that less coupling of bead-S-S-2-Py with papain-SH would occur. For this reason, in this study, coupling experiments with papain-SH were also done using the low functional group density beads which contained 0.19 μeq SH/g of beads (Table VIII).
Coupling of Low Thiol Titre Bead-S-S-Py with Papain-SH

The activated papain used in the coupling experiments with low thiol titre bead-S-S-2-Py (0.19 μeq-S-S-2-Py/g of dry beads) had a thiol content of 0.85 moles thiol per mole of papain, which was better than the literature range of 0.4-0.6 moles thiol per mole of papain (8). One reason that 2-Py-S-S-2-Py titration of activated papain did not yield results of one mole thiol per mole of papain was possibly because of some noncovalent association of 2-thiopyridone with the protein. Harris and Hodgins (55) observed unpredictable results for the formation of Py-2-SH during the reaction of protein thiol groups with 2-Py-S-S-2-Py in their experiments with BSA. Their study revealed that Py-2-SH associated noncovalently with reduced or unreduced proteins to cause a hypsochromic shift from 343 to 302 nm (55). A more important reason that 2-Py-S-S-2-Py titration of activated papain did not yield results of one thiol per mole papain is the fact that activated papain always contains some inactive protein which is not activated by treatment with thiol or KCN and which may be a sulphonic acid of papain (1).

The activated papain in this study was assayed for catalytic activity using the substrate D,L-BAPA by the procedure of Arnon (45) with some modifications. The activator used in the assay was DTE (56) instead of
cysteine. The assay was done using smaller volumes than in Arnon's procedure (45) to increase the sensitivity and the assay was carried out at pH 6.0 because the optimum pH for N-α-benzoyl-L-arginine-β-nitroanilide (L-BAPA), the true substrate, was in the pH range of 6.0-6.4 (57). The D form of D,L-BAPA, N-α-benzoyl-D-arginine-β-nitroanilide (D-BAPA) is a competitive inhibitor in the assay of papain (58), and was also found to be a competitive inhibitor in the assay of trypsin (59). The inhibitor constant, $K_i$, for D-BAPA in the assay of papain was determined to be 2.3 mM by Tokura et al. (58). By modifying the procedure of Arnon (45), the sensitivity of the assay of activated papain with D,L-BAPA was increased approximately 10-fold, as seen by comparing Fig. 9 with the assay results of Arnon's study of papain (45).

When coupling experiments of activated papain were carried out with the low functional group density bead reagent (0.19 μmoles SH/g of dry beads), the Py-2-SH release in the coupling supernatants was 15% and 8% of protein thiol groups (Table VIII). Determination of the decrease in protein content in the coupling supernatant indicated 24% and 22% coupling. For the coupling supernatant of experiment 1, protein present as papain-SH (estimated from 2-Py-S-S-2-Py titration) and protein which was absent as a result of coupling (estimated from the
Py-2-SH content) could be added to give 1.73 mg. This amount of protein was very close to the 1.75 mg of papain-SH used in the experiment. When the analogous estimations and calculations were carried out for experiment 2, the total amount of protein as papain-SH was 1.63 mg, which was less than the 1.75 mg of papain-SH used in the coupling experiment. When protein was measured in the dialyzed cleavage supernatants the percentage coupling was 13% and 8% for the 2 experiments (Table VIII). When the protein in the dialyzed cleavage supernatant of experiment 1 was added to the amount of papain-SH in the coupling supernatant, the sum (1.71 mg) was close to the amount of papain-SH used in the experiment (1.75 mg). When the analogous calculations were done for experiment 2, the sum of papain-SH (1.62 mg) was less than the amount of papain-SH used in the experiment (1.75 mg). The coupling yields of the experiments were 6 nmol/g of dry beads, and 3 nmol/g of dry beads. The coupling yields (6 nmol/g and 3 nmol/g of dry bead, respectively), of this study were slightly lower than those of Chen's bead coupling experiments with papain (2) (mentioned above), and were much lower than the coupling yields in the experiments with papain carried out by Brocklehurst et al. (1) using agarose-glutathionyl-S-S-2-Py beads (discussed above).

The coupling yields were in close agreement to the
amounts of protein in the dialyzed and undialyzed cleavage supernatants calculated from D,L-BAPA assay and the standard curve of activated papain (Fig. 9), given in Table IX. The fact that the amounts of protein in the dialyzed cleavage supernatants, calculated from D,L-BAPA assay and Fig. 9, support the $A_{278}$ values of protein in the dialyzed DTE cleavage supernatants is good because both the $A_{278}$ values in the dialyzed cleavage supernatants and the $A_{343}$ values for Py-2-SH released in the coupling supernatants were low. When the protein in the coupling supernatants and the undialyzed or dialyzed cleavage supernatants that was calculated to be catalytically active (Table IX) were added up, the sum was close to the amount of papain-SH used in the experiment (1.75 mg) (Table VIII). Both coupling yields were equal within 8% experimental error to the coupling yields indicated by Py-2-SH release (Table VIII). This gives evidence that lowering the functional group density improves the coupling results. However, even with lowering of the functional group density of the beads, the coupling yields were not increased. The 6 nmol/g and 3 nmol/g of beads coupling yields of protein indicated that only 3.2% and 1.6% total bead sites were utilized. This result, in turn, indicated that on the basis of hard sphere calculations presented earlier that these amounts of site utilization would correspond to 11% and 6% masking of the available surface
area of 25 μm diameter beads by 50 Å diameter proteins.

One reason for the low coupling yields in the coupling experiments with papain, other than possibly some adhesion of enzyme to the beads, might be enzyme leakage (60), along with some non-specific adherence of Py-2-SH on the beads. When Lasch and Koelsch (60) immobilized the enzyme leucine aminopeptidase to the conjugate Sepharose-glutathione 5-(2-nitrobenzoic acid) disulfide which resulted in release of the chromophoric thiolate ion by replacement with the thiol groups of the enzyme, there was 10-30% leakage of the totally immobilized enzyme, mainly due to steric hindrance. Lasch and Koelsch (60), consistently observed that with a single-point-attached enzyme, (a) a certain amount of the enzyme was released with a time course that followed quite closely a monoexponential process, (b) the remainder was stable in the time range studied, and (c) the absolute value of the detached and the remaining portion of the enzyme varied unpredictably from preparation to preparation and was not correlated with the velocity of the cleavage reaction. They concluded that there were (at least) two populations of the covalently attached enzyme, one liable to solvolysis, the other resistant to cleavage. In the study of Lasch and Koelsch (60), the proportions of the two populations of the covalently attached enzyme varied with the extent of cross-linking during CNBr-activation.
When the specific activity of the protein in the DTE cleavage supernatants of the coupling experiments with the lower functional group density bead reagent were determined (Table VIII), the specific activities of the 2 cleavage supernatants were 0.022 U·mg⁻¹ and 0.027 U·mg⁻¹, respectively. The specific activity values of the papain in the cleavage supernatants, above, show that the papain that was coupled retained some of its catalytic activity. However, the values were much lower than the specific activity of the activated papain started with in the coupling experiments, which was 0.065 U·mg⁻¹ (see Table VIII and Fig. 9). This could be due to one of the reasons, or a combination of them, below.

In the coupling experiments with papain (Table VIII), 15 mM DTE was used in the cleavage reaction. Dithioerythritol has been shown to cause inactivation of papain owing to autolysis of papain (54). In the study done by Sluyterman and Wijdenes, it was found that incubation of papain with 3-17 mM DTE and dithiothreitol at pH 8.5 caused inactivation of papain due to autolysis (54). Inactivation was not observed when equivalent concentrations of cysteine, mercaptoethanol, or 2,3-dimercaptoethanol were used as activators (54). Since the specific activities of papain in the cleavage supernatants of the coupling experiments (Table VIII) were much lower than the specific activity of
the papain started with, an assay was done of papain that was incubated with 15 mM DTE for 40 min to mimic the conditions of the DTE cleavage reaction of the coupling experiments. In the coupling experiments the total time for the DTE cleavage reaction was 30 min. Then the DTE cleavage supernatants were read at 278 nm and 343 nm prior to assaying them with D,L-BAPA. Thus, 40 min was the time chosen for incubation of the papain in, prior to assaying it with D,L-BAPA (Fig. 11). The results of the assay were compared with the specific activities of papain in the DTE cleavage supernatants, and to the assay done of papain in 0.10 M sodium phosphate buffer, pH 6.0 (containing 0.3 M KCl and 1 mM EDTA) (Fig. 12). The specific activity of the papain that had been incubated for 40 min in 15 mM DTE prior to being assayed was 0.033 U·mg⁻¹, which was close but slightly higher than the values of the specific activities of the protein in the DTE cleavage supernatants (Table VIII). The reason that the specific activities of the protein in the DTE cleavage supernatants were lower than the specific activity of the papain incubated in 15 mM DTE for 40 min was probably because the papain in the DTE cleavage supernatants had been treated with 5 mM DTE for 15 min to activate it, then it was put through a Sephadex G-25 column to get rid of the excess activator, whereas, the papain incubated for 40 min in 15 mM DTE (Fig. 11) had not been. The papain
in the DTE cleavage supernatants had more exposure to DTE. The DTE may have caused more inactivation of the papain in the DTE cleavage supernatants. The specific activity of the papain which had been incubated in 15 mM DTE for 40 min (Fig. 11) was much lower than that of the papain in 0.10 M sodium phosphate buffer, pH 6.0 (containing 0.3 M KCl and 1 mM EDTA) without DTE until the assay (0.063 U·mg⁻¹) (Fig. 12), which was expected since DTE has been shown to cause inactivation of papain (54).

When the dialyzed DTE cleavage supernatants were assayed for activity using D,L-BAPA, the specific activities for the 2 experiments (Table VIII) were 0.004 and 0.005 U·mg⁻¹. The values were low possibly due to the loss in activity because 15 mM DTE was present for a longer time than in the undialyzed cleavage supernatants, so there was more inactivation of the protein, although autolysis by itself for three days is also a reason why more inactivation occurred in the dialyzed cleavage supernatants than in the undialyzed cleavage supernatants. In future coupling experiments with papain, although DTE (56) is a potent activator, since inactivation occurs owing to autolysis, it is suggested that a lower concentration of DTE be used for the cleavage reaction.

Another possible reason that the specific activities of papain in the DTE cleavage supernatants were much lower than
those of the papain used in the coupling experiments (Table VIII), was the ionic strength of the buffer. Sorrentino et al. (61) revealed that reactions of monomeric enzymes with polyelectrolytic substrates were highly modulated by salt, whereas those catalyzed by dimeric enzymes were not. In their study of monomeric papain (61), at a pH 8.5 and a temperature of 37°C, at 0.3 M sodium chloride, the specific activity of monomeric papain dropped to 50% of the value it had at zero concentration of sodium chloride. At pH 7.5, the specific activity of monomeric papain was also affected by the ionic strength (61). Sorrentino et al. (61) found that the optimum activity of monomeric and dimeric papain occurred at salt concentrations close to zero. They postulated that the reason for this was the fact that in the pH region where their experiments were performed, the positively charged groups of the enzyme were solvated by the negative solute ions (61).

Although in this study a polyelectrolytic substrate was not used, ionic strength effect might still have contributed to the loss in specific activity of the papain in the DTE cleavage supernatants in comparison to that of the papain started with (Table VIII). In the DTE cleavage reaction 0.10 M sodium phosphate buffer, pH 6.0 (containing 0.3 M KCl and 1 mM EDTA) was used, whereas prior to the cleavage reaction N$_2$-saturated 50 mM acetate buffer, pH 4.5 (con-
taining 0.15 M NaCl and 1 mM EDTA) was used, so the salt concentration was 2-fold greater in the DTE cleavage reaction. The decrease in the specific activities of the protein in the dialyzed cleavage supernatants of the coupling experiments (Table VIII) in comparison to those in the undialyzed DTE cleavage supernatants might also be explained partly by the effect of ionic strength. The cleavage supernatants were in 0.10 M sodium phosphate buffer, pH 6.0 (containing 0.3 M KCl and 1 mM EDTA) and were dialyzed against N₂-saturated 50 mM acetate buffer, pH 4.5 (containing 0.15 M NaCl and 1 mM EDTA). The ionic strength would have had a longer time (3 days) to effect the papain of the dialyzed cleavage supernatants, than to effect the papain in the undialyzed cleavage supernatants. In future coupling experiments, it is suggested that the ionic strength in the buffers be significantly lowered.

For future coupling experiments with papain-SH, another substrate, for example, L-BAPA, should be used to assay papain instead of D,L-BAPA because D-BAPA is a competitive inhibitor in the assay (58), as discussed above. In addition, L-BAPA is soluble in water, whereas, D,L-BAPA is only slightly soluble in water (58).

Before concluding this discussion, a comparison will be made of the papain coupling yields of this study with some papain immobilization results which did not employ covalent
chromatography (62-65).

In 1974, Goldstein et al. (62) immobilized papain on polyisonitrile-nylon in the presence of acetaldehyde and an excess of acetate to effect binding via the protein amino groups (mainly via the lysine residues). The total bound protein was 69 mg/g of support and the active protein bound was 32 mg/g of support. In the same study, Goldstein et al. (62) coupled papain to polyisonitrile-nylon in the presence of acetaldehyde and Tris, to effect binding through the carboxyl groups of the protein. Coupling experiments via carboxyl groups of protein on polyisonitrile-nylon, yielded 47 mg/g of support and 14 mg were active. In addition, Goldstein et al. (62) immobilized papain on diazotized polyaminoaryl-nylon mainly through azo bonds with tyrosine residues. The coupling experiments yielded 86 mg/g of support and the active protein bound was 30 mg/g of support. All 3 coupling methods of Goldstein et al. (62), above yielded greater amounts of papain bound than in this study. In this study, coupling experiments with the high functional group density reagent yielded total bound papain of 0.51 mg/g and 0.46 mg/g of dry beads, respectively. In the coupling experiments with the low functional group density bead reagent, the totally bound protein yields were 0.13 mg/g and 0.07 mg/g of dry beads, respectively. The catalytically active protein yields were 0.13 mg/g and a 0.04 mg/g of dry
beads, respectively. The yields of this study were much lower than those of the study of Goldstein et al. (62), above, mainly due to area available for packing of spheres on the support. In this study beads of 10-37 \( \mu \)m diameter were used. However, in the study of Goldstein et al. (62), the diameter of the spherical nylon powder particles was in the range 0.2-0.7 \( \mu \)m which made the chemically modified nylon well suited for the covalent fixation of enzyme due to the favorable area-to-weight ratio of the support (63). However, the use of fine nylon powders for large-scale conversions in packed-bed reactors was limited by their high resistance to flow. Therefore, in 1976, Goldstein et al. (63) used derivatives of nylon fabric sheet disks for enzyme immobilization because nylon fabric sheets and nylon fibers have low hydrodynamic resistance. When papain was immobilized on acylhydrazide derivatives of nylon fabric sheets, the total bound protein was 0.35 mg/g of support and the active bound protein was 0.15 mg/g of support (63). Immobilization of papain on aminoaryl derivatives of nylon fabric sheets yielded 0.52 mg total protein bound/g of support and the active bound protein was 0.21 mg/g of support. The results, above, were not far away from the coupling yields of this study (given above). However, the results were low as compared to those from coupling protein to similarly derivatized nylon powders with papain.
The decrease in binding capacity that accompanied the change in support geometry from fine spherical particles of nylon powder (0.2-0.7 μm in diameter) to fibrous nylon filaments of mean diameter 125 μm was determined by calculations to be related to the decrease in available surface area (63). In the same study, Goldstein et al (63) immobilized papain on polyacrylamide-nylon grafts to try to increase the protein-binding capacity. It was thought that the space available for the random close packing of spheres around a fibrous filament could theoretically be enlarged by growing polymeric side chains out of the surface, and three-dimensional packing would be allowed (63). When papain was immobilized on acylhydrazide derivatives of polyacrylamide-nylon grafts, the total bound protein was 2.64 mg/g of support, of which 2.16 mg were active protein. When papain was immobilized on aminoaryl derivatives of polyacrylamide-nylon grafts the total bound protein was 2.16 mg/g of support, of which 1.53 mg were active protein. The results of papain immobilization on derivatives of polyacrylamide-nylon grafts, above, showed that the protein-binding capacity was increased by immobilization on polyacrylamide-nylon grafts as opposed to ungrafted nylon (63). In addition, there was higher retention of biological activity and enhanced thermal stability by protein bound on polyacrylamide-nylon grafts (63).
Polyacrylamide-nylon graft copolymers were also used to immobilize papain by using an azide method and a glutaraldehyde method (64). In 1979, when Beddows et al. (64) immobilized papain on polyacrylamide-nylon graft copolymers using the azide method, the yield was 1.3 mg active enzyme/g of support. When the glutaraldehyde method was used, 1.5 mg active enzyme/g of support resulted (64).

In 1981, papain was immobilized through its amino groups on an alginic acid-polyacrylamide copolymer (65). Although a large number of different supports had been used for immobilization of enzymes, Panduranga Rao et al. (65) pointed out that in those methods the contact surface of enzyme and matrix was small so there was only a slight possibility of multicenter binding of enzyme with the matrix. The alginic acid-polyacrylamide copolymer system was used for enzyme immobilization to try and eliminate the small contact surface of enzyme and matrix because the carboxyl functional groups of alginic acid and the amide groups of polyacrylamide could be converted to their corresponding azides, which would provide more sites for the binding of enzyme. When papain was immobilized on an alginic acid-polyacrylamide copolymer, the enzyme bound, as estimated by the ninhydrin method, was 236 mg/g of support and the activity was 141 U/g using N-benzoyl-L-arginine ethyl ester as the substrate (65). By weight difference, the enzyme bound was 215 mg/g of
support (65). Although the results of protein binding to alginic acid-polyacrylamide copolymers (65), above, were much higher than the results of this study, it must be emphasized that the method of immobilization was different. Papain was immobilized on alginic acid-polyacrylamide due to multiple attachment of the enzyme molecules on the carrier (65), whereas, in this study, papain was immobilized on long-arm thiolated polyacrylamide beads through single point attachment.

In concluding this discussion, although there are problems which must be overcome, the positive results of the coupling experiments are encouraging. The results of coupling of the lower thiol titre bead reagent (Table VIII) with papain are especially encouraging since the coupling indicated by Py-2-SH release was approximately equal to the protein isolated in the dialyzed cleavage supernatant, and since the papain in the undialyzed and dialyzed cleavage supernatants was catalytically active. Evaluation of the bead reagents in the isolation of cell membrane protein can be encouraged if the problem of increasing thiol titres upon recycling of the beads in 2-Py-S-S-2-Py is overcome by a method that would wash away the possible non-specifically adsorbed reagent on the beads (and in the pores), without removing the covalently-bonded reagent.
CHAPTER V

SUMMARY AND FUTURE ENDEAVOURS

In this study the synthesis, characterization, and evaluation of a long-arm thiolated polyacrylamide bead reagent was carried out. The bead reagent was prepared from beaded polyacrylamide (≤400 mesh, 10-37 μm diameter, 1800 dalton inclusion limit) using the spacer Jeffamine ED-600 (length 70-100 Å) via aminolysis, following by thiolation using DTSP and reduction using DTE. Beads of high and low functional group densities were synthesized. The thiol titres of the beads were determined spectrophotometrically using DTNB and/or 2-Py-S-S-2-Py, followed by DTE back titration. The thiol titre of the high functional group density beads was in the range of 0.96-1.84 μeq/g of dry beads and the thiol titre of the low functional group density beads was in the range of 0.10-0.57 μeq/g of dry beads. The beads were characterized using coupling experiments with the sulfhydryl protease papain after their conversion to the mixed disulfide form bead-S-S-2-Py by titration with 2-Py-S-S-2-Py.

In the two coupling experiments with papain that were performed with the high functional group density
bead reagent (1.22 μmoles SH/g of dry beads), there was a 8.5- and 9.1-fold excess of bead-S-S-2-Py over papain-SH. Thiopyridone release exceeded coupled protein released by reductive cleavage by up to 4-fold. When protein was measured in the dialyzed DTE cleavage supernatant, the percentage coupling was 14% in the two experiments, and the coupling yields were 22 nmol/g of dry beads and 20 nmol/g of dry beads. The coupling yields indicated that 1.8% and 1.6% of the total bead sites were utilized.

In the coupling experiments with the low functional group density bead reagent (0.19 μmoles SH/g of dry beads), there was a 4.9-fold excess of bead-S-S-2-Py over papain-SH. Thiopyridone release corresponded to 15% and 8% of protein thiol groups. When protein was measured in the dialyzed cleavage supernatants the percentage coupling was 13% and 8%, and the coupling yields were 6 nmol/g of dry beads and 3 nmol/g of dry beads. The coupling yields were in close agreement with the protein in the undialyzed and dialyzed cleavage supernatants that were calculated from assays with D,L-BAPA. Thiopyridone release was approximately equal to coupled protein released by reductive cleavage, which revealed that use of the low thiol titre bead reagent was preferred over that of the high thiol titre bead reagent in coupling experiments with papain-SH. However, coupling yields were still low. The coupling yields indicated that only 3.2% and 1.6% of the total
bead sites were utilized. When the cleavage supernatants were assayed for papain catalytic activity with D,L-BAPA, before and after dialysis, the specific activities of papain in the cleavage supernatants before dialysis were 0.022 U·mg⁻¹ and 0.027 U·mg⁻¹. The specific activities of the papain in the cleavage supernatants after dialysis were 0.004 U·mg⁻¹ and 0.005 U·mg⁻¹. The above specific activities for the protein in the cleavage supernatants, before and after dialysis, demonstrate that even after immobilization of papain and reductive cleavage the papain remained catalytically active.

In regard to future experiments, it would be useful to determine the thiol titre of the bead samples by radio-chemical titration using excess [1⁻¹⁴C]-iodoacetamide of known specific activity as in the work of Chen (2). The results would be compared with the thiol titres determined spectrophotometrically by 2-Py-S-S-2-Py titration, since non-specific adsorption of 2-Py-S-S-2-Py may have occurred, as evidenced by increasing thiol titres of the low functional group density beads upon recycling in 2-Py-S-S-2-Py solution followed by DTE back titration.

As far as coupling experiments with papain, it would be useful to try to couple bead-SH with papain-S-S-2-Py and compare the results with those obtained in coupling bead-S-S-2-Py with papain-SH. The results of Chen's study
(2) revealed that the coupling experiments of BSA-S-S-2-Py with bead-SH were much more successful than those achieved by coupling bead-S-S-2-Py with BSA-SH. However, one problem that might arise with coupling of bead-SH with papain-S-S-2-Py is that since the thiol beads are reducing, they might promote a premature elution of the coupled protein by forming an internal disulfide bridge, especially if the degree of substitution is high enough (22). Another problem that might arise in coupling bead-SH with papain-S-S-2-Py is that bead-SH might couple with Py-2-SH which might have adhered to the papain non-covalently when the activated papain was titrated with 2-Py-S-S-2-Py to form papain-S-S-2-Py (55).

As far as coupling the beads with other enzymes, it would be interesting to attempt to couple the beads with bovine kidney sulfhydryl oxidase to see if indeed renal sulfhydryl oxidase is only bound by cysteine-containing substrates as was discussed by Schmelzer et al. (30). Renal sulfhydryl oxidase activity was not bound by a sulfhydryl cellulose column prepared according to the procedure of Feist and Danna (31), but was bound to cysteinylsuccinamidopropyl-glass (30), as discussed in the INTRODUCTION.

The long-range goal of this project is the isolation of membrane proteins, and since coupling experiments of
the beads with papain-SH were successful, coupling experiments with cell membrane glycoproteins can be encouraged.
APPENDIX 1

A. CALCULATION OF THE FUNCTIONAL GROUP DENSITY ON BEAD SURFACES (2)

(a) For spherical beads of 25 \( \mu \)m diameter

\[
\text{Area per bead} = 4\pi r^2 = 1.96 \times 10^{-9} \text{ m}^2
\]
\[
= 1.96 \times 10^{11} \text{ Å}^2
\]

\[
\text{Volume per bead} = \frac{4\pi r^3}{3} = 8.18 \times 10^{-5} \text{ m}^3
\]

1 g of dry beads results in 3.5 ml of packed beads and of this volume 74% or 3.5 x
\[
\frac{74}{100} \times 10^{-6} = 2.59 \times 10^{-6} \text{ m}^3
\]

would be due to the beads themselves, assuming close packing of spheres.

Beads per dry g = \( \frac{\text{volume of beads}}{\text{volume per bead}} \)

\[
= 3.17 \times 10^8
\]

Total surface area per dry g of beads

\[
= \text{beads per dry g} \times \text{area per bead}
\]
\[
= 3.17 \times 10^8 \times 1.96 \times 10^{-9} \text{ m}^2
\]
\[
= 6.22 \times 10^{-1} \text{ m}^2
\]
\[
= 6.22 \times 10^{19} \text{ Å}^2
\]

(i) 1.22 \( \mu \)eq functional groups per g of beads corresponds to 1.22 \( \times 10^{-6} \) x 6.02 x 10\(^{23}\)

\[
= 7.3 \times 10^{17} \text{ sites per g}
\]

Area per site = \( \frac{\text{total surface area per/g beads}}{\text{# sites per g}} \)

\[
= \frac{6.22 \times 10^{19} \text{ Å}^2}{7.3 \times 10^{17} \text{ sites}}
\]
\[
= 85.2 \text{ Å}^2 \text{ site}^{-1}
\]

or a "patch" 9.2 Å x 9.2 Å.
1.22 μeq functional group per dry g corresponds to 7.3 x 10^{17} sites per g, and if these sites are on the bead surface this corresponds to an area per site of 85.2 Å^2 or a "patch" of 9.2 x 9.2 Å

(ii) 0.19 μeq functional groups per dry g of beads would similarly correspond to 1.1 x 10^{17} sites per g, and if these sites are on the bead surface this corresponds to an area per site of 565.4 Å^2 or a "patch" of 23.8 x 23.8 Å

(b) Similar calculations, as above, were done on spherical beads of 10 μm diameter.

Area per bead = \(3.14 \times 10^{-10}\) M^2

\[= 3.14 \times 10^{10} \text{ Å}^2\]

Volume per bead = \(5.24 \times 10^{-16}\) M^3

Beads per dry g = \(\frac{2.59 \times 10^{-6}}{5.24 \times 10^{-6}}\)

\[= 4.94 \times 10^9\]

Total surface area per dry g = 1.55 M^2

\[= 1.55 \times 10^{20} \text{ Å}^2\]

(i) 1.22 eq functional groups per dry g correspond to 7.3 x 10^{17} sites per g, and if these sites are on the bead surface this corresponds to an area per site of 212 Å^2 or a "patch" of 14.6 x 14.6 Å

(ii) 0.19 μeq functional groups per dry g would correspond to 1.1 x 10^{17} sites per g, and if these sites are on the bead surface this corresponds to an area per site of 1396 Å^2 per site or a "patch" 37.4 x 37.4 Å

B. CALCULATION OF PACKING OF SPHERICAL PROTEIN MOLECULES ON SURFACES OF SPHERICAL BEADS (2)

A spherical protein of 50 Å diameter (40-50,000 daltons) would have a cross-sectional area of 1964 Å^2 (πr^2) and if this area is taken as a "projection" of such a globular protein packed around a bead:

(a) (i) For spherical beads of 25 μm diameter the maximum amount of protein per g of dry beads
would be 53 nmol, which for beads with 1.22 μeq/g of surface functional groups

represents \( \frac{85.2}{92} \times 100 = 4.3\% \) utilization

of bead sites at saturation, for mono-attachment of each protein.

(ii) Similarly for spherical beads of 25 μm diameter, the maximum amount of protein per g of dry beads would be 53 nmol, which for beads with 0.19 μeq/g of surface functional μeq/g of surface functional groups represents 28.8% utilization of bead sites at saturation, for mono-attachment of each protein molecule.

(b) (i) For spherical beads of 10 μm diameter with 1.22 μeq/g of surface functional groups, the analogous calculation indicates 10.8% utilization of bead sites at saturation.

(ii) Similarly for spherical beads of 10 μm diameter with 0.19 μeq/g of surface functional groups, the analogous calculation indicates 71.1% utilization of bead sites at saturation.
APPENDIX 2

CALCULATION OF THE UNCERTAINTY IN SLOPES AND Y-INTERCEPTS

After linear regression analysis of Figures 9 - 12 was done, the uncertainty in the slope $\sigma_m$ and in the y-intercept $\sigma_b$ were calculated as follows:

$$\sigma_m = \left[ \frac{N}{N \sum x^2 - (\Sigma x)^2} \right]^{1/2} \sigma_y$$

$$\sigma_b = \left[ \frac{\Sigma x}{N \sum x^2 - (\Sigma x)^2} \right]^{1/2} \sigma_y$$

where $\sigma_y = \left( \frac{S}{N-1} \right)^{1/2}$, $S$ is equal to $\Sigma (y_i - \bar{y}_i)^2$, and $\bar{y}_i$ is the best $y$ for a given $x$, $i = 1 \longrightarrow N$. $N$ is the total number of points.
REFERENCES


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