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## Tryptidase: A Novel Trypsin-Like Enzyme from Rat Skin. Isolation and Characterization

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TRYPTIDASE:  
A NOVEL TRYPSIN-LIKE ENZYME FROM RAT SKIN.  
ISOLATION AND CHARACTERIZATION

by  
Vincent J. Braganza, s.j.

A Dissertation Submitted to the Faculty  
of the Graduate School of Loyola University of Chicago  
in Partial Fulfillment of the Requirements for  
the Degree of Doctor of Philosophy

August

1987

Two roads divided in the woods,  
I took the one less traveled by,  
And that has made the difference.

-Robert Frost-

## DEDICATION

I dedicate this dissertation lovingly to:  
my mother, in respect and admiration for her  
industry at dress designing and  
tailoring that made it possible  
for me to be today where I am;  
my father, with proud appreciation for his  
high thinking and simple living,  
and for letting his actions and  
his heart speak louder than  
his words.

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## VITA

The author, Vincent Joaquim Braganza, is the son of Santurnino Joaquim Braganza and Girzalina Maria Braganza. He was ordained a priest of the Jesuit order on 25 April 1981.

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In September, 1987, he will assume a faculty position at St. Xavier's College, Ahmedabad, in the Department of Biochemistry.

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## LIST OF ABBREVIATIONS

|              |  |
|--------------|--|
| $M_r$        | molecular weight   |
| PA           | plasminogen activator  |
| BAEE         | N-benzoyl-L-arginine ethyl ester   |
| ATEE         | N-acetyl-L-tyrosine ethyl ester  |
| PAB          | p-aminobenzamidine coupled with 6-aminohexanoic acid coupled with Sepharose 4B |
| Con A        | concanavalin A coupled to agarose  |
| NPGB         | p-nitrophenyl-p'-guanidinobenzoate   |
| MUGB         | methylumbelliferyl guanidinobenzoate   |
| Trasylol     | basic pancreatic trypsin inhibitor   |
| TLCK         | tosyl lysine chloromethylketone  |
| TPCK         | tosyl phenylalanine chloromethylketone   |
| Z-PCK        | benzyloxycarbonyl phenylalanine chloromethylketone                             |
| p-CMB        | p-chloromercuribenzoate  |
| DFP          | diisopropylphosphofluoridate   |
| LBTI         | lima bean trypsin inhibitor  |
| SBTI         | soybean trypsin inhibitor  |
| OMTI         | ovomucoid trypsin inhibitor  |
| BANA.HCl     | benzoyl arginine naphthylamide hydrochloride                                   |
| BAA.HCL      | benzoyl arginine amide hydrochloride   |
| TAME         | tosyl arginine methyl ester  |
| BAPNA (BAPA) | benzoyl arginine p-nitroanilide  |
| AGLME        | acetyl-glycyl-lysyl methyl ester   |

|                                      |  |
|--------------------------------------|--|
| KPTI                                 | Kunitz pancreatic trypsin inhibitor  |
| EDTA                                 | ethylenediamine tetraacetic acid   |
| A-Gly-Gly-PAB                        | Agarose-glycyl-glycyl-p-aminobenzamidine                                       |
| BAME                                 | benzoyl arginine methyl ester  |
| BSA                                  | bovine serum albumin   |
| DTT                                  | dithiothreitol   |
| BIS                                  | N,N'-methylene-bis-acrylamide  |
| TEMED                                | N,N,N',N'-tetramethylethyldiamine  |
| PAG-plates                           | precast polyacrylamide gels  |
| phos-EDTA-7.5                        | 0.025 M sodium phosphate containing 0.001 M EDTA, pH 7.5                       |
| phos-EDTA-1 M NaCl-7.0               | 0.025 M sodium phosphate containing 0.001 M EDTA, 1 M NaCl, pH 7.0             |
| phos-EDTA-0.5 M NaCl-6.0             | 0.025 M sodium phosphate containing 0.001 M EDTA, 0.5 M NaCl, pH 6.0           |
| phos-Na <sub>3</sub> -0.5 M NaCl-6.0 | 0.025 M sodium phosphate containing 0.02% Na <sub>3</sub> . 0.5 M NaCl. pH 6.0 |
| TFA                                  | trifluoroacetic acid   |
| TCA                                  | trichloroacetic acid   |
| S-2288                               | H-D-Ile-Pro-Arg-pNA  |
| S-2238                               | H-D-Phe-pip-Arg-pNA  |
| Chromo. PL                           | CBZ-Gly-Pro-Arg-pNA  |
| Spec. Ca                             | Tos-Lys-(Cbo)-Pro-Arg-pNA  |
| -pNA                                 | p-nitroanilide   |
| Bz                                   | benzoyl  |
| Tos                                  | tosyl  |
| pip                                  | piperidyl  |
| CHG                                  | cyclohexylglycine  |

|      |                   |
|------|-------------------|
| HHT  | hexahydrotyrosine |
| Nleu | norleucine        |
| CHA  | cyclohexylalanine |
| Z    | benzyloxycarbonyl |

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## CHAPTER I

### INTRODUCTION

#### A. Classes of Proteases

The field of proteases covers enzymes that are capable of cleaving proteins and peptides. Within this class there are sufficiently significant differences between groups of enzymes to allow for their further classification. This classification has recently been reviewed by McDonald (1985), and is briefly summarized below.

Broadly speaking, proteases as a term, is reserved for a more general use to refer to both endopeptidases (proteinases) and exopeptidases (peptidases). The former comprise that class of proteases that have been shown to cleave an internal bond in a polypeptide chain away from its terminal end. Exopeptidases on the other hand, need to have a free charged group and can cleave at or near the N-terminal or C-terminal end of a protein or peptide. This primary classification of proteases references their specificity.

An additional specificity-based classification is also applied to exopeptidases. Thus aminopeptidases, dipeptidyl peptidases and tripeptidyl peptidases attack the N-terminal of the substrate and remove one or two or three amino acids at a time, respectively. Similarly, one can

have carboxypeptidases and peptidyl dipeptidases which attack the C-terminal end and remove one amino acid at a time or two amino acids at a time, respectively. There are also dipeptidases that are restricted to cleaving unsubstituted dipeptides, and tripeptidases that can remove an amino acid from either end of an unsubstituted tripeptide. A new class called omega peptidases has also been defined (McDonald and Barret, 1985) and is made up of enzymes which are capable of removing N-terminal residues that lack a free alpha-amino group, or C-terminal residues that lack a free alpha-carboxyl group. This class also includes exopeptidases that hydrolyze isopeptide bonds and other peptide bonds that do not link alpha-amino and alpha-carboxyl groups.

Within each of these two broad classes, endopeptidases and exopeptidases, further subdivisions have been attempted that are based on the realization that all proteases utilize one of four catalytic mechanisms (Hartley, 1960). Amendments to this original scheme have been proposed for naming and classifying endopeptidases (proteinases) (Barrett, 1980a). These define the following five classes: a) serine proteinases (EC 3.4.21; diisopropylphosphofluoridate (DFP)-sensitive, active at pH 7-9), b) cysteine proteinases (EC 3.4.22; acetate-sensitive, active at pH 2-7), c) aspartic proteinases (EC 3.4.23; pepstatin-sensitive, active at pH 2-7), d) metallo

proteases (EC 3.4.24; EDTA-sensitive active at pH 7-9), e) proteases of unknown catalytic mechanism (EC 3.4.99). This catalytic classification is also applied to exopeptidases (peptidases), though to date no aspartic peptidase has been reported (McDonald, 1985).

Endo- and exopeptidases that are classified as cysteine proteases include papain, bromelain, ficin and cathepsin B. Pepsin and rennin belong to the aspartic proteases class, whereas collagenase, carboxypeptidase A, and angiotensin converting enzyme and the aminopeptidases are good examples of metallo proteases.

The class that has perhaps been most widely studied and which probably constitutes the biggest group of known enzymes is the serine class. Within this class it is customary to refer to a few families of enzymes. One family is composed of trypsin-like enzymes which are distinguished by their preference for basic amino acid residues (discussed below). Another family is made up of chymotrypsin-like enzymes which have a preference for hydrophobic amino acid residues and include chymase I, chymase II, and cathepsin G. Enzymes specific for proline comprise another family. The kallikrein family is a group of serine proteases that are involved in generating kinin activity, etc. These enzymes may not have evolved from each other, but may demonstrate common characteristics because they arose from a common progenitor gene (Woodbury and Neurath, 1980).

Some of the members of the trypsin-like family of proteases are the enzymes of the coagulation pathway (e.g. thrombin, factor Xa), the complement pathway (e.g. C1 esterase, factor D), the fibrinolytic pathway (e.g. plasmin, urokinase, tissue plasminogen activator), as well as tryptase (though only a recently identified enzyme). Although enzymes within the trypsin-like family all have a primary specificity for cleaving substrates on the carboxyl side of the amino acids arginine or lysine, they often exhibit very high substrate specificity. They may be able to hydrolyze only one bond in one protein of the coagulation cascade, for example. The specificity arises in part because of the requirement for a specific amino acid sequence on either side of the basic amino acid where cleavage occurs. This requirement is called secondary specificity. Frequently, a small synthetic substrate which has an amino acid sequence similar to that of the natural substrate in the region of the scissile bond can also be hydrolyzed. Related substrates with slightly altered sequence may not be cleaved. Thus, synthetic substrates can be used to define both the primary and secondary specificities of the protease and can be used to distinguish one protease from another even when their natural substrate is unknown.

The present project will focus on a serine protease that is trypsin-like which has been isolated from the skin

of rat. A number of synthetic substrates will be used to gain a preliminary insight into the primary and secondary specificities of the enzyme.

#### B. Physiological Functions of Proteases

Proteolytic enzymes have been shown to play a role in some very important cellular and physiological processes. These include: chemotaxis, endocytosis, exocytosis, angiogenesis, tumorigenesis and fertilization (Woodbury & Neurath, 1980). They also play a role in inflammatory events such as blood clotting, clot lysis, degradation of cells and tissue matrix, cell transformation and chemotaxis of inflammatory cells (Barrett & McDonald, 1980). They are also involved in intracellular protein and peptide degradation, and metabolism of hormones and neurotransmitters (McDonald & Barrett, 1986). More recently these enzymes have also been implicated in prohormone processing (Schwartz, 1986). The study of proteases continues therefore to be an area of ceaseless and intense biochemical interest.

#### C. Skin as a Source of Proteases

Some of the specific features of the skin add further elements of significance to the study of these enzymes in this organ.

The skin is the main organ that interfaces between an animal and its environment. This role requires among other things that replacement of cells in the skin take place much

more continuously than in other organs of the body. In fact the rate of cell division in the epidermis is much greater than in any internal organ (Spearman, 1973). Proteolytic enzymes have been implicated in cell proliferation (Schnebli, 1975; Fraki *et al.*, 1983) and in regular cell turnover (Dean, 1980). One can therefore expect to find a range of proteases in the skin associated with its regular protein catabolism and its rapid proliferation.

Another aspect of the skin that sets it apart from other organs is its size in terms of both area and weight. The skin is the largest organ of the body, comprising up to 15-20 % of the weight of the body in rats and humans (Harkness, 1971). This is an advantage as far as using it as a source for extracting proteases since much more tissue per animal is available and consequently more enzyme in some instances.

A third feature of the skin that contributes to its uniqueness is its cellular composition. The skin is made up of at least seven different cell types that can trace their embryologic origins back to either the ectoderm, the neural crest, the mesoderm and even the bone marrow (Gilchrist, 1984). Hence, there is a great likelihood that the proteases found in the skin may well be similar to proteases found in other organs of the body that derive from similar origins or share some of these cell types. Studies of the proteolytic enzymes of the skin can contribute to our understanding of

this class of enzymes in several parts of the body.

D. Significance of Studying Skin Proteases

The skin is most definitely a visual marker for aging and indeed has been accepted as a model for studying the aging process (Gilchrist, 1984). Its easy accessibility in live animals as well as some of its features discussed above are the strengths of this model. Since proteases are involved in cell turnover and proliferation, studies on the proteolytic activity of the skin may contribute to our understanding of the aging phenomenon.

Epidermal serine proteases have been studied recently in patients with psoriasis (Dubertret et al., 1984), a skin disease that may affect up to 2% of a country's population (Mier & Cotton, 1976). The study suggests that these enzymes may be responsible for initiating the psoriatic lesion.

In view of all of the special features of the skin described above, the investigation of proteases in the skin is of major significance. This study presents the isolation to homogeneity and characterization of a proteolytic enzyme from rat skin. The profile of its various characteristics suggests strongly that it is a hithertofore unknown trypsin-like enzyme. In view of this "trypsin-like" nature of the enzyme and in order to facilitate discussion about this enzyme, we have decided to refer to it as "tryptidase".

## CHAPTER II

### LITERATURE SURVEY

#### A. Proteolytic Enzymes in Skin

Investigations into the proteolytic activity of the skin began with the identification of caseinolytic activity in saline extracts of animal and human skin (Sexsmith and Petersen, 1917). Since then an entire range of proteases have been partially purified and characterized from the skin of the human, rat, rabbit, toad, cat and guinea pig.

##### 1. Conditions for Extraction of Skin Proteases

Jansen and Hopsu-Havu (1969) made a detailed study to determine the optimal conditions required to extract the caseinolytic activity of rat skin. Their findings are summarized by Hopsu-Havu et al. (1977), who have also reviewed extraction methods for other endopeptidases and exopeptidases from the skin. The main conclusion is that salt concentration in the extracting buffer plays an important role in extracting one or another kind of protease.

##### 2. Sources of Proteases in Skin

Three classes of proteases have been identified by Mier & Cotton (1976) based on their cellular origin: one class is constitutive and free in the skin, the second class consists of enzymes that are of lysosomal origin; and the

third class comprises those that are induced under specific circumstances such as wounding and burning.

### 3. Protease Inhibitors in Skin

Inhibitors to a number of the proteases found in the skin have been identified and purified. These have been reviewed in relation to diseased states by Hopsu-Havu et al. (1981). The presence of these inhibitors in the skin is a further indication of the completeness and significance of the proteolytic enzyme system in the skin.

### 4. Proteases Identified, Isolated and Characterized from Skin

The chemical, physical, and catalytic properties of the known proteolytic enzymes of the skin have been reviewed in detail by Hopsu-Havu et al. (1977) and Fraki et al. (1983). These proteases include representatives from the entire spectrum of classes and subclasses of proteolytic enzymes described by McDonald (1985). This extensive review data is not recapitulated here, but will be referred to as needed when "tryptidase" is compared with other enzymes in the Discussion. Instead the properties of skin proteases not covered in previous reviews are summarized briefly below.

A neutral proteinase was purified and characterized from goat skin (Sivaparvathi et al., 1975). Based on amino acid analysis, its  $M_r$  was 40-45,000 (Sivaparvathi et al., 1980). A metalloproteinase that is active against the

elastase substrate succinyl-(Ala)<sub>3</sub>-p-nitroanalide (SAPNA) but will not cleave insoluble elastin has been identified in human skin fibroblasts (Szendroi et al., 1984; Paller et al., 1985; Schwartz et al., 1986) and in blistering diseases (Oikarinen et al., 1986). A neutral cathepsin that may be different from a chymotrypsin-like cathepsin isolated from whole skin (Schechter et al., 1983) has also been detected in human skin fibroblasts (Paller et al., 1985; Schwartz et al., 1986).

An aminoendopeptidase ( $M_r = 400,000$ ) has also been separated from cathepsins B, D, H, and L in newborn rat epidermis (Harvima et al., 1987). While this study demonstrated the presence of cathepsin L in skin tissue for the first time, cathepsin H had been identified earlier as the BANA-hydrolase purified from rat skin by Jarvinen & Hopsu-Havu (1975) (Rinne et al., 1985). The purification and properties of aminoendopeptidase in rat epidermis were studied by Ito et al. (1984).

Melanocytes isolated and grown from newborn human foreskin have been shown to have a 72,000 dalton plasminogen activator (PA) (Hashimoto et al., 1986). The PA activity in the culture medium was inhibited by anti-tissue PA IgG.

Angiotensin converting enzyme was partially purified and characterized in mouse and human skin (Hara et al., 1982). The solubilized enzyme in both mouse and human tissue had a  $M_r$  of 430,000 in the presence of Triton X-100.

A soluble form of  $M_r = 330,000$  was also found.

Prolidase and prolinase were previously demonstrated in skin tissue. Another proline specific enzyme Proline iminopeptidase was also shown to be present in cultured skin fibroblasts in a single form (Butterworth and Priestman, 1984a).

An interesting enzyme that has an  $\alpha$ -amidating activity dependent on copper and ascorbate was purified to homogeneity from Xenopus laevis skin (Mizuno et al., 1986). The enzyme was also partially purified from skin secretions of the organism (Mollay et al., 1986).

#### 5. Specific Proteases in Skin which Cleave the Trypsin Substrate, BAEE and the Chymotrypsin Substrate, ATEE

In terms of the focus of the present project, our original interest was in those proteases that have trypsin-like and/or chymotrypsin-like activities on the basis of cleavage of benzoyl-arginine ethyl ester (BAEE) and acetyl-tyrosine ethyl ester (ATEE), respectively. This interest derived from earlier studies on an enzyme from toad skin which has both activities. Therefore, trypsin-like and chymotrypsin-like enzymes will be discussed below in some detail.

##### a. Carboxamidopeptidase (Cathepsin A) from Toad Skin

An enzyme having both BAEE and ATEE activity has been purified to homogeneity from toad skin in our laboratory (Simmons & Walter, 1980). p-Aminobenzamidine-CH-Sepharose

(PAB) and concanavalin A-agarose (con A) specific affinity chromatography was used to purify the enzyme 3800-fold with a 22% recovery. The dual trypsin/chymotrypsin activities of the enzyme was seen with peptide substrates as well as with ester substrates. The enzyme hydrolyzes both of the neurohypophyseal hormones, oxytocin and vasopressin at the Leu<sup>8</sup>-Gly<sup>9</sup>-NH<sub>2</sub> and Arg<sup>8</sup>-Gly<sup>9</sup>-NH<sub>2</sub> bonds, respectively, thus suggesting the name carboxamidopeptidase. The pH optimum of the enzyme is 7.5. The enzyme is a serine protease and is significantly inhibited by the trypsin inhibitors p-nitrophenol-p'-guanidinobenzoate (NPGb) (10 μM), 4-methylumbelliferyl-p-guanidinobenzoate (MUGb) (10 μM), basic pancreatic trypsin inhibitor (Trasylol) (0.1 mg/ml), tosyl lysine chloromethylketone (TLCK) (1.0 mM), leupeptin (44 μg/ml), antipain (4.6 μg/ml) and by the chymotrypsin inhibitors, tosyl phenylalanine chloromethylketone (TPCK) (1.0 mM) benzyloxycarbonyl phenylalanine chloromethylketone (ZPCK) (0.2 mM) and chymostatin (65 μg/ml). A sulfhydryl group is apparently near the active site since the enzyme is inhibited by p-chloromercuribenzoate (p-CMB) (0.05 mM). This effect is reversed by cysteine (Simmons & Walter, 1980). The molecular weight of carboxamidopeptidase was estimated to be 100,000 by gel filtration in the presence of high salt. However SDS-polyacrylamide gel electrophoresis of the enzyme under reducing and non-reducing conditions indicated that the enzyme is composed of at least two

subunits ( $M_r = 48,000$ ) each having a light and heavy chain each of molecular weights 19,000 and 28,000, respectively. The enzyme active site resides in the heavy chain.

More detailed substrate specificity studies using vasopressin analogs indicated that the enzyme has a specificity for the penultimate amino acid which is broader than both trypsin and chymotrypsin (Simmons, 1981). Finally "carboxamidopeptidase" was also shown to have carboxypeptidase-like activity on non-amidated peptide substrates, with a pH optimum of 5.5 (Simmons, 1981). Thus the enzyme can be classified as a serine carboxypeptidase with properties similar to those described for yeast carboxypeptidase Y (Hayashi, 1976) and mammalian cathepsin A (Logunov and Orekhovich, 1972).

b. Rat Skin Carboxamidopeptidase?

An enzyme that hydrolyzes the trypsin substrate BAEE and the chymotrypsin substrate ATEE, as well as other amino acid esters, was also purified 54-fold from rat skin (Seppa, 1976). This enzyme is like the toad enzyme in that it has a molecular weight of 125,000 and is inhibited by diisopropylphosphofluoridate (DFP) (0.1 mM) and p-CMB (0.1 mM). However, TLCK, lima bean trypsin inhibitor (LBTI), soybean trypsin inhibitor (SBTI), Trasylol and ovomucoid (OMTI) do not have any significant inhibitory effect on the enzyme. Arginine-Sepharose chromatography was used to purify the enzyme. NaCl was required for optimal BAEE

activity of the enzyme. The low purification factor indicates that the final preparation does not contain pure enzyme, possibly accounting for some of the differences from the toad enzyme.

Based on the few similarities between the two enzymes, it was suggested that the rat skin enzyme, like the enzyme from toad skin, might turn out to be similar to cathepsin A on further study (Simmons, 1979).

In the rat, cathepsin A has been identified and characterized only in liver lysosomes (Taylor and Tappel, 1973a; Taylor and Tappel, 1973b; Taylor and Tappel, 1974; Matsuda and Misaka, 1974; Matsuda and Misaka, 1975; Matsuda, 1976), brain (Grynbaum and Marks, 1976; Marks et al., 1981). and muscle (Obled et al., 1980). To date, cathepsin A has not been described in rat skin. The only study which showed cathepsin A in mammalian skin is that of Volden et al., (1976) which demonstrated CBZ-Glu-Tyr cleavage by guinea-pig epidermis.

In order to broaden the study of enzymes having the dual BAEE and ATEE activities and to test the cathepsin-A hypothesis, we sought to purify this enzyme to homogeneity from rat skin using protocols developed in our laboratory for the toad enzyme. In attempting to do this, we isolated an enzyme that hydrolyzed BAEE but that had no activity against ATEE (Braganza & Simmons, 1986) and was not affected by inhibitors of chymotrypsin (Braganza & Simmons, 1987).

No such enzyme had been previously purified and characterized from rat skin. We therefore shifted our attention to the trypsin-like enzymes in the skin.

#### 6. Trypsin-Like Enzymes In The Skin

A protease having "the pH activity curve of trypsin" was first identified in rat skin by Beloff & Peters (1945). The enzyme was shown to have optimum caseinolytic activity in the presence of 2.5% KCl, while the use of 5% KCl resulted in greater efficiency of extraction of the proteinase activity.

Histochemical studies were conducted on skin sections from rat, rabbit, guinea pig, mouse, dog and man. Trypsin-like activity was detected only in the mast cells of dog and man (Glennner & Cohen, 1960). The observations were based on the hydrolysis of benzoyl-arginine B-naphthylamide hydrochloride (BANA.HCl) and its inhibition by both DFP and benzoyl-arginine amide hydrochloride (BAA.HCl).

Proteolytic activity of human skin slices was studied using the substrates BAEE, tosyl-arginine methyl ester (TAME), ATEE, and acetyl-tryptophan ethyl ester (ATrEE). Maximal BAEE activity was found in the epidermis, although considerable activity was also present in all skin layers (Yamura & Cormia, 1961). Extracts of the human skin slices also demonstrated high BAEE and TAME activity.

##### a. Tryptase From Human Skin

Fraki & Hopsu-Havu (1975) separated a BAEE

hydrolysing activity from an ATEE hydrolysing activity in human skin using Sephadex G-100 gel filtration and hydroxyapatite chromatography. The BAEE enzyme, which was partially purified 30-fold could split benzoyl-arginine p-nitroanilide (BAPA) and histone proteins with an optimum pH of 7.5-8.2. While Trasylol (330 IU/ml) and TLCK (1.0 mM) inhibited the enzyme, SBTI, LBTI and OMTI did not. Significantly enough, no major effects of a serine protease inhibitor E-600 or of p-CMB were reported. The activity of the enzyme was considerably higher against TAME and acetyl-glycyl-lysyl methyl ester (AGLME) than against BAEE. The molecular weight of the enzyme was 120,000, although in dilute buffer aggregates ranging from 340,000 to 930,000 were formed (Fraki & Hopsu-Havu, 1972a).

This human skin enzyme has been referred to as tryptase and its subsite specificity has been studied (Tanaka et al., 1983). The enzyme has an extended substrate-binding site with a high specificity for two basic residues. A role in the processing of prohormones and proproteins has been suggested for the enzyme. The enzyme was also found to be labile in the absence of salt in this study, although high salt concentrations had an inhibitory effect.

A monoclonal anti-tryptase antibody raised against tryptase from human pulmonary mast cells binds to dermal mast cells and to washed dispersed keratinocytes (Schwartz

et al., 1985), thus indicating the cellular origin of the enzyme in the skin. Mastocytosis extracts of human skin have also been shown to contain markedly increased levels of tryptic proteinases suggesting a mast cell origin (Fraki et al., 1986)

b. Characteristics of Tryptase from Other Human Tissue Sources

Tryptase has been identified as the predominant neutral protease of human mast cell secretory granules in studies conducted on dispersed pulmonary mast cells (Schwartz et al., 1981). The enzyme has also been well purified from this source (Schwartz et al., 1981; Schwartz, 1985) and based on SDS-polyacrylamide gel electrophoresis has been shown to have a tetrameric subunit structure composed of two subunits of molecular weights 37,000 and 35,000. Both units apparently contain active sites (Schwartz et al., 1981) and are immunogenically related (Smith et al., 1984; Schwartz, 1985). Heparin, a component of rat and human mast cells has a stabilizing effect on the enzyme. The stabilization is through direct interaction with the enzyme rather than by an indirect ion-binding mechanism. In Tris-buffered saline in the absence of heparin an inactive 34,000 dalton form is detected (Schwartz and Bradford, 1986a).

The enzyme has the ability to rapidly inactivate human high molecular weight kininogen (HMWK) (Maier et al.,

1983). Tryptase cleaves HMWK without generating any kinin contractile activity. On the other hand, no significant cleavage of low molecular weight kininogen (LMWK) or destruction of its reactivity with kallikrein was detected (Schwartz et al., 1984). Tryptase can also destroy fibrinogen in the presence and absence of heparin with minimum fibrinolytic activity. The enzyme does not hydrolyze plasminogen (Schwartz et al., 1985). Tryptase has no kallikrein or kininase activities and neither activates nor destroys prekallikrein (Schwartz et al., 1985)

Immunocytochemical studies have further confirmed the localization of this enzyme to the mast cell of the lung (Smith et al., 1984). These authors have developed a procedure to isolate the enzyme from whole human lung after autopsy. The enzyme obtained by their procedure is similar to the pulmonary mast cell tryptase, except that it has a slightly lower molecular weight. The enzyme activity decreases with increasing NaCl concentration. Tryptase is apparently present in both the types of mast cells identified in human tissue (Irani et al., 1986). One type (TC) has both tryptase and chymotryptic activity and the other type (T type) has only tryptase activity. This is an interesting observation in light of earlier discoveries that there are two types of mast cells in the rat, type T and type AT, which contain the chymotrypsin-like enzymes RMCPI and RMCPII, respectively (Woodbury et al., 1981).

A tryptase that is immunologically identical to the one from human lung was identified in the mast cells of human pituitary connective tissue (Cromlish, et al., 1987). Using Western blotting, at least five different size forms of the enzyme were shown to be present in human tissue. These authors also showed that the first four amino acids of the NH<sub>2</sub>-terminal sequence of the enzyme are homologous to that of trypsin and other serine proteases.

An enzyme partially purified from human uterine cervix (Ito et al., 1980) has been considered to be tryptase (Cromlish et al., 1987). The molecular weight of the enzyme and its inhibitor profile and proteinase activity compare favorably with the tryptases from other sources mentioned above.

### c. Tryptase from Rat Mast Cells

A rat mast cell tryptase has also been isolated and characterized recently (Kido et al., 1985a). The enzyme is associated with an inhibitor (trypstatin) that affects its activity above pH 7.5. In the absence of the inhibitor, it is activated by 10 mM CaCl<sub>2</sub> and is inhibited by the trypsin inhibitors BPTI, SBTI and  $\alpha_1$ -antitrypsin. With trypstatin present, however, only  $\alpha_1$ -antitrypsin had an effect on the enzyme. The molecular weight of the enzyme-inhibitor complex was determined to be approximately 144,000 by gel filtration, while that of trypstatin is about 7600. A factor Xa substrate, Boc-Ile-Glu-Gly-Arg-MCA was the most

rapidly cleaved of the substrates tried with the enzyme. However, TAME, an important tryptase substrate was not assayed. It has been suggested (Kido et al., 1985b) that the enzyme is activated together with mast cell chymotrypsin-like proteases by IgE-receptor bridging, a process which plays a role in triggering the process of mast cell degranulation.

d. Other Trypsin-Like Enzymes In the Skin

Hydrolysis of the trypsin-like substrate BAEE has been detected in guinea pig skin and the activity has been partially purified and characterized (Song et al., 1969). The enzyme is inhibited by SBTI ( $1.67 \times 10^{-5}$  M), Trasylol (300 units) and TLCK (5.12 mM). Injection of the preparation intradermally resulted in an inflammatory reaction and prolonged increase in vascular permeability to plasma proteins.

A permeability factor of  $M_r = 35,000$  with serine protease-like activity was also extracted from guinea pig skin. The activity was purified 292-fold using hydroxyapatite, diethylaminoethyl cellulose and Sephadex G-75 column chromatography (Yamamoto and Kambara, 1978). Activation of the factor is adversely affected at high salt concentrations on Sephadex G-100. At high salt concentrations the  $M_r$  of the enzyme is approx. 80,000. It is activated by dialysis against a 67 mM phosphate buffer at pH 5.8-6.4 (Yamamoto et al., 1978). SBTI (0.5 mM) and DFP

(0.5 mM) inhibit the activity but EDTA (1 mM) and pepstatin (1 mM) do not. It has a pI of 4.5, and the evidence indicates that the factor can activate prekallikrein and may be similar to the plasma Hageman factor (Kozono et al., 1980).

Soybean trypsin inhibitor affinity chromatography was used to purify (254-fold) a proteinase from human skin of  $M_r = 28,000$  (Hatcher et al., 1977). Complete inhibition of the enzyme occurs in the presence of  $\alpha_2$ -macroglobulin (1  $\mu$ M),  $\alpha_1$ -antitrypsin (13  $\mu$ M), C1 inactivator (8  $\mu$ M), DFP (5  $\mu$ M), and SBTI (9  $\mu$ M). TLCK (5  $\mu$ M) inhibits the enzyme significantly. No effect was seen of Trasylol (1000 KI units), Kunitz pancreatic trypsin inhibitor (KPTI) (30  $\mu$ M), EDTA (2 mM), or TPCK (5  $\mu$ M). Injection of the preparation into rabbit skin induced polymorphonuclear leukocyte infiltration into the cutis and edema (Levine et al., 1976).

#### B. Goals and Objectives of this Project

It is clear from the discussion above that there is trypsin-like activity in skin tissue arising from different enzymes. These have not been purified to homogeneity and characterized from rat skin tissue. Since our earlier studies indicated that we may have discovered a new trypsin-like enzyme in rat skin, our goal has been to purify this enzyme to homogeneity and to characterize it chemically, physically, enzymatically and structurally. A purification scheme has been optimized to give maximum yield of the

enzyme in minimum time. In addition, the molecular weight, isoelectric point, N-terminal sequence, inhibitor profile, assay conditions, stability of the enzyme with respect to pH and temperature, the pH activity optimum and substrate specificity of the enzyme has been studied. These data will be used to substantiate our claims concerning the novelty of this enzyme.

## CHAPTER III

### MATERIALS AND METHODS

#### A. Materials

The sources of the animals, reagents and chemicals are listed below according to the experiment in which they were used. Reagents and materials used in more than one experiment are listed only once.

##### 1. Purification Scheme

Male rats (Sprague Dawley), 45-60 days old were from Holtzman, Madison, Wi. NaCl was obtained from Mallinckrodt, St. Louis, Mo. while  $\text{NaHPO}_4$  and  $\text{NaH}_2\text{PO}_4$  were from Mallinckrodt or Fisher Sci. Co., Pittsburg, Pa. Ethylenediaminetetraacetic acid (EDTA), agarose-glycyl-glycyl-p-aminobenzamidine gel (A-Gly-Gly-PAB) (lot # 54F-9580 & 84F-9610), benzamidine.HCl and  $\text{NaN}_3$  were bought from Sigma Chem. Co., St. Louis, Mo. Enzyme grade  $(\text{NH}_4)_2\text{SO}_4$  was obtained from Schwarz Mann Biotech. Div. of ICN Biomedicals, Inc., Cambridge, Ma. Dialysis tubing was from Spectrum Medical Inds., Inc., Los Angeles, Ca. Concanavalin A-agarose gel (lot # 10355) was obtained from Bethesda Research Laboratories, Gaithersburg, Md. The Centricon-30 microconcentrator units were from Amicon Division, W. R. Grace & Co., Danvers, Ma.

## 2. Substrates Used in Standard Assays and Substrate Profile Studies

All amino acids are of the L-configuration unless otherwise indicated. BAEE, benzoyl-L-arginine (BA), ATEE, acetyl-L-tyrosine (AT), TAME, tosyl-L-arginine (TA), benzoyl-L-arginine methyl ester (BAME), benzoyl-L-arginine p-nitroanilide (BAPNA), benzoyl-L-arginine B-naphthylamide (BANA) were from Sigma Chemical Co. S-2484, S-2444, S-2266, S-2288, S-2251, S-2238 were from Kabi Vitrum, Stockholm, Sweden or Helena Laboratories, Beaumont, Tx. Spectrozyme Ca, Spectrozyme pKal and Spectrozyme PCa were from American Diagnostica Inc., Greenwich, Ct. Bz-Pro-Phe-Arg-p-nitroanilide acetate, Spectrozyme PL, H-D-Val-Gly-Arg-p-nitroanilide hydrochloride, Chromozym P were from Pentapharm Ltd. Substrate C<sub>1</sub>E-1 was from Immuno Diagnostics, Vienna, Austria. CBS 31.39 and CBS 34.47 were from Diagnostica Stago, Ansieres, France. Chromozym TH, Chromozym PL, and Chromozym U were from Boehringer Mannheim, Indianapolis, In. (The majority of the p-nitroanilide substrates was the kind gift of Dr. J. Fareed, Dept. of Pathology, Loyola Univ. of Chicago, Maywood, Il.)

## 3. Protein Assays

Bradford Reagent was the product of Bio Rad Laboratories, Richmond, Ca., and bovine serum albumin (BSA) was purchased from Sigma Chemical Co.

## 4. Storage Experiments

Carbowax (20 M) was a gift of Dr. Richard Schultz, Dept. of Biochemistry, Loyola Univ. of Chicago. Acetone was supplied by American Sci. Prods., McGaw Park, Il.

#### 5. pH Studies

All anionic buffers were prepared from sodium salts. Sodium borate, sodium citrate, sodium phosphate (mono and dibasic), succinic acid and sodium hydroxide were from Fisher Sci. Co. Sodium acetate was from J.T. Baker Chemical Co., Phillipsburg, NJ and Tris (base and HCl) was from Sigma Chemical Co.

#### 6. Inhibitors

SBTI, LBTI, ovoinhibitor, OMTI,  $\alpha$ -1 antitrypsin, hirudin, concanavalin A, MUGB, TPCK, ZPCK, TLCK, p-CMB,  $MnCl_2$ , antipain, leupeptin, chymostatin, elastatinal, pepstatin, bestatin, dithiothreitol (DTT), 2-mercaptoethanol, and  $\epsilon$ -amino caproic acid were all obtained from Sigma Chemical Co. NPGB was purchased from Nutritional Biochemical Corp., Cleveland, O. DFP was obtained from Aldrich Chemical Co., Milwaukee, Wi and stored as a 240 mM solution in isopropanol at  $-20^\circ$ . Trasylol was a product of Farbenfabriken Bayer AG.  $CaCl_2$ ,  $CoCl_2$ , and  $MgCl_2$  were from Fisher Sci. Co. and  $HgCl_2$  was from J. T. Baker Chem. Co.

#### 7. Gel Filtration

The molecular weight standards, rabbit muscle aldolase, ovalbumin, glutamate dehydrogenase and ribonuclease were obtained from Pharmacia Fine Chemicals.

B-Amylase was from Sigma Chemical Co.

#### 8. SDS Gel Electrophoresis

Acrylamide and N,N'-methylene-bis-acrylamide (BIS) were purchased from Biorad Laboratories. Lauryl Sulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), bromophenol blue, Dalton Mark VII-L (molecular weight standards mixture), and coomassie brilliant blue were the products of Sigma Chemical Co. Acetic acid was from Mallinckrodt and methanol from American Sci.Prods. [<sup>3</sup>H]-DFP (3.9 Ci per mmol) was from New England Nuclear Co., Boston, Ma. Scintillation fluid, Ecoscint, was from National Diagnostics, Somerville, NJ.

#### 9. Isoelectric Focusing

Precast Ampholine-containing polyacrylamide gels (pH 3.5-9.5) (PAG-plates) were the product of LKB-produkter AB, Bromma, Sweden and the isoelectric focusing standard kit was from Pharmacia Fine Chemicals (pH 3.0-6.5 with colored dye marker) or from Sigma Chemical Co. (pH 3.0-9.5).

#### 10. Peptide and Protein Digestion

Bradykinin, oxytocin and vasopressin were purchased from Bachem Inc, Torrance, Ca. or Vega Biotechnologies, Tucson, AZ. Azocoll, casein, and haemoglobin were the products of Sigma Chemical Company.

### B. Methods

#### 1. Purification Scheme

The following steps were used to purify the trypsin-

like enzyme from rat skin:

a. Tissue Preparation

Male Sprague Dawley rats, 45-60 days old were sacrificed and their skins removed. Skins not immediately used for enzyme extraction were stored frozen at  $-20^{\circ}$  and were thawed out overnight at  $4^{\circ}$  before use. The fur was then clipped away as close as possible to the epidermis using a pair of Oster Professional Animal Grooming Clippers (Model A-5) with a No. 80, size 40 blade. Rats have a cutaneous muscle called the m. cutaneus trunci covering the lateral thoracic and abdominal wall from the shoulder to the base of the tail (Hebel and Stromberg, 1976). This extremely thin muscle sheet which is very closely appended to the dermis was first scraped off using sharp anatomical razors.

The skins were next rinsed and washed in 0.65% saline and then minced in a Universal No. 3 meat grinder through two cycles.

b. Extraction

The minced skin was then homogenized in 0.025 M sodium phosphate containing 0.001 M EDTA and 0.5 M NaCl, pH 7.0 (phos-EDTA-0.5 M NaCl-7.0) (1:9, w/v) in a Waring blender (Model PB-5). Homogenization was carried out at high speed at  $4^{\circ}$  C for six, 20 sec intervals with 20 sec rest intervals in between.

c. Ammonium Sulfate Precipitation

The fur and other tissue remnants were removed from the homogenate by filtration under vacuum, in a cold room, through a Buchner funnel lined with a nylon mesh. The filtrate was collected and brought to 25%  $(\text{NH}_4)_2\text{SO}_4$  by the addition of 138 g of the solid salt per liter of filtrate. The solution was then allowed to stand undisturbed for between 4-6 h. It was then centrifuged at  $13,700 \times g$  for 15 min at  $4^\circ \text{C}$ . The sediment was discarded and the resulting supernatant was brought to 80%  $(\text{NH}_4)_2\text{SO}_4$  saturation by the rapid addition of 361.5 g per liter (of 25% saturated supernatant) (Taylor, 1953). This solution was allowed to stand unstirred for 14 h and then centrifuged. The sediment was collected and resuspended in 100 ml 0.025 M sodium phosphate containing 0.001 M EDTA and 0.5 M NaCl, pH 6.0 (phos-EDTA-0.5 M-NaCl-6.0), and then dialyzed against two changes of 6.5 l each of the same buffer. Insoluble materials in the retentate were removed by centrifugation at  $23,700 \times g$  for 15 min at  $4^\circ$ .

d. Agarose-Glycyl-Glycyl-p-Aminobenzamidine

Chromatography

The centrifuged retentate was then applied at room temperature to an agarose-glycyl-glycyl-p-aminobenzamidine affinity column (1.5 x 20 cm) which had been equilibrated with phos-EDTA-0.5 M-NaCl-6.0. An LKB peristaltic pump was used to maintain a steady rate of 25 ml/h. The sample reservoir was maintained on ice during the procedure. When

the sample application was completed, the column was washed with one column volume (25 ml) of 0.025 M sodium phosphate containing 0.02 %  $\text{NaN}_3$  and 0.5 M NaCl, pH 6.0 (phos- $\text{NaN}_3$ -0.5 M NaCl-6.0). The column was then placed at 4° C for 1 h and then washed with one column volume of cold phos- $\text{NaN}_3$ -0.5 M NaCl-6.0. The enzyme was then eluted using phos- $\text{NaN}_3$ -0.5 M NaCl-6.0 containing 0.1 M benzamidine.HCl.

e. Concanavalin A-Agarose Chromatography

When one third of a column volume (approx. 10 ml) of the elution buffer had been passed through the A-Gly-Gly-PAB column, the eluent was directed into a concanavalin A-agarose column (1 x 10 cm) equilibrated with phos- $\text{NaN}_3$ -0.5 M NaCl-6.0 and the flow rate was immediately reduced to 10 ml/h. The two affinity columns connected in series were further washed with 40 ml of the benzamidine-containing buffer. The first column was subsequently removed from the system and the concanavalin A-agarose column was then washed with one column volume (10 ml) of phos- $\text{NaN}_3$ -0.5 M-NaCl-6.0 at a flow rate of 5 ml/h. One third column volume (3.5 ml) of a solution of 100 mg/ml of  $\alpha$ -methyl-D-mannoside in phos- $\text{NaN}_3$ -0.5 M-NaCl-6.0 was passed through the column and the flow was stopped. One hour later, the elution of the enzyme from the column was continued using the same buffer. Fractions of 1 ml were collected in polystyrene tubes. Fractions containing BAEE activity were pooled and then concentrated to 25  $\mu$ l in each unit, using four Centricon-30

microconcentrator units. The buffer of the sample was then changed to phos-EDTA-0.5 M-NaCl-6.0 by two dilution-concentration cycles using 2 ml of this buffer for each dilution. Enzyme from all four microconcentrator units was pooled. Each unit was then washed three times with 200 ul of the buffer which was then added to the enzyme pool to give a final volume of 2.5 ml pure enzyme. The concentrated enzyme was stored at 4<sup>0</sup> in a polystyrene tube.

f. Regeneration of Specific Affinity Columns

At the end of each purification the A-Gly-Gly-PAB and the con-A column were regenerated for future use. The A-Gly-Gly-PAB column was washed with two column volumes (50 ml) of phos-EDTA-0.5 M-NaCl-6.0 containing 8 M urea, followed by 4 column volumes (100 ml) of the same buffer without urea. Both solutions were applied to the column under gravity at room temperature.

The con-A column was regenerated using a modification of the method of Pitlick and Nemerson (1976). Two column volumes (20 ml) of a buffer consisting of 100 mM sodium acetate, 0.5 M NaCl, 10 mM calcium chloride, 10 mM magnesium chloride, 0.02% NaN<sub>3</sub>, pH 6.0 were pumped into the column at 10 ml/h at 4<sup>0</sup>. Both columns were then stored at 4<sup>0</sup> till further use.

2. Enzyme Assays

a. Esterase Activity

Hydrolysis of BAEE was monitored spectrophotometri-

cally (Schewert and Takenaka, 1955). A 1 ml assay volume was used consisting of 0.9 ml of enzyme (100-150 ng) in 0.025 sodium phosphate, 1 mM EDTA, pH 7.5 (phos-EDTA-7.5) and 0.1 ml of 10 mM BAEE in distilled deionized H<sub>2</sub>O. The change of absorbance with time was recorded at 253 nm at 25<sup>0</sup> in a Gilford Response spectrophotometer for intervals of 5 or 15 mins. One unit of activity is defined as 1 umole of substrate hydrolysed per minute and was calculated by the formula:

$$\text{unit activity} = \frac{\Delta A_{253}/\text{min}}{\Delta A_{253} \text{ for total cleavage}} \times \frac{1 \text{ umole BAEE}}{1}$$

where  $\Delta A_{253}$  for total cleavage is the difference in absorbance between 1mM BA and 1mM BAEE ( 0.95 Abs. units) Specific activity is defined as the units of activity per mg of protein. The cuvettes were washed between assays with phos-EDTA-1 M-NaCl-7.0 buffer to ensure removal of any residual activity due to enzyme adsorbed to the surface of the cuvettes.

#### b. Amidase Activity

Hydrolysis of the amide substrates S-2288, S-2238, Spectrozyme PCa and Chromozym PL, was monitored spectrophotometrically in a manner similar to BAEE. The change in absorbance over time was monitored at 405 nm (Svendsen et al., 1972) at 25<sup>0</sup> in a Gilford Response spectrophotometer. The use of the Gilford Response spectrophotometer was convenient for these assays since it made comparisons with the BAEE assays described above easy.

However, its use was necessitated due to the fact that assays for the 23,700 x g step of the purification scheme had particulate matter in them and so could not be assayed on the Multistat III<sup>R</sup> centrifugal analyzer which was used to obtain a substrate profile for the amidase activity of the enzyme (see below). A unit of activity is defined identically as for the esterase activity, and is given by the formula:

$$\text{unit activity} = \frac{\Delta A_{405}/\text{min}}{\Delta A_{405} \text{ for total cleavage}} \times \frac{1 \text{ umole subst.}}{1}$$

where  $\Delta A_{405}$  for total cleavage is the difference in absorbance between 1 mM of p-nitroaniline (p-NA) and 1mM of the pertinent amide substrate used. The specific activity is again defined as activity units per mg of protein.

### 3. Protein Determination

The Bradford protein micro assay was used in order to determine the protein concentration of the enzyme (Bradford, 1976). Bovine serum albumin was used as a standard.  $A_{595}$  was determined on a Gilford Response spectrophotometer.

### 4. NaCl Concentration Dependence of Extraction of BAEE Activity

Three rat skins were processed and minced as described above in the tissue preparation step of the enzyme purification scheme. The minced tissue was then divided into two equal halves by wet weight. The first half was homogenized in phos-EDTA-0.5 M-NaCl-7.0 and the second half

was homogenized in phos-EDTA-1 M-NaCl-7.0 as described in step 1 b of the purification scheme above. An aliquot from each half was then centrifuged at 23,700 x g. The supernatant was assayed for BAEE activity using the standard assay described above.

#### 5. Time Course of Extraction of BAEE Activity

The uncentrifuged 0.5 M NaCl extracts and the 1 M NaCl extract from the study above were allowed to stand at 4<sup>0</sup> in a cold room. An aliquot was removed from each extract at intervals of 2 h and centrifuged at 23,700 x g at 4<sup>0</sup>. The amount of BAEE activity in each supernatant was then determined using the BAEE assay described above except that phos-EDTA-1 M-NaCl-7.0 was used as the assay buffer. Aliquots were removed at 0 h, 2 h, 4 h, 6 h, and 8 h respectively.

#### 6. Pilot Study for Ammonium Sulfate Precipitation

A pilot study was conducted in order to determine the cutoff limits for the percent  $(\text{NH}_4)_2\text{SO}_4$  so that this purification step could be optimized (Clark & Switzer, 1977). The uncentrifuged 1 M NaCl extract was used in this study. The extract was filtered under vacuum through cheese cloth in a Buchner funnel to remove tissue fragments and particulate matter. Aliquots (50 ml) of the filtrate were then each subjected to precipitation by the addition of  $(\text{NH}_4)_2\text{SO}_4$  to different percentages of saturation. The salt was mixed until it had dissolved completely and the mixtures

were allowed to sit undisturbed overnight at 4<sup>0</sup> C. The mixtures were then centrifuged at 13,800 x g at 4<sup>0</sup> C for 15 min. After discarding the supernatant, the precipitate was resuspended in 10 ml of phos-EDTA-0.5 M-NaCl-6.0 and centrifuged at 23,500 x g at 4<sup>0</sup> C for 15 min. The supernatant was then assayed for protein and BAEE activity as described above. BAEE activity and protein concentration expressed as a percent of the values for the untreated filtrate, were both plotted as functions of % (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation on the same graph. The optimum cutoff points which give maximum activity recovery with minimum protein recovery were determined. The resuspended (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was not dialyzed and no attempt was made to compensate for possible (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> inhibition at higher concentrations of the salt.

#### 7. Determination of Storage Conditions

The ability of the enzyme to survive a range of storage conditions was determined. In all cases the activity of enzyme stored at 4<sup>0</sup> C in phos-EDTA-0.5 M-NaCl-6.0 was used as a standard to compare residual activity.

##### a. Storage at RT in Phos-EDTA-0.5 M-NaCl-6.0

A sample of purified enzyme in phos-EDTA-0.5 M-NaCl-6.0 was allowed to sit at room temperature. Aliquots of the enzyme were then removed to determine residual activity. Aliquots were removed initially every 2 h for up to 10 h and then once every 24 h for up to 8 days. The standard BAEE assay was used to determine activity.

b. Storage at -20° in Phos-EDTA-0.5 M-NaCl-6.0

Purified enzyme in phos-EDTA-0.5 M-NaCl-6.0 was stored in a -20° freezer. At intervals of 24 h for up to 8 days the enzyme was removed from storage, allowed to thaw, and assayed for residual BAEE activity as described in section a above and then refrozen.

c. Effect of Lyophilization

An aliquot of enzyme was quickly frozen using a dry ice-acetone bath and then lyophilized in a Labconco Freeze Dryer. The lyophilized sample was stored at 4° for 8 days and then reconstituted in distilled deionized H<sub>2</sub>O and assayed for residual BAEE activity. A sample of enzyme frozen in the dry ice-acetone bath but not lyophilized was used as a control.

d. Effect of Vacuum Drying in the Presence of 2%

Carbowax

Two aliquots of enzyme containing 2% 20 M Carbowax were dried under vacuum in a Savant Speedvac Concentrator model RT-100. One aliquot was then stored at room temperature and another at 4° C. After 8 days, both samples were reconstituted in distilled deionized H<sub>2</sub>O and assayed for residual BAEE activity as described above.

8. Effect of pH

Citrate (pH 2.5-4.0), acetate (pH 4.0-5.5), succinate (pH 5.0-6.0), phosphate (pH 6.0-8.0), Tris-HCl (pH 7.0-9.0), and borate (pH 8.5-10.0) buffers were prepared at 25° C.

Each buffer was 0.1 M and no attempt was made to equalize ionic strengths. Sodium salts were used in all the anionic buffers.

a. pH Stability

A fixed aliquot of enzyme was brought to 0.1 ml in each of the buffers described above and incubated for 2 h at 25° C. Assay buffer (0.8 ml of 0.1 M phosphate, pH 7.5) was then added to each incubation mixture. Enzyme activity was then determined following the addition of 0.1 ml of 10 mM BAEE.

b. pH Optimum

The buffers mentioned above were used in place of the standard buffer in the BAEE assay to determine the pH optimum of the enzyme. The reference cuvette contained BAEE in 0.1 M phosphate, pH 7.5, in each case. Non-enzymatic hydrolysis of BAEE was determined for each buffer in the absence of enzyme and then subtracted from the activity observed with enzyme. The  $\Delta A_{253}$  for total cleavage, used to calculate the activity units, was determined in each buffer. Average rates were determined over a 15 min period.

9. Temperature Studies at pH 7.5

An aliquot of enzyme was brought to 0.1 ml in phosphate-EDTA-7.5 and then preincubated at various temperatures for 30 min. The enzyme mixture was then assayed for residual BAEE activity at 25° using the standard assay described above.

## 10. Effect of NaCl Concentration on BAEE Activity

Phos-EDTA-7.5 and phos-EDTA-1 M-NaCl-7.5, were prepared as stock buffers. The stock buffers were then mixed in appropriate proportions to give buffers containing no NaCl, 0.2 M NaCl, 0.4 M NaCl, 0.6 M NaCl, 0.8 M NaCl, and 1 M NaCl. The purified enzyme was then brought to 0.9 ml in each of these buffers and assayed for BAEE activity as described in the standard assay above. With the maximum activity recovered serving as a standard, the % activity was plotted as a function of NaCl concentration.

## 11. Inhibition Profile Studies

A set of chemical modification agents, protein protease inhibitors, microbial protease inhibitors, and metal ions were tested for their effect on the enzyme. In each case the standard BAEE assay was used to determine the effect of the inhibitor on the enzyme.

### a. Chemical Modification Agents

The effect of chemical modification agents on the enzyme was studied by preincubating enzyme and inhibitor in phos-EDTA-7.5 in a total volume of 0.04 ml at 25<sup>0</sup> C for 10 min in the assay cuvette. The entire mixture was then assayed for residual activity by adding assay buffer and BAEE to the cuvette. Enzyme incubated similarly but without inhibitor, was used as a control. In cases where an organic solvent was needed to prepare a solution of an inhibitor, the pertinent solvent was included in the control incubation

mixture.

b. Protein Protease Inhibitors

All protein protease inhibitors used were water soluble. Enzyme and inhibitor were preincubated in phos-EDTA-7.5 in a total volume of 0.1 ml at 25<sup>0</sup> for 5 min. The entire mixture was then used as above to determine activity. An incubation mixture without inhibitor served as a control.

c. Microbial Protease Inhibitors

A reaction mixture of inhibitor and enzyme was prepared in a total volume of 0.9 ml and immediately assayed for activity. A reaction mixture without enzyme was used as a control. When an inhibitor required an organic solvent to dissolve it, a corresponding amount of solvent was usually included in the control.

d. Effect of Metal Ions

The following chloride salts were used to study the effect of a series of heavy metal ions on the purified enzyme: MgCl<sub>2</sub>, MnCl<sub>2</sub>, CoCl<sub>2</sub>, CaCl<sub>2</sub>, and HgCl<sub>2</sub>. Stock solutions (200 mM) of each of these salts were prepared in distilled deionized H<sub>2</sub>O. An aliquot of each stock solution, sufficient to give a concentration of 10 mM (2 mM for HgCl<sub>2</sub>) in a final assay volume of 1 ml, was brought, together with enzyme, to 0.9 ml in phos-EDTA pH 7.5 and assayed for BAEE activity as described in the standard assay above. Blanks contained the corresponding amount of metal ion concentration in each case (except for the HgCl<sub>2</sub> assay).

## 12. Time Course of Inactivation by DFP

The enzyme was preincubated with 0.1 M DFP in phospho-EDTA-0.5 M-NaCl-6.0 in a total volume of 0.04 ml for time periods ranging from 0 to 30 min. At each time point, the entire preincubation mixture was assayed for residual BAEE activity using the standard assay described above. Enzyme preincubated in the absence of DFP and in the presence of 2-isopropanol (the organic solvent used to dissolve and store DFP) was used as a control. The percent residual activity compared to the control was determined at each time point. The log percent residual activity was then plotted as a function of time of preincubation.

The concentration of DFP in the preincubation mixture was much greater than the concentration of the enzyme in each case. The reaction was therefore pseudo-first order and under these conditions, a plot of the log percent residual activity as a function of the time of preincubation was a straight line. The following equation was used to calculate the pseudo-first order rate constant ( $k_{\text{obsd}}$ ):

$$k_{\text{obsd}} = \frac{\ln \frac{e_1}{e_2}}{T_2 - T_1}$$

where  $e_1$  is the enzyme activity at time  $T_1$  and  $e_2$  is the enzyme activity at time  $T_2$ . The second order rate constant (expressed as  $\text{M}^{-1} \text{sec}^{-1}$ ) was obtained from  $\frac{k_{\text{obsd}}}{[\text{I}]}$ , where  $[\text{I}]$  is the concentration of DFP (Powers, 1977).

## 13. Molecular Weight Determination by High Performance

### Gel Permeation Chromatography

In determining the molecular weight of the enzyme by high performance gel permeation chromatography (Andrews, 1965), a Toya Soda TSK-gel SW 3000 column was used. The column was equilibrated overnight in phos-EDTA-6.0 buffer containing NaCl. Molecular weight determinations were made in the presence of either 0.05 M NaCl, 0.5 M NaCl, or 1.0 M NaCl. In each case the column was run at 7.2 ml/h and fractions were collected every 0.5 min. Fractions collected for the enzyme run were assayed for BAEE activity using the standard assay. Protein peaks were monitored at 214 nm and 280 nm. The following proteins were run as standards: rabbit muscle aldolase ( $M_r = 158,000$ ), bovine serum albumin ( $M_r = 68,000$ ), ovalbumin ( $M_r = 45,000$ ), B-amylase ( $M_r = 200,000$ ), glutamate dehydrogenase ( $M_r = 290,000$ ) and ribonuclease ( $M_r = 13,700$ ).

#### 14. Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel

##### Electrophoresis

Sodium dodecyl sulfate (SDS) gel electrophoresis was performed on the enzyme using the method of Laemmli (1970). A vertical discontinuous slab gel system was set up using an LKB vertical gel electrophoresis unit and an LKB high voltage power supply. The stock solutions needed for each of the gel components and the corresponding amounts used to prepare the gels is given in Appendix, Table A and Table B. Appendix, Table C details the buffers used for the

electrophoresis. The running gels (approx. 12 cm in length) contained 12.5% polyacrylamide. These were overlaid by a spacer component (approx 3.5 cm in length) containing 3% polyacrylamide. Enzyme samples (5-10 ug) were brought to 5 M urea and 2% SDS (and also 2% 2-mercaptoethanol for samples to be electrophoresed under reducing conditions) and heated to 100° C for 5 min. A few microliters of bromophenol blue dye solution were then added to the samples which were then layered into wells in the spacer gel. Electrophoresis was performed at room temperature at 10 ma per gel until the marker was 1 cm deep into the stacking gel. At this point the current was raised to 20 ma per gel and electrophoresis continued until dye had reached the lower edge of the gel. Gels were removed, and the migration distance of the dye was measured together with the length of the gel. They were then stained for 16 h in coomassie brilliant blue stain (0.1% coomassie blue, 50% methanol, 10% acetic acid). Destaining was done using several changes of a solution containing 7.5% acetic acid and 7.5% methanol.

A series of molecular weight standards were also electrophoresed in the gels in order to determine the molecular weight of the enzyme. The standards were prepared for electrophoresis as described in Sigma technical bulletin MWS-877 and brought to 6 M urea and 2% 2-mercaptoethanol and incubated at 37° C for 2 h. Aliquots were then stored at -20° C and thawed for use. The Dalton Mark VII-L mixture

(Sigma) containing the following standards was used: bovine albumin ( $M_r = 66,000$ ), egg albumin ( $M_r = 45,000$ ), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase ( $M_r = 36,000$ ), bovine erythrocyte carbonic anhydrase ( $M_r = 29,000$ ), bovine pancreas PMSF-treated trypsinogen ( $M_r = 24,000$ ), soybean trypsin inhibitor ( $M_r = 20,100$ ), bovine milk lactalbumin ( $M_r = 14,200$ ). The mobility of the enzyme and each standard in the gel was calculated as follows:

$$\text{mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration.}}$$

A plot of the mobilities as a function of log molecular weight was then used to determine the molecular weight of the enzyme.

#### 15. SDS Gel Electrophoresis of [ $^3\text{H}$ ]-DFP-labelled

##### Enzyme

The enzyme was incubated with 25  $\mu\text{M}$  [ $^3\text{H}$ ]-DFP at 25 $^{\circ}$ C in a total volume of 0.5 ml for 72 h in phos-EDTA-0.5 M-NaCl-6.0. Complete inhibition was further ensured by the addition of 2  $\mu\text{l}$  of 250 mM cold DFP. Removal of free [ $^3\text{H}$ ]-DFP was achieved by repeated dilution and filtration in a Centricon-30 microconcentrator unit. The enzyme was then subjected to SDS gel electrophoresis under both reducing and non-reducing conditions as described above. The gels were cut into 2 mm slices and each slice was placed into 0.3 ml 1 N NaOH for 12 h. Each NaOH extract was then diluted with

0.35 ml of 1 N HCl. This solution was then added to 10 ml of scintillation fluid (Ecoscint) in a vial and counted in a Beckman Model LS-7500 liquid scintillation counter.

#### 16. Isoelectric Focusing On Polyacrylamide Gel

Isoelectric focusing of the enzyme using an LKB Multiphor Flat Bed Electrophoresis Unit was carried out on precast polyacrylamide gels containing Ampholine carrier ampholytes. PAG-plates in the pH range 3.0 to 9.5 from LKB were used. The 0.5 mm gels were supported on a flat bed cooling plate maintained at 10<sup>0</sup> by an Lauda Thermostatic Circulator. Glycerol was used to effect thermal contact between the gel and the cooling plate. The enzyme was concentrated in phos-EDTA-6.0 buffer using a Centricon-30 microconcentrator unit, and spotted on top of the gel away from either electrode in 10-15 ul aliquots. 1 M NaOH was used as the cathode solution and 1 M H<sub>3</sub>PO<sub>4</sub> as the anode solution. The electrophoresis was carried out for 2 h with the following initial settings: voltage: 1500 V, current: 50 mA, and power: 30 W. An LKB high voltage stabilized power supply was used.

In order to ascertain the pI of the enzyme a mixture of proteins of known isoelectric points was spotted on the gel as described for the enzyme above and electrophoresed. A colored marker dye in this mixture served to monitor completion of migration of the standards and the enzyme. The migration distance of the dye from the cathode and the

length of the gel was recorded at the end of the experiment. The solutions used for fixing, staining, destaining and preserving the gel are described in Appendix, Table D. On completion of electrophoresis the PAG-plate was immediately placed in the fixing solution for 45 min, followed by destaining solution for 5 min. The gel was then stained with Coomassie brilliant blue staining solution preheated to 60° C. It was then destained with several changes of the destaining solution. The destained gel was then placed in preserving solution for 1 h, removed, dried at room temperature and stored.

The following standards of known pI were used in the experiment: amyloglucosidase (pI = 3.50), methyl red (pI = 3.75), soybean trypsin inhibitor (pI = 4.55), B-lactoglobulin A (pI = 5.13), bovine carbonic anhydrase B (pI = 5.65), human carbonic anhydrase B (pI = 6.57), myoglobin (pI = 6.76), L-lactic dehydrogenase (pI = 8.55) and trypsinogen (pI = 9.3).

#### 17. Active Site Titration

A stock solution of MUGB (10 mM) dissolved in DMF was prepared for use to titrate an aliquot of the purified enzyme (Jameson et al., 1973). Enzyme was brought to 0.9 ml in phos-EDTA-7.5 and 0.1 ml of the stock MUGB serially diluted in 0.001 N HCl to 0.1 mM was added to it to start the reaction. The release of the fluorescent methyl umbelliferone (MU) was monitored on a Perkin Elmer MPF-44B

fluorescence spectrophotometer. The excitation wavelength was 365 nm and the emission wavelength was 445 nm. The percent fluorescence corresponding to the enzyme was equal to the intercept on the y-axis obtained by extrapolating the linear portion following the "burst" in fluorescence due to the enzyme, back to the axis on a plot of percent fluorescence as a function of time (Hartley & Kilby, 1954, Gutfreund, 1955, Schonbaum et al., 1961) A stock solution of a sodium salt of MU (50  $\mu$ M) was also prepared in double distilled deionized H<sub>2</sub>O. The percent fluorescence of a series of dilutions of this stock was measured and plotted as a function of concentration. The concentration of the enzyme used for the experiment was then determined from this standard curve. The experiment was repeated for various concentrations of MUGB in the assay.

#### 18. Hydrolysis of Synthetic Substrates

More than 20 tri- and tetra p-nitroanilide substrates were assayed for amidolytic activity with tryptidase using a Multistat III<sup>R</sup> centrifugal analyzer, in order to determine the specificity of the enzyme. An aliquot of enzyme was inserted in one chamber of each cuvette, in the 20 cuvette rotor, while phos-EDTA-7.5 buffer was inserted in the other cuvette together with each substrate. The final volume for each assay was 250  $\mu$ l and concentration of the substrate in the assay was 1 mM. The rotor was first incubated at 30<sup>0</sup> for a few seconds during which time the contents of the two

compartments of each cuvette were mixed by centrifugal force. Changes in absorbance were then immediately monitored at 405 nm at intervals of 0.5 min for a period of 5.5 min. Linear regression plots of the data were then obtained by use of a computer program and the change in absorbance per min for each substrate was determined from the slope of the curve. This quantity was then used to calculate the activity of the enzyme using the formula for amidase activity given above.

Substrates which were most rapidly hydrolyzed by the pure enzyme were further used to assay the crude extract and the  $(\text{NH}_4)_2\text{SO}_4$  precipitation step described in the purification scheme, in order to find a more specific substrate for monitoring purification. The method followed for these latter assays is described in section 2b above.

#### 19. Hydrolysis of Larger Peptides

The following larger peptides were assayed for cleavage by the enzyme. In each case, 10 mM stock solutions of the peptide were prepared in distilled deionized water. The peptide was then diluted in phos-EDTA-0.5 M-NaCl-7.5 to 1 mM in a final assay volume of 0.1 ml. Enzyme was added and the reaction was incubated at 25<sup>0</sup> for 16-18 h. At the end of this period the reaction was terminated by the addition of 30 ul of 10 mM heptane sulfonic acid-phosphate, pH 2.5. The volume was brought to a total of 0.213 ml with assay buffer. The mixture was then filtered through an

Amicon PM-30 membrane and the cleaved fragments detected by HPLC. Controls included 1) an aliquot of enzyme incubated in the assay buffer but in the absence of peptide, and 2) identical concentration of peptide in the assay buffer in the absence of enzyme.

a. Vasopressin and Oxytocin

A uBondapak C<sub>18</sub> reversed-phase column (3.9 mm x 30 cm) (10 μm) and a Spectra Physics Model SP8700 solvent delivery system was used in order to separate cleaved products. The separation was performed isocratically using a mobile phase consisting of 0.1% trifluoroacetic acid (TFA) and acetonitrile in a ratio of 16:84 for vasopressin and 20:80 for oxytocin. Samples were injected by a Waters WISP Autosampler and the gradient flow rate was 1 ml/min. Peptides were detected using a UV absorbance monitor set at 206 nm. The following standards were run: vasopressin and desglycinamide vasopressin in the case of vasopressin, and oxytocin and desglycinamide oxytocin in the case of oxytocin.

b. Bradykinin

In the case of bradykinin, a C<sub>18</sub> Novapak, Radialpak cartridge (10 cm x 5 mm) (10 μm) was used. Potential products were separated using a gradient between the equilibration buffer, 10 mM heptanesulfonate-phosphoric acid, pH 2.55 (A), and methanol (B) (Orawski et al., 1987). The gradient was developed as follows: 0 min-100% A, 10 min-

95% A, 65 min-40% A. This system is capable of separating about 30 metabolites of bradykinin.

## 20. Protein Degradation Studies

The ability of the enzyme to cleave macromolecules was determined using casein, azocol, and haemoglobin. Casein was assayed using the sensitive casein agar plate assay. The details of the procedure are described in the Biorad bulletin accompanying the Biorad protease detection kit. In the assays for azocol and haemoglobin, stock solutions of the protein substrates, buffers and reagents and assay techniques were as described in detail in Bergmeyer (1983). The latter procedures involved the incubation of the enzyme with a given protein substrate at 25° for 30 min in potassium phosphate buffer pH 7.5. The mixture was then treated with trichloroacetic acid (TCA) (0.19 mM final concentration in the assay mixture) and thoroughly mixed. The precipitate was removed by centrifugation or filtration (Kunitz, 1947; Anson, 1938; Moore, 1969). The TCA soluble peptides were then measured by the method of Folin and Ciocalteu (1927).

## 21. Kinetic Studies

In order to determine the kinetic constants of the enzyme with respect to various substrates, each of the substrates in question was assayed on a Gilford Response Spectrophotometer at its characteristic wavelength as indicated above. Various concentrations of substrate (0.5

$K_m - 5 K_m$ ) were used and the change in absorbance over time was monitored. The initial velocities corresponding to each substrate concentration were then calculated by a built-in computer program on the spectrophotometer. Hanes-Woolf plots were then drawn for this data and the kinetic parameters determined using a computer program called Enzpak produced by Elsevier Publishing Co. (Hanes, 1932; Hanes et al., 1972).

## 22. Partial N-Terminal Sequence of Tryptidase

About 30 ug (based on Bradford protein assay) of pure enzyme was brought to doubly distilled deionized H<sub>2</sub>O by repeated filtration in a Centricon-30 microconcentrator unit. The sample was then passed through a 0.4 micron membrane filter and vacuum dried. Doubly distilled deionized H<sub>2</sub>O subjected to the same process with no enzyme was also prepared as a control. The amino terminal sequence of tryptidase through 25 residues was obtained through the courtesy of Applied Biosystems, Inc., Forest City, CA. The data was obtained by automated sequential Edman degradation using a 477A Pulsed Liquid Phase Protein/Peptide Sequencer.

## CHAPTER IV

### EXPERIMENTAL RESULTS

#### A. Pilot Studies for Purification Scheme

A number of pilot studies corresponding to possible steps in the purification scheme of tryptidase were done in order to set up optimal conditions for isolating the enzyme.

##### 1. Salt Extraction Study

The extractability of the BAEE activity from rat skin using two different concentrations of salt in neutral pH phos-EDTA buffer was studied. As can be seen from Table I no appreciable increase of BAEE activity occurred at 1.0 M NaCl in comparison with extraction in 0.5 M NaCl. Although a 1 M NaCl extraction had been used in our early work on tryptidase to determine some of the conditions for the purification scheme, the salt concentration used in the majority of the work reported here was therefore 0.5 M NaCl. Extraction of the enzyme in phos-EDTA-0.5 M-NaCl-pH 7.0 constitutes the first major step in the purification of tryptidase from rat skin. The choice of the lower concentration of salt has the advantage of preventing the extraction of most of the ATEE activity as shown in Table I and also decreases the volume of extract that must be processed in the subsequent  $(\text{NH}_4)_2\text{SO}_4$  step.

##### 2. Time Course of Extraction of BAEE Activity

TABLE I  
EXTRACTABILITY OF BAEE AND ATEE ACTIVITY  
FROM RAT SKIN BY NaCl

| SUBSTRATE | [NaCl] (M) | ACT. (U/ml) |
|-----------|------------|-------------|
| BAEE      | 0.5        | 0.044       |
|           | 1.0        | 0.045       |
| ATEE      | 0.5        | 0.027       |
|           | 1.0        | 0.150       |

Both BAEE and ATEE assays were done in duplicates on a Gilford Response spectrophotometer at 25<sup>0</sup> in an assay volume of 1 ml. Concentration for both substrates in the assay was 1 mM. Phos-EDTA-1 M NaCl pH 7.0 was used as the assay buffer. Assays for ATEE were carried out at 237 nm.

Skin tissue is rich in mast cells. In the event that the enzyme of interest to this project could be from this source, it was important to determine whether the enzyme yields could be increased by allowing for the degranulation of the mast cells over time. Attempts were therefore made to monitor the release of BAEE and ATEE activity over time into the homogenate. Table II contains the results of such an experiment. Over a period of eight hours no appreciable increase in the yield of BAEE or ATEE activity was seen in a 1 M NaCl extract. There was a 20% increase in BAEE activity but almost an 80% increase in ATEE activity over eight hours in the 0.5 M NaCl extract. It was therefore of advantage not to allow for any incubation period immediately after the homogenization step so that BAEE activity was not further contaminated by ATEE activity.

### 3. Pilot Study for Ammonium Sulfate Precipitation

With a view to concentrating down the BAEE activity and also achieving a measure of purification, a pilot ammonium sulfate study was undertaken. The results of such an experiment are portrayed in Figure 1. The overlap for the protein recovery curve and the BAEE activity recovery curve occurs at 40% and 70%. While these limits afforded an early practical starting point, the optimizing of recoveries, especially of activity in bulk studies, required extending the range to 25% to 80%.

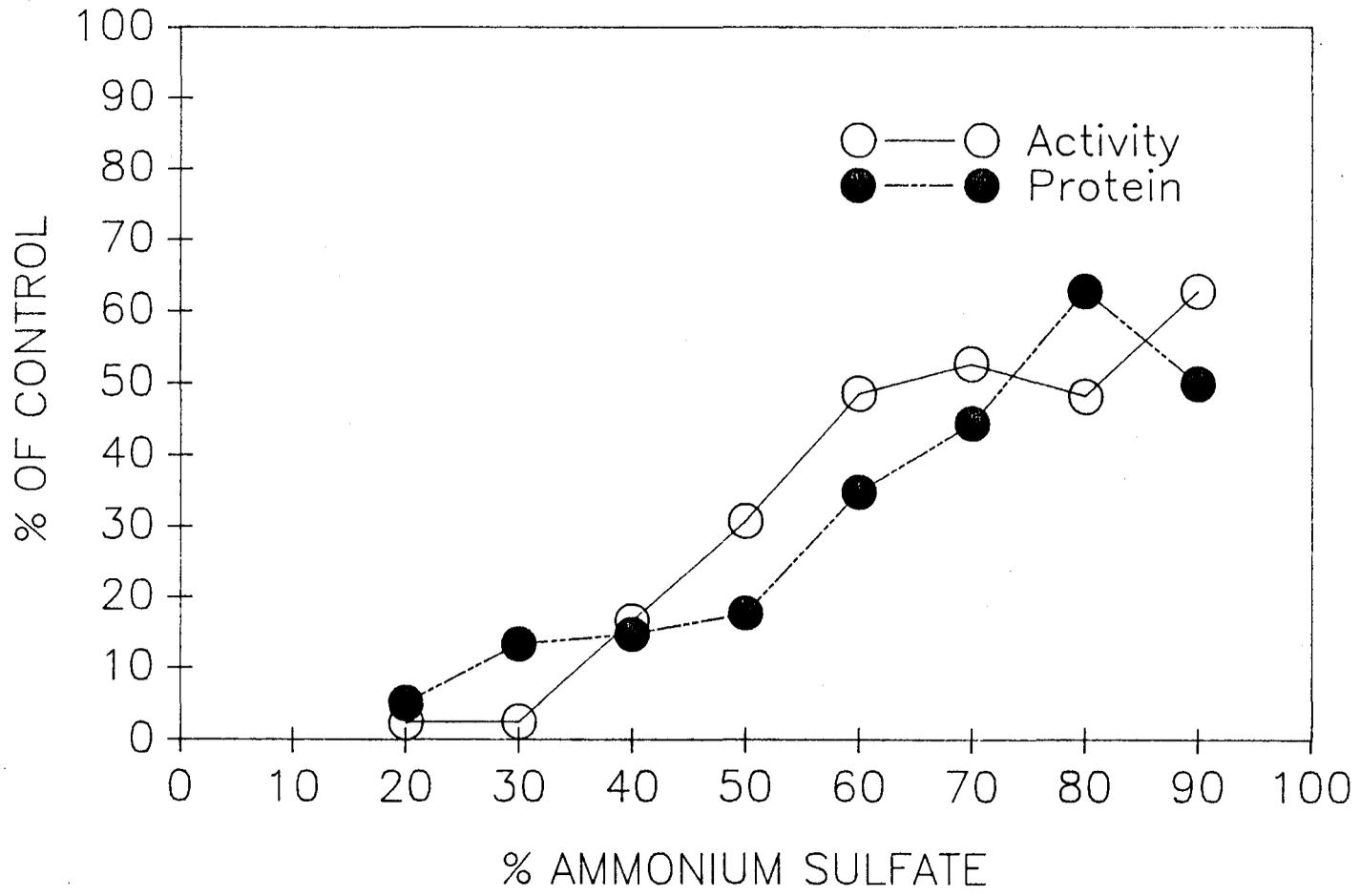
### 4. DEAE and CM Ion Exchange Chromatography

TABLE II  
 TIME COURSE OF EXTRACTION OF BAEE AND ATEE ACTIVITY  
 FROM RAT SKIN

| TIME (h) | BAEE Units/ml |          | ATEE Units/ml |          |
|----------|---------------|----------|---------------|----------|
|          | 0.5 M NaCl    | 1 M NaCl | 0.5 M NaCl    | 1 M NaCl |
| 0        | 0.044         | 0.045    | 0.027         | 0.150    |
| 2        | 0.045         | 0.047    | 0.031         | 0.143    |
| 4        | 0.046         | 0.052    | 0.037         | 0.148    |
| 6        | 0.051         | 0.049    | 0.026         | 0.140    |
| 8        | 0.054         | 0.048    | 0.046         | 0.132    |

Both BAEE and ATEE assays were conducted in phos-EDTA-1 M NaCl-7.0 buffer at 25<sup>0</sup> in 1 ml assay volumes on a Gilford Response spectrophotometer. Concentration for both substrates in the assay was 1 mM. Assays for ATEE were done at 237 nm. All assays were done in duplicate.

Figure 1. Pilot ammonium sulfate precipitation study. The experiment was performed using 50 ml aliquots of a 1 M NaCl, pH 7.0 extract filtered through a Buchner funnel to remove particulate matter. Aliquots were brought to 20, 40, 60, 70, 80 and 90 percent ammonium sulfate (Taylor, 1953) and were allowed to sit overnight at 4<sup>0</sup>. They were then centrifuged at 13,700 x g. The precipitate was resuspended in 25 ml phos-EDTA-6.0 buffer and assayed for BAEE activity (O) and protein (●) (Bradford, 1976). The activity and protein of the aliquot used for the experiment served as the control value. The % protein and % activity of the control were then plotted as a function of % ammonium sulfate.



Test tube studies (Pharmacia publication) were conducted on the BAEE activity to test the usefulness of ion exchange chromatography as a purification step. Precipitated enzyme from the 30-70% ammonium sulfate step was reconstituted in 0.5 M phosphate buffer, at either pH 6.0, 6.5, 7.0, 7.5 or 8.0. The data obtained (not shown) indicated that in experiments using DEAE A-50 ion exchange medium, over 90% of the BAEE activity adsorbed at pH 7.5. When CM-Sephadex A-50 chromatography medium was tested using acetate buffers at either pH 4.0, 4.5, 5 or 5.5, maximum adsorption occurred at pH 5.0. However, it was also found that about 50% of the BAEE activity was lost in converting to pH 5.0 before application to the medium. While ion exchange chromatography conditions were established at pH 6.0 for bulk experiments using a combination of DEAE and CM-Sephadex, the technique had the following disadvantages: a) the step necessitated the conversion of the enzyme preparation to a no NaCl state which resulted in significant loss of BAEE activity, b) equilibration, application, elution and reconcentration of eluent required an average of two days. Since a later experiment showed that it was possible to go to a specific affinity step directly, no further use was made of ion exchange chromatography in the final purification scheme proposed.

5. Agarose-Glycyl-Glycyl-p-Aminobenzamidine Affinity Chromatography Pilot Study

Since our initial goal was to purify a carboxamidopeptidase-like enzyme from rat skin, the p-aminobenzamidine-CH-Sepharose (PAB-CH-Sepharose) affinity column was tried using conditions described by Simmons & Walter (1981). However, while 34% of the activity from a previous ion exchange step adsorbed to the column, it was not possible to elute activity using 50 mM D-tryptophan methyl ester as described for the toad enzyme by the authors above.

Commercially available agarose-glycyl-glycyl-p-aminobenzamidine (A-Gly-Gly-PAB) (p-aminobenzamidine attached to agarose through a glycyl glycine spacer arm) was then obtained. It was decided to try benzamidine in the elution buffer to recover BAEE activity that adsorbed to this column. When initial results proved encouraging using 0.2 M benzamidine, a systematic study was conducted to optimize conditions. Table III indicates that while the overall recovery for this step is independent of the temperature at which the experiment is performed, temperature does have a significant impact on the purification achieved. The purification factor using room temperature is nearly 10-fold higher than that performed under identical conditions at 4<sup>0</sup>. The pH conditions are also seen to be an important factor in achieving better purifications. The purification achieved at pH 6.0 is nearly double that achieved at pH 7.0 when the experiment is performed at room temperatures. However at 4<sup>0</sup> the opposite trend is in effect, with the purification

TABLE III  
 AGAROSE-GLY-GLY-PAB AFFINITY COLUMN  
 PILOT STUDY

| pH <sup>a</sup> | TEMP.          | SP. ACT. <sup>b</sup><br>OF ELUTED ENZYME<br>(U/MG) | PURIF.<br>FACTOR | % APPLIED<br>ENZYME<br>ELUTED |
|-----------------|----------------|---|------------------|-------------------------------|
| 6.0             | 4 <sup>0</sup> | 3.84  | 48.1             | 27                            |
| 6.0             | RT             | 42.8  | 521              | 20                            |
| 7.0             | 4 <sup>0</sup> | 4.38  | 62.4             | 28                            |
|                 | RT             | 20.2  | 311              | 22                            |

<sup>a</sup>Enzyme was brought to the corresponding pH before application to the column at either pH. The buffer used at each pH was Phos-EDTA-0.5 M NaCl.

<sup>b</sup>BAEE activity and the Bradford protein assay were used to calculate the specific activity.

The enzyme was eluted with 0.1 M benzamidine in the application buffer. Fractions containing BAEE activity were pooled and benzamidine was removed by ultrafiltration through a PM-30 Amicon membrane before estimation of BAEE activity.

factor being about 50% higher.

Based on this pilot study it was decided to purify the enzyme using A-Gly-Gly-PAB specific affinity chromatography at pH 6.0 and room temperature. While the column is maintained at room temperature, the enzyme reservoir is kept on an ice bath to prevent proteolytic loss or temperature denaturation of the BAEF activity before adsorption to the column. Collection of eluent fractions was also done at 4<sup>0</sup> for the same reasons.

Application of the reconstituted dialyzed ammonium sulfate precipitate directly to the column did not reduce the degree of purification achieved or the overall recovery of the enzyme. This made it possible to skip the ion exchange step.

#### 6. Concanavalin-A Agarose Affinity Chromatography

Since carboxamidopeptidase from toad skin (Simmons & Walter, 1981) was known to be a glycoprotein, and our original focus was to identify and isolate this enzyme in rat skin, concanavalin-A agarose affinity chromatography was tried on the BAEF activity eluted from the A-Gly-Gly-PAB column. Adsorption of the enzyme to the column was achieved in 25mM phosphate buffer at pH 6.0 in the presence of 0.5 M NaCl. The enzyme was eluted with the same buffer containing 100 mg/ml  $\alpha$ -methyl-D-mannoside .

#### B. Final Purification Scheme

A final purification scheme utilizing the information

derived from numerous pilot studies, some of which were discussed above, was developed which could yield an enzyme preparation which was homogeneous. The procedure is outlined in the flow chart given in Table IV. The purification of the enzyme is achieved in just two major steps. Following homogenization of the skin tissue, the first major step consists of the 25-80% ammonium sulfate precipitation followed by dialysis. The second major step is specific affinity chromatography utilizing two sequential columns of different specificity. The first column is the A-Gly-Gly-PAB column which has affinity for the active site of tryptidase and the second column is con A-agarose which binds carbohydrate moieties attached to the enzyme. Since the specific affinities of the two columns are directed to different parts of the enzyme molecule, it was possible to couple the two columns as indicated in Table IV. The 1 mM EDTA which was added to the buffers in the earlier part of the purification scheme was eliminated, since EDTA would remove the calcium and magnesium ions required for the binding properties of concanavalin A.  $\text{NaN}_3$  (0.02%) was used instead as a bacteriostatic agent in the column buffers.

#### 1. Purification of Tryptidase

Results of two representative purifications of tryptidase are expressed in Tables V and VI and are based on BAEE activity rather than on activity of more specific substrates (see below).

TABLE IV

PURIFICATION SCHEME FOR TRYPTIDASE

## I. TISSUE PREPARATION AND HOMOGENIZATION

- a. Removal of fur, muscle tissue and fatty layer, and fascia
- b. Mincing: twice through # 3 meat grinder
- c. Homogenization in phos-EDTA-0.5 M NaCl-7.0 (1:9 v/v)
- d. Filtration through nylon mesh and Buchner funnel under vacuum

II. 25-80%  $(\text{NH}_4)_2\text{SO}_4$  PRECIPITATION

- a. Filtrate brought to 25%  $(\text{NH}_4)_2\text{SO}_4$ , allowed to sit for 4-6 h and centrifuged at 13,700 x g.
- b. Supernatant brought to 80%  $(\text{NH}_4)_2\text{SO}_4$ , left unstirred for 14-16 h and then centrifuged as above.
- c. Reconstitution of 80% ppt. in phos-EDTA-0.5 M NaCl-6.0
- d. Dialysis against reconstitution buffer
- e. Centrifugation of dialysate. Supernatant saved.

## III. SPECIFIC AFFINITY CHROMATOGRAPHY

- a. Application of sample to Gly-Gly-PAB-agarose column at RT followed by wash with equilibration buffer.
- b. Equilibration of column to 4° followed by a 2<sup>nd</sup> wash and finally elution with phos- $\text{NaN}_3$ -0.5 M NaCl-6.0 containing 0.1 M benzamidine.
- c. Eluent pumped onto concanavalin A-agarose column
- d. Columns disconnected and concanavalin A column washed with phos- $\text{NaN}_3$ -0.5 M NaCl-6.0, followed by elution with the same buffer containing 100 mg/ml  $\alpha$ -methyl-D-mannoside.

## IV. CONCENTRATION AND STORAGE OF ENZYME

- a. Conversion to phos-EDTA-0.5 M NaCl-6.0 in Amicon microconcentrators.
- b. Storage at 4°.

TABLE V  
PURIFICATION OF TRYPTIDASE BASED ON BAAE ACTIVITY

| Treatment                    | Total Activity (U) <sup>a</sup> | Total Protein (mg) <sup>b</sup> | Specific Act. (U/mg) | Percent Recovery | Purification Factor |
|------------------------------|---------------------------------|---------------------------------|----------------------|------------------|---------------------|
| 13,700 x g<br>Supernatant    | 252                             | 5460                            | 0.046                | 100              | 1                   |
| 25-80% Amon<br>Sulf. Ppt.    | 140                             | 2160                            | 0.065                | 55.6             | 1.4                 |
| Gly-Gly-PAB;<br>Con A Affin. | 15.8                            | 0.14                            | 117.0                | 6.3              | 2550                |

<sup>a</sup>All assays were performed at 25° C.

<sup>b</sup>Bradford microassay was used to estimate protein.

TABLE VI  
PURIFICATION OF TRYPTIDASE BASED ON BAEE ACTIVITY

| Treatment                    | Total Activity (U) <sup>a</sup> | Total Protein (mg) <sup>b</sup> | Specific Act. (U/mg) | Percent Recovery | Purification Factor |
|------------------------------|---------------------------------|---------------------------------|----------------------|------------------|---------------------|
| 13,700 x g<br>Supernatant    | 153                             | 3320                            | 0.046                | 100              | 1                   |
| 25-80% Amon<br>Sulf. Ppt.    | 86.8                            | 1500                            | 0.058                | 56.8             | 1.3                 |
| Gly-Gly-PAB;<br>Con A Affin. | 12.1                            | 0.073                           | 166                  | 7.9              | 3610                |

<sup>a</sup>All assays were performed at 25° C.

<sup>b</sup>Bradford microassay was used to estimate protein.

a. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> Fractionation

A significant proportion of the BAEE activity is lost in the 25-80% ammonium sulfate precipitation step. The loss seems to occur mainly in the 80% step since the 25% supernatant was seen to repeatedly contain 85-90% of the original activity. No significant purification is achieved in this step. However, it does serve to eliminate over 50% of the contaminating protein and results in significant concentration of the enzyme which is required for the subsequent steps.

b. Specific Affinity Chromatography

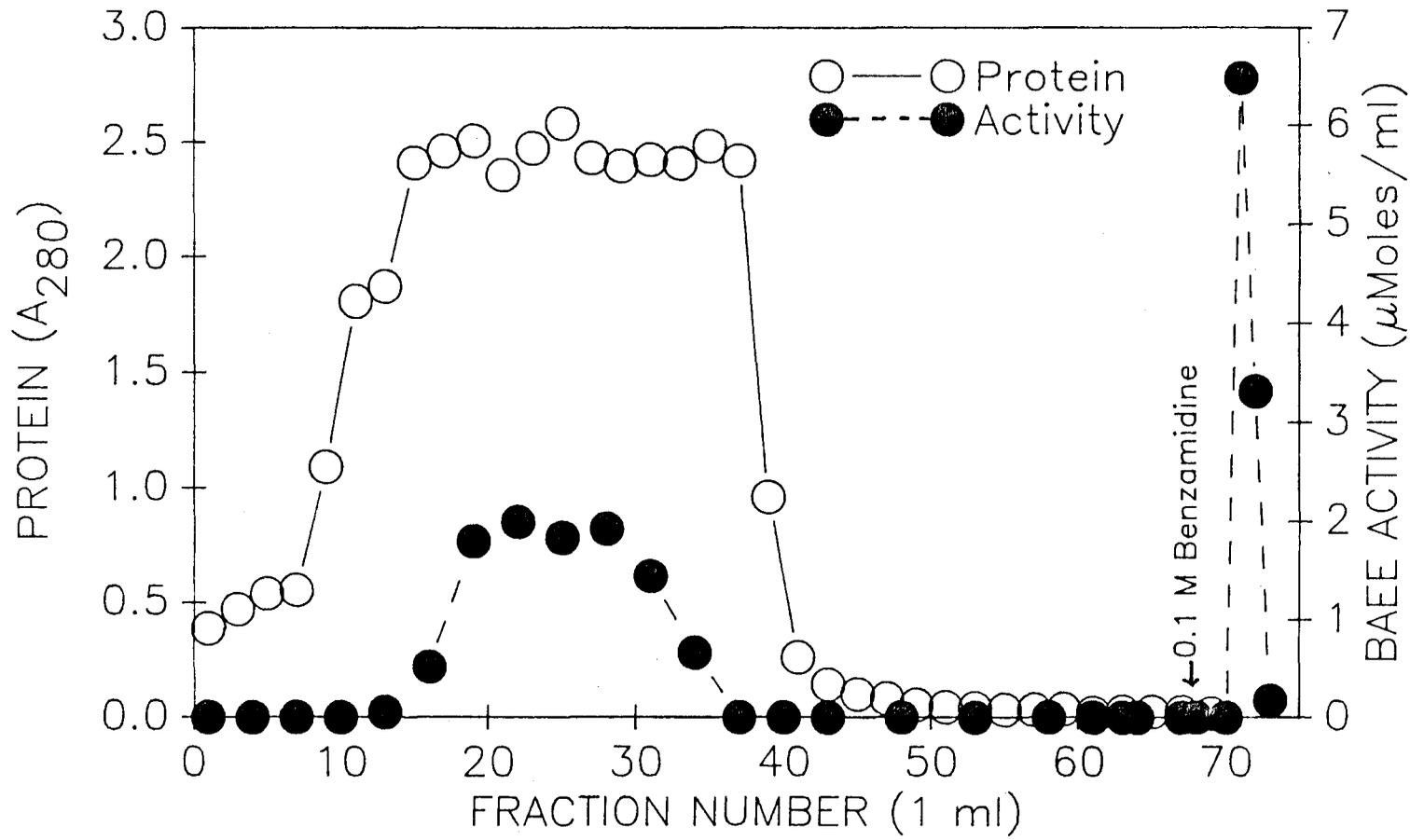
i. A-Gly-Gly-PAB-Chromatography

The affinity of tryptidase for the A-Gly-Gly-PAB-column is demonstrated in Figure 2. The bulk of contaminating protein as well as non-tryptidase BAEE activity flowed through the column when a concentrated enzyme solution from a previous step was applied in phos-EDTA-0.5 M-NaCl-6.0 buffer. Only about 20-30% of the applied BAEE activity bound to the column. Most of the adsorbed activity could be eluted with 0.1 M benzamidine. In Figure 2, the eluted enzyme was collected in fractions and assayed to give a complete chromatogram. Normally, however, the eluted enzyme was pumped directly onto the con A-agarose column.

ii. Concanavalin-A-Agarose Chromatography

When enzyme solution from the A-Gly-Gly-PAB column

Figure 2. Agarose-Glycyl-Glycyl-p-Aminobenzamide chromatography. Enzyme from the previous purification step was applied at a flow rate of 25 ml/h to a 1.5 x 20 cm agarose-glycyl-glycyl-p-aminobenzamide column equilibrated with phos-EDTA-0.5 M NaCl-6.0 and 1 ml fractions were collected. The enzyme reservoir was maintained at 4<sup>0</sup>C on an icebath and while the column itself was kept at room temperature. After application of the enzyme, the column was washed with 1.5 column volumes of the equilibration buffer. The enzyme was then eluted with the same buffer containing 0.1 M benzamide.HCl and 1 ml fractions were collected. Protein (Bradford, 1976) (O); BAEE activity (●).



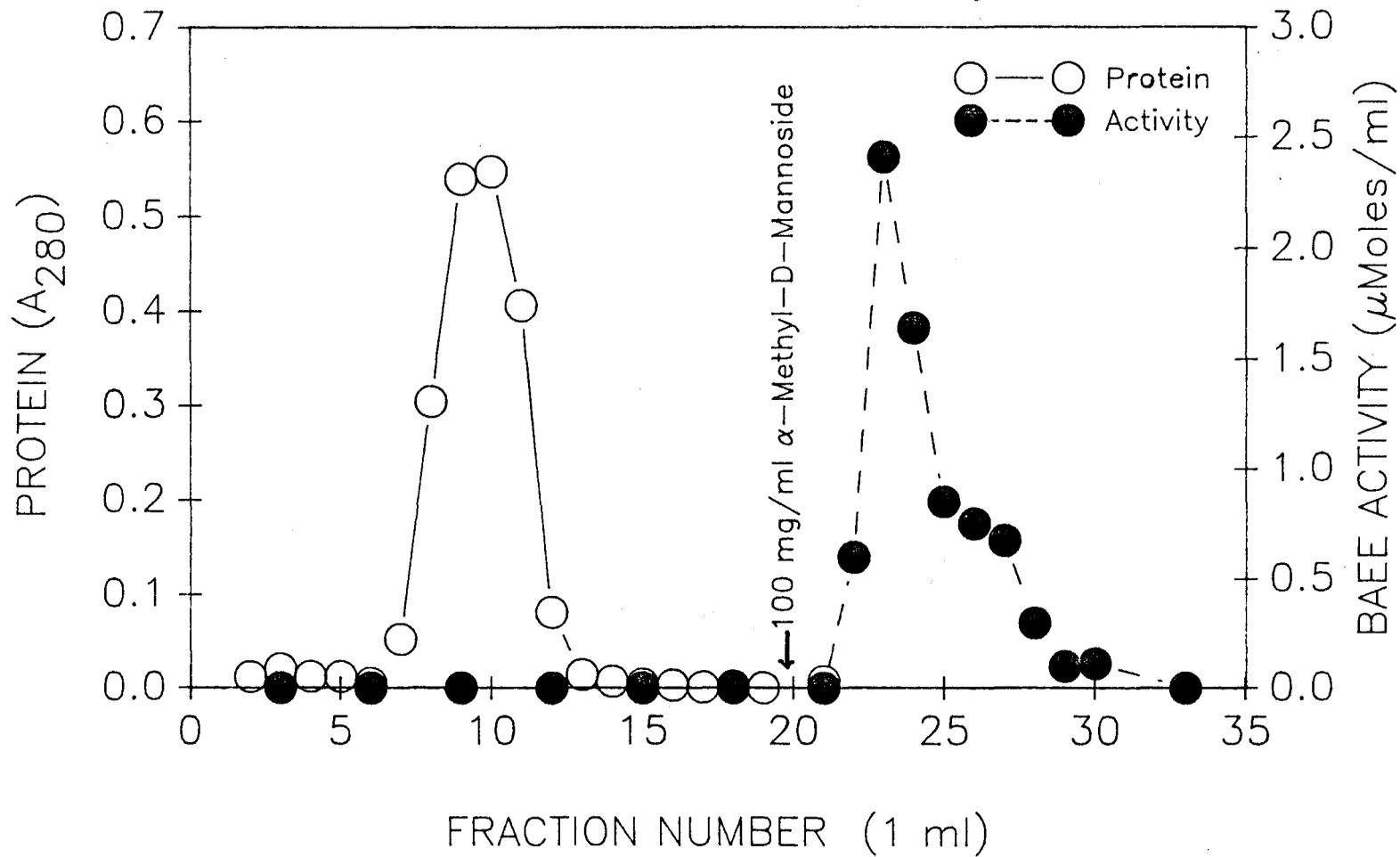
was concentrated and then applied to a con A-agarose column in 25 mM phos-0.5 M-NaCl-6.0, the majority of the BAEE activity was retained on the column while inactive protein eluted in the breakthrough peak (Figure 3). Upon applying 100 mg/ml of  $\alpha$ -methyl-D-mannoside in equilibration buffer to the column, tryptidase was eluted in an initial sharp peak followed by some trailing.

The overall purification achieved in this two step purification, based on BAEE activity, is in the range 2500 to 3600 with overall recoveries ranging from 6% to 9%.

c. Purification Based on Correction for Non-tryptidase BAEE activity

Mention was made above of the fact that the bulk of BAEE activity passed through the A-Gly-Gly-PAB column. In order to ascertain that this effect was not due to overloading of the column, the A-Gly-Gly-PAB column was subjected to the routine cleaning procedure consisting of two column volumes of phos-EDTA-0.5 M-NaCl-6.0 buffer containing 8 M urea followed by at least four column volumes of the buffer without urea. The pooled breakthrough was then applied to the column using conditions identical to those for the original enzyme solution application. Fractions were collected and assayed for BAEE activity. It was found that no adsorption of activity to the column occurred. All of the activity could be accounted for in the breakthrough. Furthermore, benzamidine failed to elute any

Figure 3. Concanavalin A-agarose chromatography. The enzyme preparation from the agarose-glycyl-glycyl-p-aminobenzamide chromatography step was concentrated and applied at a flow rate of 10 ml/h to a 0.75 x 10 cm concanavalin A-agarose column equilibrated in 25 mM phosphate-0.5 M NaCl-pH 6.0 buffer, and 1 ml fractions were collected. The column was then washed with 1 column volume of the equilibration buffer and eluted in the same buffer containing 100 mg/ml  $\alpha$ -methyl-D-mannoside. The flow rate was decreased to 5 ml/h. After passing through 1 column volume of the elution buffer, the flow was stopped and the column was allowed to incubate for 1 h. Elution was then continued. The entire experiment was carried out at 4<sup>0</sup> C. Protein (Bradford, 1976) (O); BAEE activity (●).



additional activity from the column.

Since the breakthrough of the A-Gly-Gly-PAB column contains non-tryptidase BAEE activity, the results shown in Table VII are reconfigured to reflect this. In this table, the breakthrough BAEE activity from the A-Gly-Gly-PAB column is subtracted from the values for both the 13,600 x g supernatant and the ammonium sulfate precipitation steps to give activity values which more nearly approximate the actual tryptidase activity present at the end of the two steps. When the calculations are redone using such an approach, the enzyme is seen to be purified 6900-fold with a recovery of nearly 16%.

d. Purification Based on More Selective Synthetic Substrates

The experiments described above made it apparent that the homogenate from rat skin contained more than one BAEE hydrolyzing enzyme. In order to monitor the purification of tryptidase more accurately a more selective substrate was necessary. Several different peptidyl-p-nitroanilides were therefore tested as potential substrates using a purified preparation of the enzyme. A number of these p-nitroanilides were observed to be hydrolyzed. Aliquots from each step of one purification were then assayed for their ability to cleave 20 of these peptides. Of these, the nine listed in Table VIII held promise as selective substrates for tryptidase. All nine yielded final recoveries for the

TABLE VII  
 PURIFICATION OF TRYPTIDASE BASED ON BAEE  
 AND CORRECTED FOR BREAKTHROUGH ACTIVITY

| Treatment                 | Total Activity (U) <sup>a, c</sup> | Total Protein (mg) <sup>b</sup> | Specific Act. (U/mg) | Percent Recovery | purification Factor |
|---------------------------|------------------------------------|---------------------------------|----------------------|------------------|---------------------|
| 13,700 x g Supernatant    | 77.8                               | 3320                            | 0.024                | 100              | 1                   |
| 25-80% Amon Sulf. Ppt.    | 11.7                               | 1500                            | 0.008                | 15.1             | 0.3                 |
| Gly-Gly-PAB; Con A Affin. | 12.1                               | 0.073                           | 166                  | 15.6             | 6920                |

<sup>a</sup>All assays were performed at 25° C.

<sup>b</sup>Bradford microassay was used to estimate proteins.

<sup>c</sup>Activity reflects breakthrough activity subtracted from total activity measured.

TABLE VIII  
SELECTIVE SUBSTRATES FOR MONITORING  
PURIFICATION OF TRYPTIDASE

| SUBSTRATE         | TOTAL ACTIVITY (Units) <sup>a</sup> |          |      | % RE-<br>COVERY<br>(overall) |
|-------------------|-------------------------------------|----------|------|------------------------------|
|                   | 13,700 x g<br>Supernatant           | 80% Ppt. | Pure |                              |
| S-2238            | 153                                 | 79.3     | 36.4 | 23.8                         |
| S-2251            | 69.76                               | 10.94    | 5.3  | 7.6                          |
| S-2266            | 151.88                              | 36.5     | 13.0 | 8.6                          |
| S-2288            | 418.26                              | 205.7    | 90.0 | 21.5                         |
| S-2444            | 164.2                               | 72.4     | 32.1 | 19.6                         |
| Chromozym Try     | 49.3                                | 14.8     | 8.1  | 16.5                         |
| Chromozym P       | 199.97                              | 96.9     | 43.6 | 21.8                         |
| Spectrozyme<br>Ca | 270.8                               | 145.37   | 77.6 | 28.7                         |
| S-2222            | 98.7                                | 51.8     | 21.8 | 22.04                        |

Assays were performed in a Gilford Response spectrophotometer at 405 nM at 25<sup>0</sup>, and were run for 1 min.

S-2238, S-2251, S-2266, S-2288 and S-2222 were 1mM in the assay, while S-2444, Spectrozyme Ca, and Chromozym P was 0.5 mM and Chromozym Try was 0.25 mM.

S-2238 = H-D-Phe-pip-Arg-pNA; S-2251 = H-D-Val-Leu-Lys-pNA; S-2266 = H-D-Val-Leu-Arg-pNA; S-2288 = H-D-Ile-Pro-Arg-pNA; S-2444 = Pyro-Glu-Gly-Arg-pNA; Chromozym Try = H-D-Val-Gly-Arg-pNA; Chromozym PL = Tos-Gly-Pro-Lys-pNA; Spectrozyme Ca = H-D-Pro-Pro-Arg-pNA; S-2222 = Bz-Ile-Glu(-O-R)Gly-Arg-pNA. R = CH<sub>3</sub> or H

<sup>a</sup>Each value is a mean of duplicate assays.

enzyme that were better than when BAEE was used as a substrate. Three of the substrates, S-2238, S-2288 and Chromozym PL, as well as Spectrozyme PCa, were then selected for monitoring two additional tryptidase purifications. The data is shown in Tables IX and X. Chromozym PL, and S-2288 yielded the highest recoveries and purification factors. Both were more selective than S-2238, since the breakthrough from the A-Gly-Gly-PAB column contained less than 10% of the activity put on with respect to Chromozym PL and S-2288 but over 35% with respect to S-2238. This is also reflected in the final recoveries and purification factors for each of them. The mean recovery from the two enzyme preparations based on Chromozym PL 29%, while S-2288 gave 35% and S-2238, 22%. The average of purification factor for Chromozym PL was 10,600, for S-2288, 12,400 and for S-2238, 8000. By comparison BAEE gave 7% recovery and only 3080-fold purification (mean based on Tables V and VI).

The results using Spectrozyme PCa in both tables are disappointing when compared with the results for Spectrozyme Ca shown in Table VIII. The reason for the disparity in results is evident from the fact that the two substrates have somewhat different sequences. The sequence for Spectrozyme Ca is H-D-Pro-Pro-Arg-pNA while that for Spectrozyme PCa is Tos-Lys-(Cbo)-Pro-Arg-pNA. It was not possible to obtain additional quantities of Spectrozyme Ca because the manufacturer had substituted it with Spectrozyme

TABLE IX  
PURIFICATION OF TRYPTIDASE BASED ON p-NA SUBSTRATES

| Treatment                 | Substrate               | Total Activity (U) <sup>a</sup> | Total Protein (mg) <sup>b</sup> | Specific Act. (U/mg) | Percent Recovery | Purification Factor |
|---------------------------|-------------------------|---------------------------------|---------------------------------|----------------------|------------------|---------------------|
| 13,700 x g Supernatant    | Chromo. PL <sup>c</sup> | 142                             | 3320                            | 0.043                | 100              | 1                   |
|                           | Spec. PCa <sup>d</sup>  | 1000                            | "                               | 0.303                | "                | "                   |
|                           | S-2288 <sup>e</sup>     | 340                             | "                               | 0.102                | "                | "                   |
|                           | S-2238 <sup>f</sup>     | 326                             | "                               | 0.098                | "                | "                   |
| 25-80% Amon Sulf. Ppt.    | Chromo. PL              | 85.5                            | 1500                            | 0.057                | 60.3             | 1.3                 |
|                           | Spec. PCa               | 314                             | "                               | 0.209                | 31.3             | 0.7                 |
|                           | S-2288                  | 197                             | "                               | 0.131                | 58.0             | 1.3                 |
|                           | S-2238                  | 227                             | "                               | 0.151                | 69.6             | 1.5                 |
| Gly-Gly-PAB; Con A Affin. | Chromo. PL              | 37.8                            | 0.073                           | 518                  | 26.6             | 12000               |
|                           | Spec. PCa               | 68.0                            | "                               | 932                  | 6.8              | 3070                |
|                           | S-2288                  | 95.7                            | "                               | 1310                 | 28.1             | 12800               |
|                           | S-2238                  | 61.8                            | "                               | 847                  | 18.9             | 8600                |

<sup>a</sup>All assays were performed at 25<sup>0</sup> in a Gilford Response spectrophotometer

<sup>b</sup>Bradford microassay was used to estimate proteins.

<sup>c</sup>Chromo. PL = CBZ-Gly-Pro-Lys-pNA

<sup>d</sup>Spec. PCa = Tos-Lys-(CHO)-Pro-Arg-pNA

<sup>e</sup>S-2288 = H-D-Ile-Pro-Arg-pNA

<sup>f</sup>S-2238 = H-D-Phe-pip-Arg-pNA

TABLE X  
PURIFICATION OF TRYPTIDASE BASED ON p-NA SUBSTRATES

| Treatment                 | Substrate               | Total Activity (U) <sup>a</sup> | Total Protein (mg) <sup>b</sup> | Specific Act. (U/mg) | Percent Recovery | Purification Factor |
|---------------------------|-------------------------|---------------------------------|---------------------------------|----------------------|------------------|---------------------|
| 13,700 x g Supernatant    | Chromo. PL <sup>c</sup> | 135                             | 2240                            | 0.060                | 100              | 1                   |
|                           | Spec. PCa <sup>d</sup>  | 682                             | "                               | 0.305                | "                | "                   |
|                           | S-2288 <sup>e</sup>     | 283                             | "                               | 0.127                | "                | "                   |
|                           | S-2238 <sup>f</sup>     | 319                             | "                               | 0.143                | "                | "                   |
| 25-80X Amon Sulf. Ppt.    | Chromo. PL              | 87.7                            | 1320                            | 0.067                | 65.1             | 1.1                 |
|                           | Spec. PCa               | 276                             | "                               | 0.210                | 40.5             | 0.7                 |
|                           | S-2288                  | 236                             | "                               | 0.179                | 83.4             | 1.4                 |
|                           | S-2238                  | 217                             | "                               | 0.165                | 68.0             | 1.2                 |
| Gly-Gly-PAB; Con A Affin. | Chromo. PL              | 41.6                            | 0.076                           | 547                  | 30.9             | 9120                |
|                           | Spec. PCa               | 71.7                            | "                               | 943                  | 10.5             | 3090                |
|                           | S-2288                  | 116                             | "                               | 1520                 | 40.9             | 12000               |
|                           | S-2238                  | 80.7                            | "                               | 1060                 | 25.3             | 7400                |

<sup>a</sup>All assays were performed at 25<sup>0</sup> in a Gilford Response spectrophotometer

<sup>b</sup>Bradford microassay was used to estimate proteins.

<sup>c</sup>Chromo. PL = CBZ-Gly-Pro-Lys-pNA

<sup>d</sup>Spec. PCa = Tos-Lys-(CHO)-Pro-Arg-pNA

<sup>e</sup>S-2288 = H-D-Ile-Pro-Arg-pNA

<sup>f</sup>S-2238 = H-D-Phe-pip-Arg-pNA

PCa.

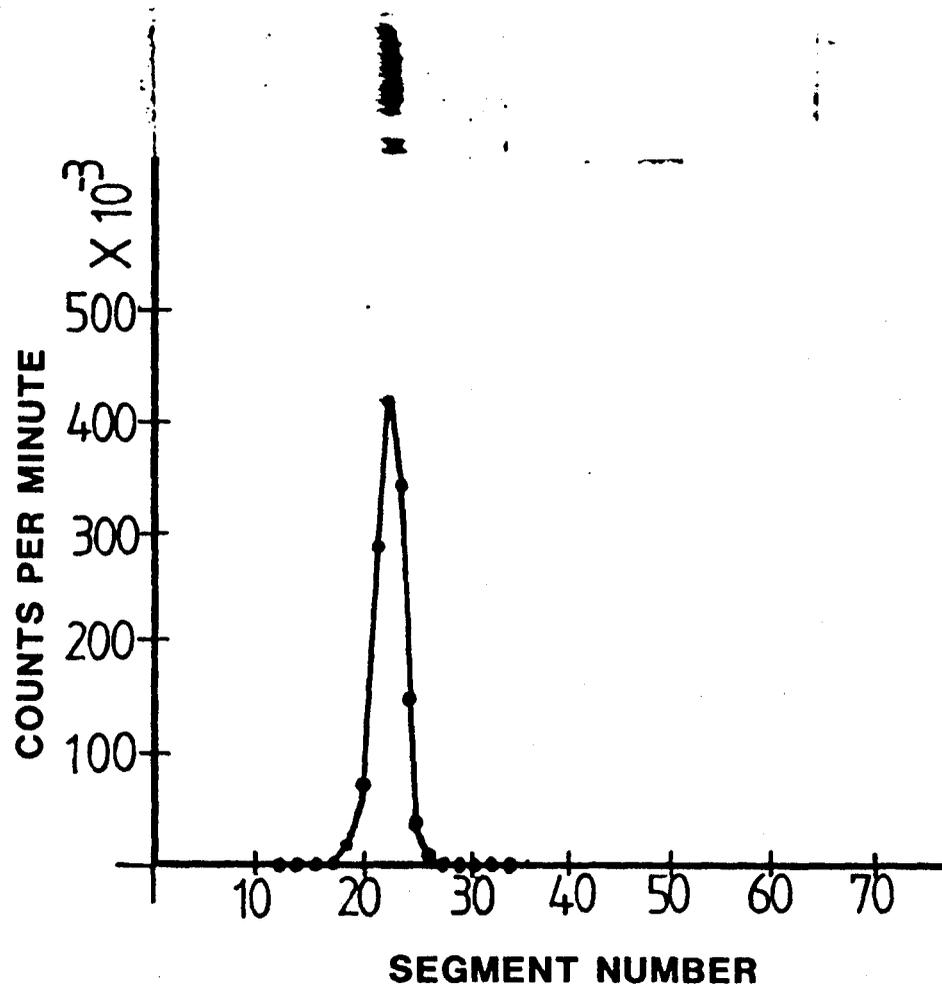
e. Purity of Tryptidase

In order to determine the purity of the tryptidase obtained through the purification scheme described, some of the enzyme was subjected to SDS polyacrylamide gel electrophoresis in 12.5% gels according to the method of Laemmli (1970). Figure 4 shows a single band for tryptidase under both reducing (lane A) and non-reducing (lane B) conditions. Visualization of the gels was achieved by coomassie brilliant blue staining.

Inhibitor studies had shown that the enzyme was inactivated by DFP. The enzyme was therefore labelled with [<sup>3</sup>H]-DFP and then subjected to SDS electrophoresis as described in METHODS. The gels were dissected into 2 mm sections which were then processed as described in METHODS to release the radioactive label and counted using a liquid scintillation counter. Figure 5 shows that the peak of radioactivity corresponded with the single protein band seen by Coomassie brilliant blue staining. The results indicate therefore that the stained band is, in fact, tryptidase and that the purification scheme therefore results in apparently homogeneous enzyme as determined by this technique. Symmetrical single protein peaks (A<sub>214</sub>) were also seen when tryptidase was subjected to analytical high performance gel permeation chromatography using both low and high salt conditions (see below). This is further corroboration of

Figure 4. Determination of purity and molecular weight of tryptidase by SDS gel electrophoresis. The enzyme was electrophoresed in SDS polyacrylamide gels under both reducing (lane A) and non-reducing (lane B) conditions as described in METHODS. Molecular weight markers were a) bovine albumin, b) egg albumin, c) glyceraldehyde-3-phosphate dehydrogenase, d) carbonic anhydrase, e) trypsinogen, f) soybean trypsin inhibitor and g)  $\alpha$ -lactalbumin. The mobility of the markers was calculated as described in METHODS and plotted against their corresponding log molecular weight to generate a standard curve.

Figure 5. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of radiolabeled tryptidase. SDS gel electrophoresis was performed as described in METHODS using non-reducing conditions. <sup>3</sup>H-DFP was used to prelabel the enzyme. Gels were stained for protein using Coomassie brilliant blue. The gel was then cut into 2 mm sections, extracted as described in METHODS, and counted for radioactivity.



the apparent homogeneity of the enzyme. When the enzyme was recently subjected to microbore reversed phase HPLC in preparation for N-terminal sequencing, two peaks were detected. Since the concanavalin A affinity of the enzyme establishes its glycoprotein nature, the two peaks possibly differ with respect to their carbohydrate moieties. The microheterogeneity detected during isoelectric focussing of the enzyme supports such a view (see below). When a mixture of the two peaks were sequenced to identify the first 25 amino acids of the N-terminal of the enzyme, a homologous sequence was obtained, substantiating that the two peaks corresponded to one and the same enzyme, and verifies the homogeneity of the tryptidase purified.

## 2. Storage Conditions for Tryptidase

The purified enzyme is stable for prolonged periods of time extending over six months in phos-EDTA-0.5 M-NaCl-6.0 at 4<sup>0</sup>. One attempt to preserve the enzyme at 4<sup>0</sup> in phosphate-EDTA buffer at neutral pH without NaCl proved costly, since the preparation assayed after three months had lost more than 50% of its activity. On other occasions when experiments with tryptidase required the elimination of salt from the preparation, even overnight storage of the enzyme without NaCl at pH 6.0 was found to result in severe losses of activity. SDS gel electrophoresis failed to reveal any autolysis fragments, although the system was incapable of detecting molecular weight fragments smaller than 11,000

daltons. A significant band was seen corresponding to that for active enzyme and may indicate that the enzyme undergoes slow denaturation without autolysis.

Tryptidase also proved to be stable at room temperature in phos-EDTA-0.5 M-NaCl-6.0 buffer in tests done for time periods ranging up to eight days. The enzyme can also survive freezing at  $-20^{\circ}$ . In fact repeated freezing and thawing of the enzyme through six 24 h cycles had no significant effect on the activity of the enzyme.

Table XI presents the results of some of the other storage conditions tried on the enzyme. The higher activities seen on day 1 compared to day 0 presumably represent more complete reconstitution of dried enzyme by day 1. Vacuum drying in the presence of 2% 20 M Carbowax using a Savant Speed-vac concentrator resulted in survival of approximately 80% activity in contrast to lyophilization where only 51% survived. The vacuum dried enzyme was just as stable at room temperature as at  $4^{\circ}$ . Tryptidase seems to survive freezing at  $-70^{\circ}$  since up to 80% of activity was recovered after freezing in an acetone-dry ice bath.

### 3. Effect of NaCl on Tryptidase Activity

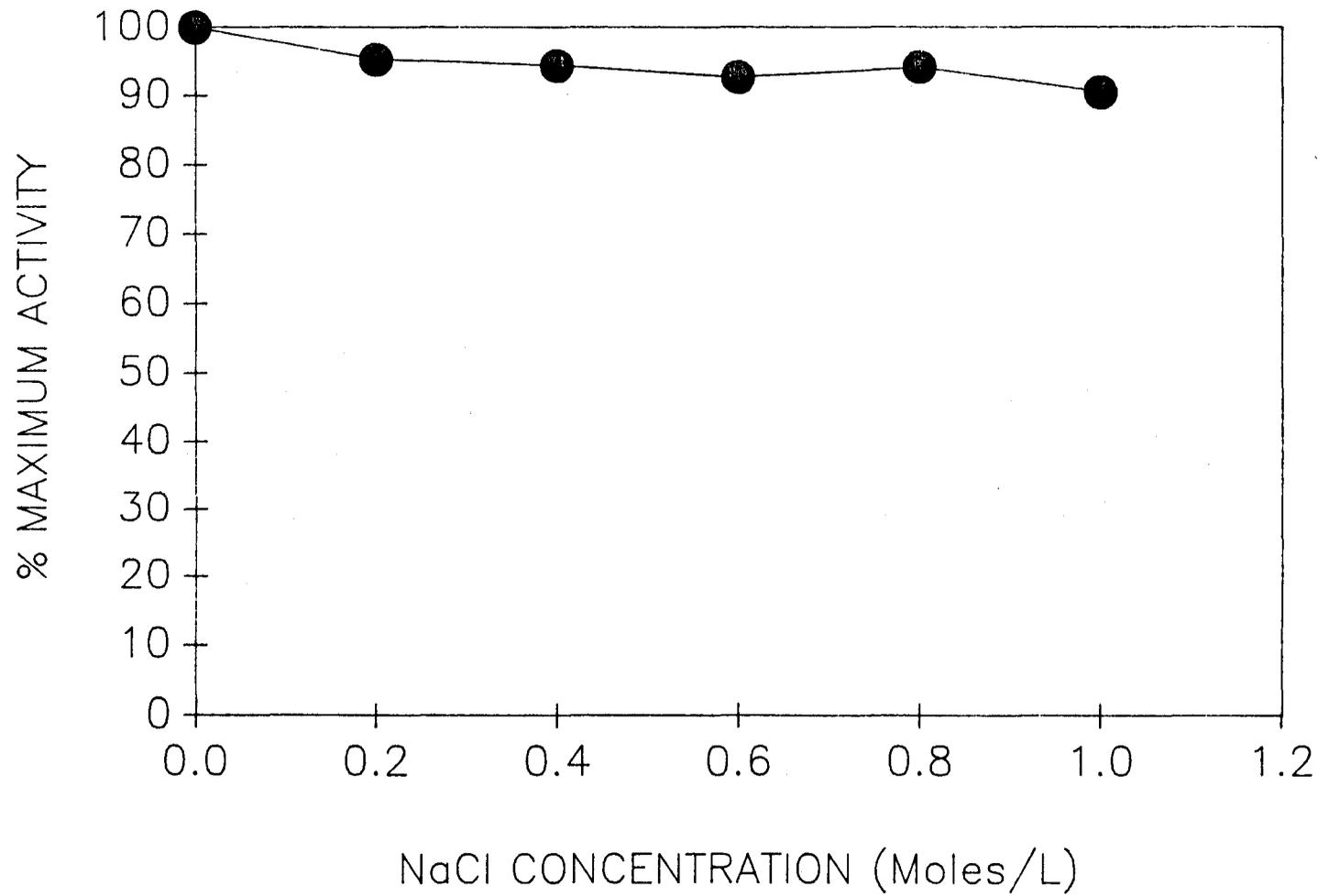
Although NaCl was seen to be important for the long term storage of tryptidase, Figure 6 suggests that its presence is not required for optimal BAEE activity of the enzyme. Concentrations of up to 1 M NaCl in the standard assay buffer had no apparent effect on activity.

TABLE XI  
EFFECT OF STORAGE CONDITIONS ON  
PURIFIED TRYPTIDASE

| STORAGE CONDITION         | % RECOVERY |       |
|---------------------------|------------|-------|
|                           | DAY 0      | DAY 1 |
| Vacuum dried stored at RT | 48         | 81    |
| Vacuum dried stored at 4° | 61         | 78    |
| Lyophilized               | 44         | 51    |
| Dry ice-acetone Frozen    | 80         | 78    |
| Stored at -20°            | 100        | 112   |

The BAEE activity of the original aliquot of enzyme put into storage was used as the 100% control. All assays for activity were performed in triplicate. Enzyme stored in phos-EDTA-0.5 M NaCl-6.0 was used in each experiment. Reconstitution was achieved in distilled deionized H<sub>2</sub>O. Dry ice-acetone bath frozen sample preserved at 4° was used as the control for the lyophilized sample.

Figure 6. Effect of NaCl on BAEE activity. The enzyme was assayed for BAEE activity for 15 min at 25<sup>0</sup> in 0.1 M phosphate buffer, pH 7.5, containing concentrations of NaCl ranging from no NaCl to 1 M NaCl. Concentration of BAEE in the assay was 1 mM. A plot of % maximum activity vs. NaCl concentration was drawn.



Consequently, it was decided not to use any NaCl in the standard buffer. It may be noted that the concentration of substrate used was 1 mM. In the light of later results showing that the  $K_m$  for BAEE is 0.0128 mM, 1 mM BAEE may be masking any effect of the NaCl on  $K_m$ .

#### 4. pH Stability

The effect of preincubating the enzyme at various pH's for 2 h at 25<sup>0</sup> is seen in Figure 7. Tryptidase is at least partially stable over the pH range 3.5 to 7.5 but retains virtually no activity following preincubation at pH's below 3.0 or above 8.0. The enzyme demonstrated maximal stability in the pH range 5.5 to 7.5.

#### 5. pH Optimum

Studies on tryptidase to determine its pH optimum for BAEE activity showed that the enzyme has optimal activity in the range pH 7.0-8.0 (Figure 8). Activity dropped rapidly at pH's below 6.0 even though the enzyme is stable at low pH's. Severe loss of activity was also observed at pH's of 8.5 and above. The loss of activity at the higher pH's is explainable in part by the instability of the enzyme at these pH's (Figure 7). Since maximum activity was observed at pH 7.5 in phosphate buffer, these conditions were used for the standard assay. No loss of activity was discernable over the routine 5-15 min assay period using these assay conditions.

#### 6. Temperature Stability at pH 7.5

Figure 7. pH stability. An aliquot of enzyme was preincubated in different 0.1 M buffers at 25<sup>0</sup> for 2 h in a 0.1 ml volume. The mixture was then assayed in the preincubation cuvette for BAEE activity using the standard assay.

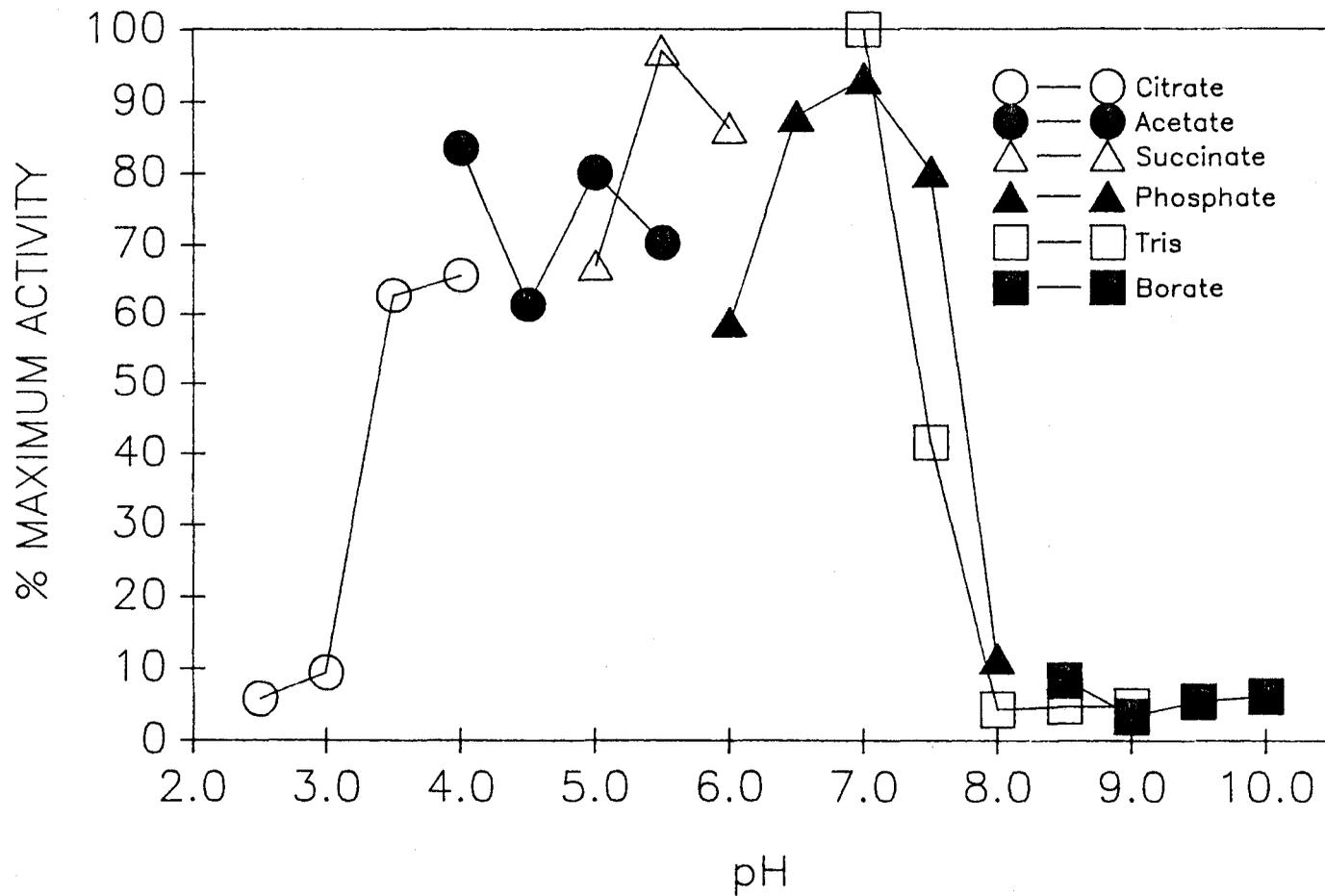
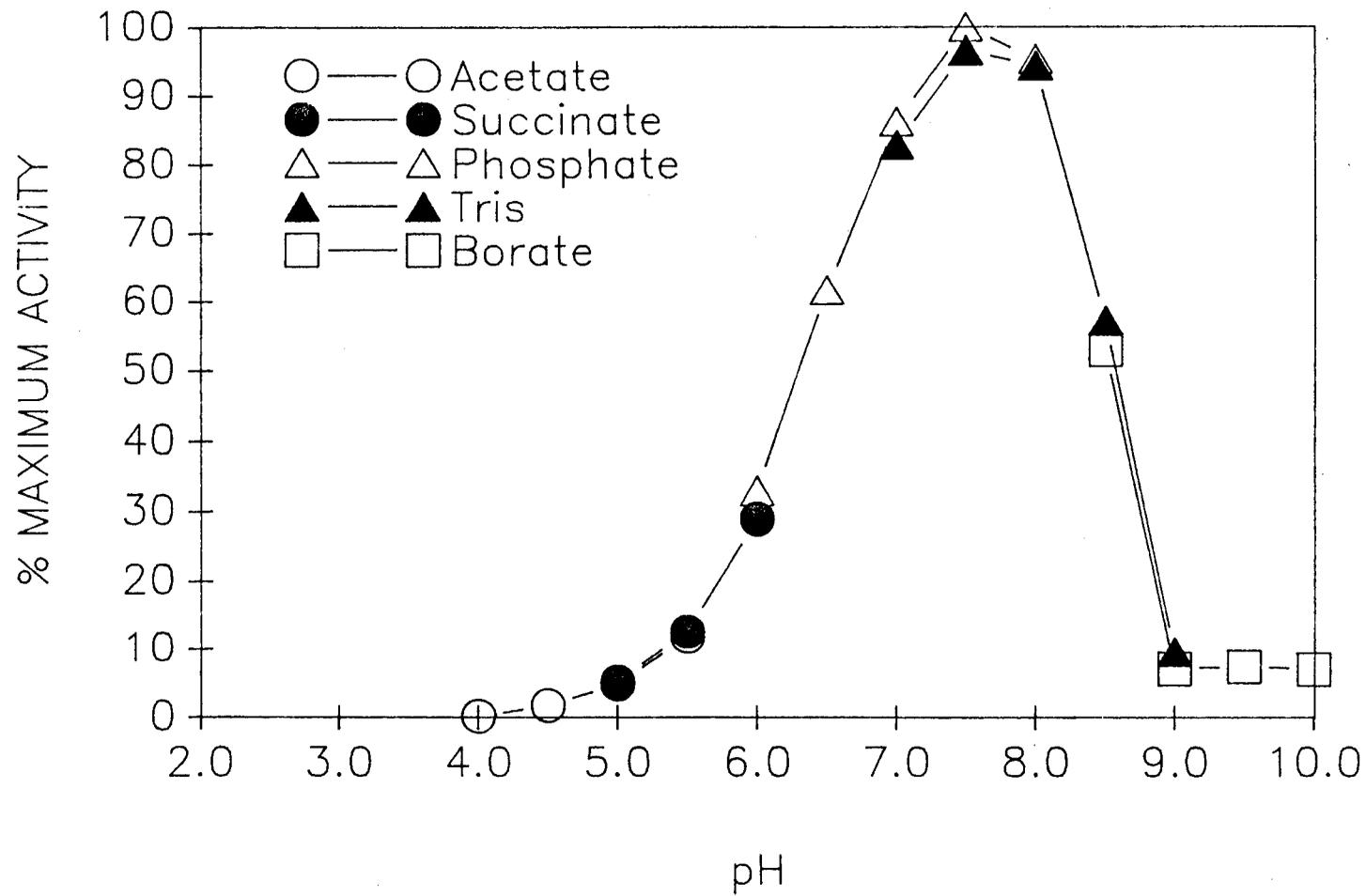


Figure 8. pH optimum. An aliquot of enzyme was assayed for BAEF activity in different 0.1 M buffers as described in METHODS.



The effect of temperature on tryptidase stability at pH 7.5 is shown in Figure 9. Half of the enzyme's maximum activity is irreversibly lost when the enzyme is preincubated for 30 min at 30<sup>0</sup>. The enzyme is rapidly inactivated when preincubated for 30 min at temperatures above 35<sup>0</sup>. Thus tryptidase is quite heat labile at pH 7.5. Routine assays were therefore done at 25<sup>0</sup> for time periods not exceeding 15 mins to keep the temperature effects to a minimum. It is possible that the enzyme may be more heat stable at lower pH's where it is more pH stable (see Figure 7).

#### 7. Effect of Protease Inhibitors on Tryptidase

##### Activity.

A number of natural protein protease inhibitors were screened in order to determine their effect on tryptidase. It is evident from Table XII that Trasylol (basic pancreatic trypsin inhibitor, bovine) is the only one of the large protein trypsin inhibitors to inhibit tryptidase. Significantly, no inhibitory effect was seen with concanavalin A. Although the enzyme can be purified using concanavalin A-agarose chromatography, the results suggest that the binding of concanavalin A does not result in a conformational change or steric hindrance sufficient to prevent accessibility of the active site to the BAEE substrate. Hirudin, an inhibitor of thrombin extracted from leeches, had no effect on tryptidase at 1 unit/ml.

Figure 9. Temperature stability at pH 7.5. An aliquot of enzyme was preincubated in phos-EDTA-7.5 at various temperatures for 30 min. in 0.1 ml volume. The entire mixture was then assayed in the preincubation cuvette for BAEE activity at 25<sup>0</sup>.

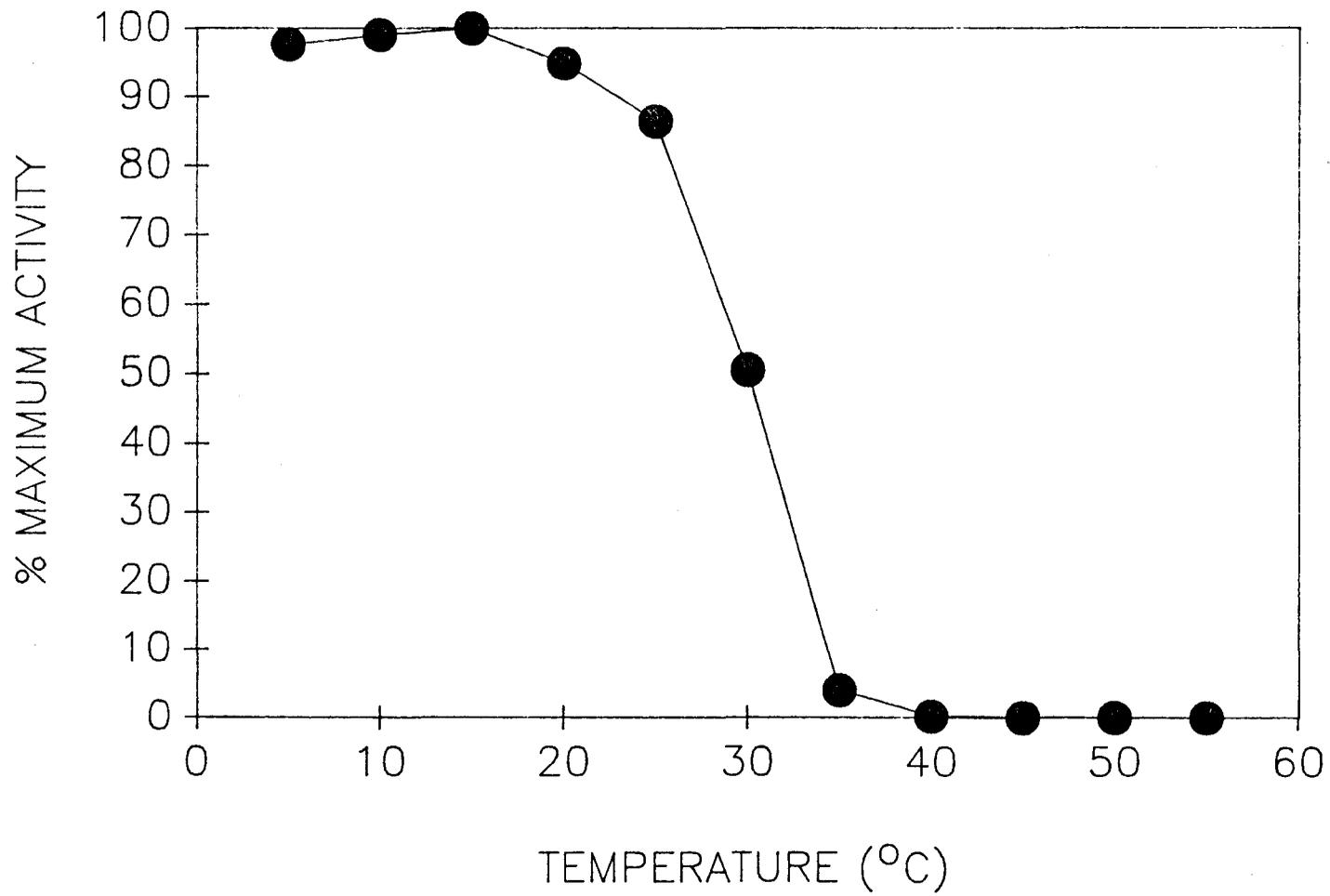


TABLE XII  
EFFECT OF PROTEINASE INHIBITORS ON TRYPTIDASE ACTIVITY

| Inhibitor  | Concentration<br>mg/ml   | Percent<br>Inhibition |
|--|--------------------------|-----------------------|
| Trasylol (basic pancreatic trypsin inhibitor)    | 0.1                      | 94                    |
| Lima Bean Trypsin Inhibitor                      | 1.0                      | 0                     |
| Ovomucoid  | 1.0                      | 0                     |
| Soybean Trypsin Inhibitor (Kunitz)               | 1.0                      | 0                     |
| $\alpha_1$ -Antitrypsin                          | 1.0                      | 0                     |
| Concanavalin A                                   | 1.0 <sup>a,b</sup>       | 0                     |
| Hirudin  | 1.0 unit/ml <sup>a</sup> | 0                     |
| NPGB (p-nitrophenyl-p'-guanidino-benzoate)       | 10.0 (uM) <sup>a</sup>   | 100                   |
| MUGB (p-methylumbelliferyl-p'-guanidinobenzoate) | 10.0 (uM) <sup>a</sup>   | 99                    |

Enzyme and inhibitor were preincubated for 5 min at 25° C and pH 7.5. Reaction was started by addition of BAEE to 1 mM. All assays were performed in triplicate.

<sup>a</sup>Concentration in final assay.

<sup>b</sup>Preincubation volume was 0.9 ml.

Two of the other inhibitors in Table XII which were effective inhibitors of tryptidase were p-nitrophenyl-p'-guanidinobenzoate (NPGB) and 4-methyl-umbelliferyl-p'-guanidinobenzoate (MUGB). Both were able to cause complete inhibition at 10  $\mu$ M. When MUGB (50 nM) was tested as a potential substrate for the enzyme using a fluorometric assay no turnover of the enzyme was seen at pH 7.5 over a 5 min period following the initial "burst" indicating that the substrate binds irreversibly to the enzyme.

Table XIII shows the effect of several microbial proteinase inhibitors (Umezawa and Ayogi, 1977) on tryptidase. Antipain and leupeptin were the best inhibitors of this class causing 80% inhibition at 50  $\mu$ g/ml. The chymotrypsin inhibitor failed to inhibit the enzyme at 100  $\mu$ g/ml as did elastatinal (the elastase inhibitor), pepstatin A and bestatin at concentrations as high as 200  $\mu$ g/ml.

#### 8. Effect of Chemical Modification Reagents

Among the inhibitors that act by chemically modifying the enzyme, diisopropylphosphofluoridate (DFP) inhibited tryptidase 93% at 1mM when preincubated with the enzyme for 10 min at 25<sup>0</sup> and pH 7.5 (Table XIV). Essentially complete inhibition could be achieved under identical conditions in 30 min. Tos-Lys-CH<sub>2</sub>Cl which is known to act by alkylating the active site histidine of trypsin was an effective inhibitor of the enzyme. Significantly less inhibition was seen for Tos-Phe-CH<sub>2</sub>Cl (22%) and Z-Phe-CH<sub>2</sub>Cl (10%), both of

TABLE XIII  
EFFECT OF MICROBIAL PROTEINASE INHIBITORS ON TRYPTIDASE

| Inhibitor                | Concentration<br>ug/ml | Percent <sup>a</sup><br>Inhibition |
|--------------------------|------------------------|------------------------------------|
| Antipain                 | 50                     | 80                                 |
| Leupeptin                | 50                     | 79                                 |
| Chymostatin              | 100                    | 0                                  |
| Elastatinal              | 200                    | 4                                  |
| Pepstatin A <sup>b</sup> | 50                     | 3                                  |
|                          | 200                    | 0                                  |
| Bestatin <sup>b</sup>    | 200                    | 0                                  |

<sup>a</sup>Inhibition measured at pH 7.5 using BAEE as substrate. Final concentration of the substrate in the assay was 1 mM. All assays were done in triplicate.

<sup>b</sup>control and inhibitor assay contained 10% methanol.

TABLE XIV  
EFFECT OF CHEMICAL MODIFICATION REAGENTS ON  
TRYPTIDASE ACTIVITY

| Reagent                                    | Concentration<br>(mM) | Percent<br>Inhibition |
|--|-----------------------|-----------------------|
| DFP <sup>a</sup>                           | 1.0                   | 93                    |
| Tos-Lys-CH <sub>2</sub> Cl <sup>a</sup>    | 1.0                   | 54                    |
| Tos-Phe-CH <sub>2</sub> Cl <sup>a, b</sup> | 1.0                   | 22                    |
| Z-Phe-CH <sub>2</sub> Cl <sup>a, c</sup>   | 0.2                   | 10                    |
| p-CMB <sup>a</sup>                         | 0.05                  | 85                    |
| DTT <sup>d</sup>                           | 10.0 <sup>e</sup>     | 0                     |
| 2-Mercaptoethanol <sup>d</sup>             | 10.0 <sup>e</sup>     | 0                     |
| ε-Aminocaproic Acid <sup>d</sup>           | 1.0 <sup>e</sup>      | 11                    |
| E-64 <sup>a</sup>                          | 10.0 (uM)             | 0                     |

<sup>a</sup>Enzyme was preincubated with test reagent for 10 min at 25° C and pH 7.5. The entire aliquot was then assayed for residual activity using BAEE as a substrate and compared to control sample preincubated in the absence of reagent.

<sup>b</sup>Preincubation mixture contained 20% methanol (v/v).

<sup>c</sup>Preincubation mixture and control contained 10% DMSO (v/v).

<sup>d</sup>Preincubation was for 5 min.

<sup>e</sup>Concentration in final assay.

which are known to act by a similar mechanism against chymotrypsin. The small degree of inhibition by these two compounds can be explained by the presence of organic solvents in the preincubation mixture. The most effective chemical modification reagent was p-chloromercuribenzoate (p-CMB) which covalently modifies sulfhydryl groups. p-CMB inhibited tryptidase 85% at 0.05 mM. E-64, which inhibits SH-enzymes, had no effect on tryptidase. DTT and 2-mercaptoethanol neither inhibited nor stimulated activity.  $\epsilon$ -Aminocaproic acid, which inhibits plasmin, also did not inhibit the enzyme.

#### 9. Time Course of Inhibition of Tryptidase by DFP

When tryptidase was preincubated for increasing periods of time with DFP, its residual activity decreased as a function of time of preincubation as evidenced in Figure 10. The pseudo-first order rate constant ( $K_{\text{obsd}}$ ) for the reaction of the enzyme with DFP was  $0.076 \text{ sec}^{-1}$ . The second order rate constant,  $K_{\text{obsd}}/[\text{I}]$ , was  $76 \text{ M}^{-1} \text{ sec}^{-1}$ .

#### 10. Effect of Metal Ions on Tryptidase Activity

Table XV shows that none of the heavy metals,  $\text{Mg}^{++}$ ,  $\text{Mn}^{++}$ ,  $\text{Co}^{++}$ ,  $\text{Ca}^{++}$ , and  $\text{Hg}^{++}$ , have any stimulatory effect on the enzyme. The only significant effect on tryptidase was by  $\text{Hg}^{++}$  which completely inactivates the enzyme at 2mM. The inhibition by mercury seen here is consistent with the inhibition seen with p-CMB above.

#### 11. Molecular Weight Determination by Gel Permeation

TABLE XV  
EFFECT OF HEAVY METAL IONS ON TRYPTIDASE ACTIVITY

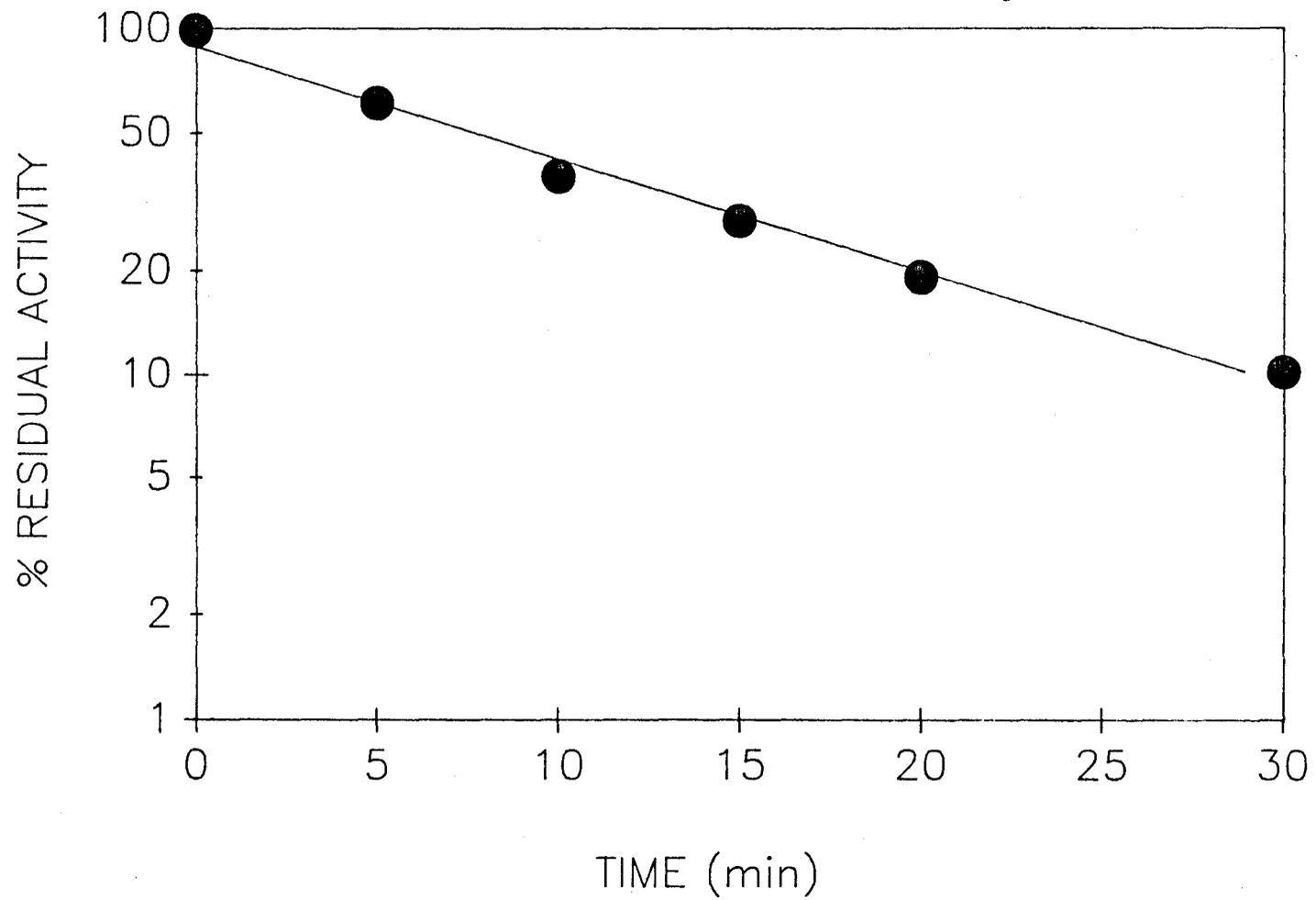
| Metal Ion                     | Concentration <sup>a</sup><br>(mM) | Percent <sup>b</sup><br>Inhibition | pH <sup>c</sup> |
|-------------------------------|------------------------------------|------------------------------------|-----------------|
| Magnesium (Mg <sup>++</sup> ) | 10                                 | 0                                  | 7.42            |
| Manganese (Mn <sup>++</sup> ) | 10                                 | 0                                  | 7.40            |
| Cobalt (Co <sup>++</sup> )    | 10                                 | 12                                 | 7.27            |
| Calcium (Ca <sup>++</sup> )   | 10                                 | 0                                  | 7.46            |
|                               | 20                                 | 10                                 | 7.48            |
| Mercury (Hg <sup>++</sup> )   | 2                                  | 98                                 | 7.34            |

<sup>a</sup>All stock solutions of metal ions were prepared in distilled deionized H<sub>2</sub>O except calcium (20 mM) which was prepared in 0.1 M Tris, pH 7.5 buffer.

<sup>b</sup>Inhibition was measured using BAEE as substrate in a 1 ml assay. Concentration of BAEE was 1 mM.

<sup>c</sup>pH's represent measured values at the end of the reaction.

Figure 10. Time course of inactivation of tryptidase by DFP. An aliquot of enzyme was preincubated with 100  $\mu$ M DFP in phos-EDTA-7.5 in a 40  $\mu$ l volume for 0, 5, 10, 15, 25 and 30 min and assayed in the preincubation cuvette for BAEE residual activity. Enzyme preincubated in the absence of DFP served as a control. The log % residual activity was plotted as a function of time.



## Chromatography

The molecular weight of tryptidase was estimated to be 158,000 by the method of Andrews (1965) when analytical high performance gel permeation chromatography was performed using a Toya Soda TSK SW-3000 column equilibrated with 1 M NaCl (Figure 11). Moreover, the enzyme eluted in a single symmetrical sharp band (Figure 12 A).

Chromatography of the enzyme on the same column equilibrated with either 0.5 M NaCl (Figure 12 B) and 0.05 M NaCl (Figure 12 C) produced no major shift in the elution time of the enzyme. This suggests that in the absence of high salt, there is neither a formation of higher molecular weight aggregates nor dissociation of the enzyme into lower molecular weight monomeric subunits.

## 12. Molecular Weight Determination Based on SDS

### Polyacrylamide Gel Electrophoresis

When SDS polyacrylamide gel electrophoresis was performed on tryptidase, a single band was observed with a  $M_r = 40,000$  in both reducing and non-reducing gels (cf. Figure 4 and Figure 13). When the enzyme was inactivated with [ $^3\text{H}$ ]-DFP and run under non-reducing conditions, the label corresponded with the protein band (cf. Figure 5). This was also the case when labelled enzyme was run under reducing conditions. These results suggested that there was only one DFP-sensitive enzyme which corresponded to the majority of the protein in the preparation. The absence of

Figure 11. Molecular weight of tryptidase by high performance gel permeation chromatography. Tryptidase, as well as the molecular weight standards, glutamate dehydrogenase, rabbit muscle aldolase, bovine serum albumin, egg albumin and ribonuclease were applied to a Toya Soda TSK-gel SW-3000 column equilibrated with phos-EDTA-1 M NaCl-6.0 and fractions were collected at 5 min intervals. A standard curve was generated by plotting the molecular sieve coefficient ( $\sigma$ ) as a function of the log of the molecular weight of the standards.

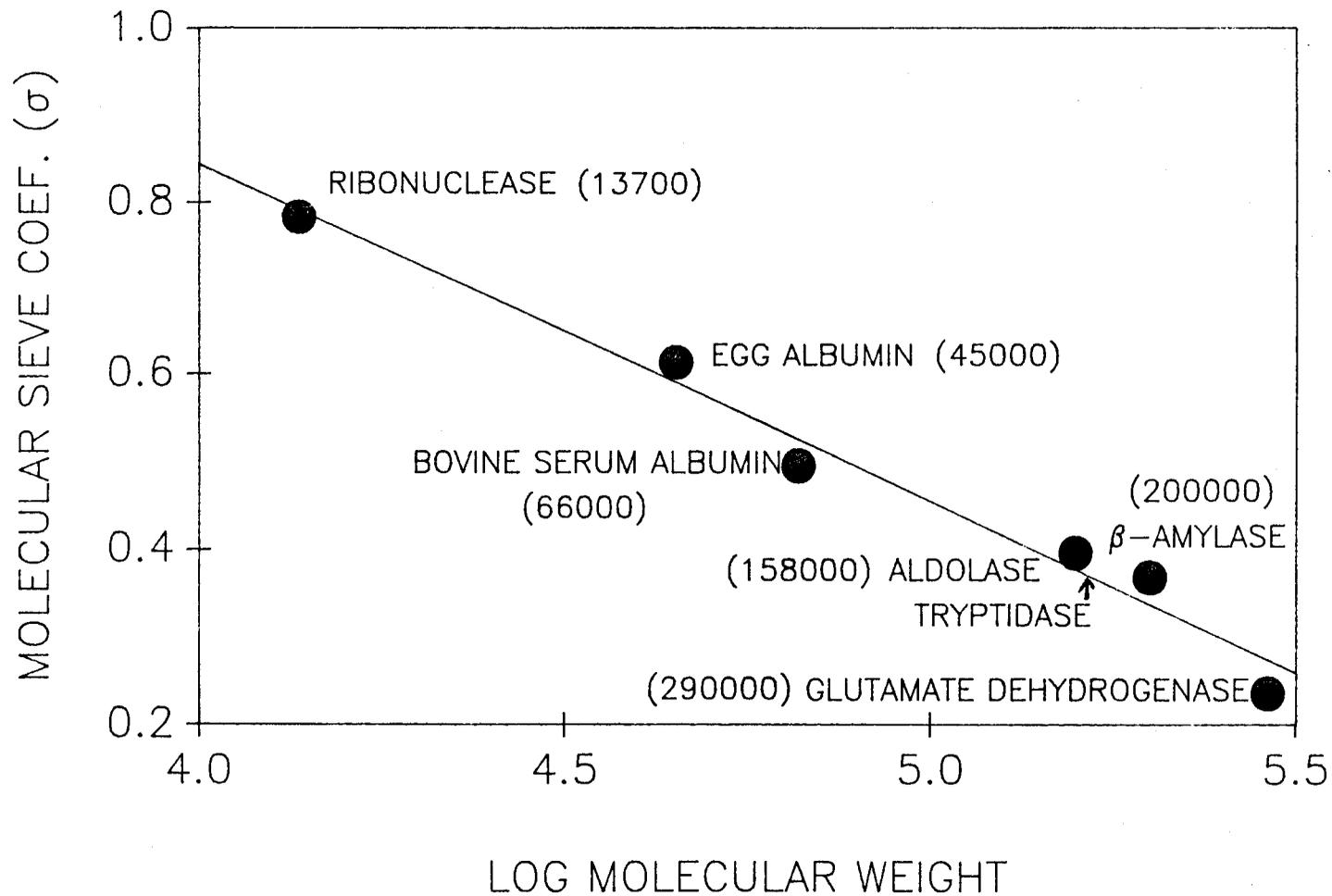


Figure 12. Molecular weight of tryptidase by high performance gel permeation chromatography at high, medium, and low NaCl concentrations. Tryptidase was applied to a Toya Soda TSK-gel SW 3000 column equilibrated in (A) phos-EDTA-1 M NaCl-6.0, (B) phos-EDTA-0.5 M NaCl-6.0 and (C) phos-EDTA-0.05 M NaCl-6.0. The enzyme was converted to the corresponding equilibration buffer before injection onto the column. The  $A_{214}$  of the fractions collected at 5 min intervals was monitored as a function of time. All other conditions for the experiment were similar to those described for Fig. 11.

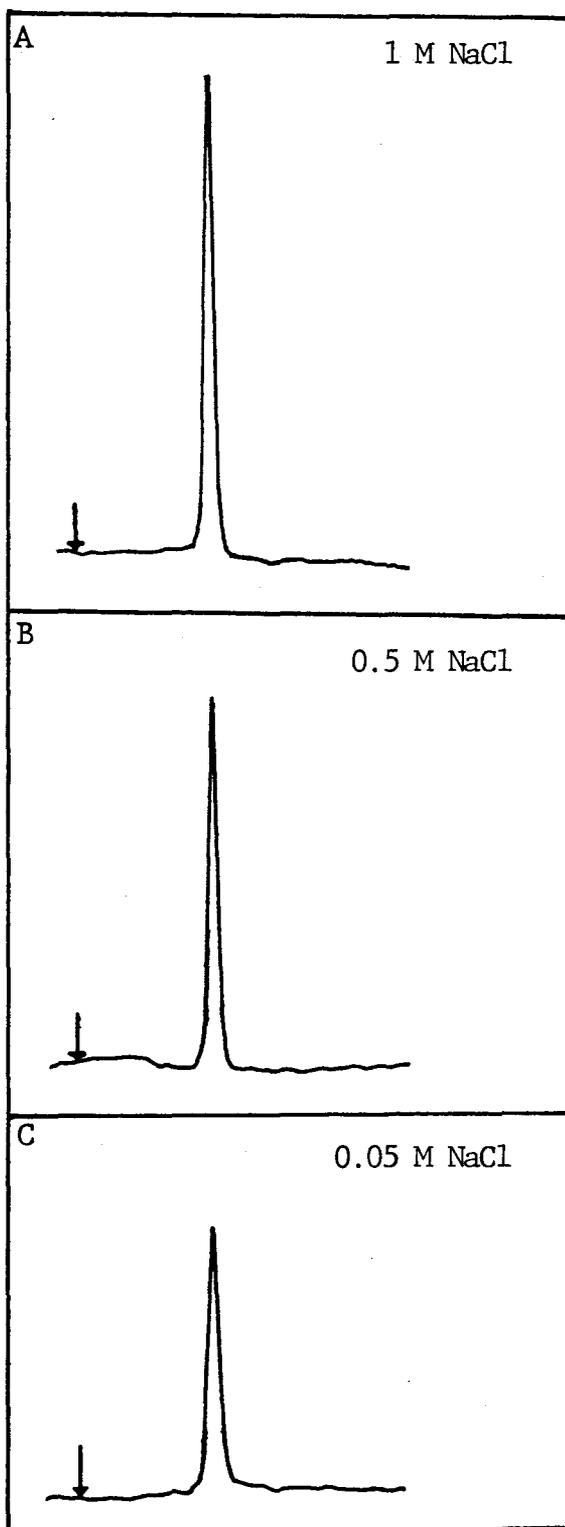
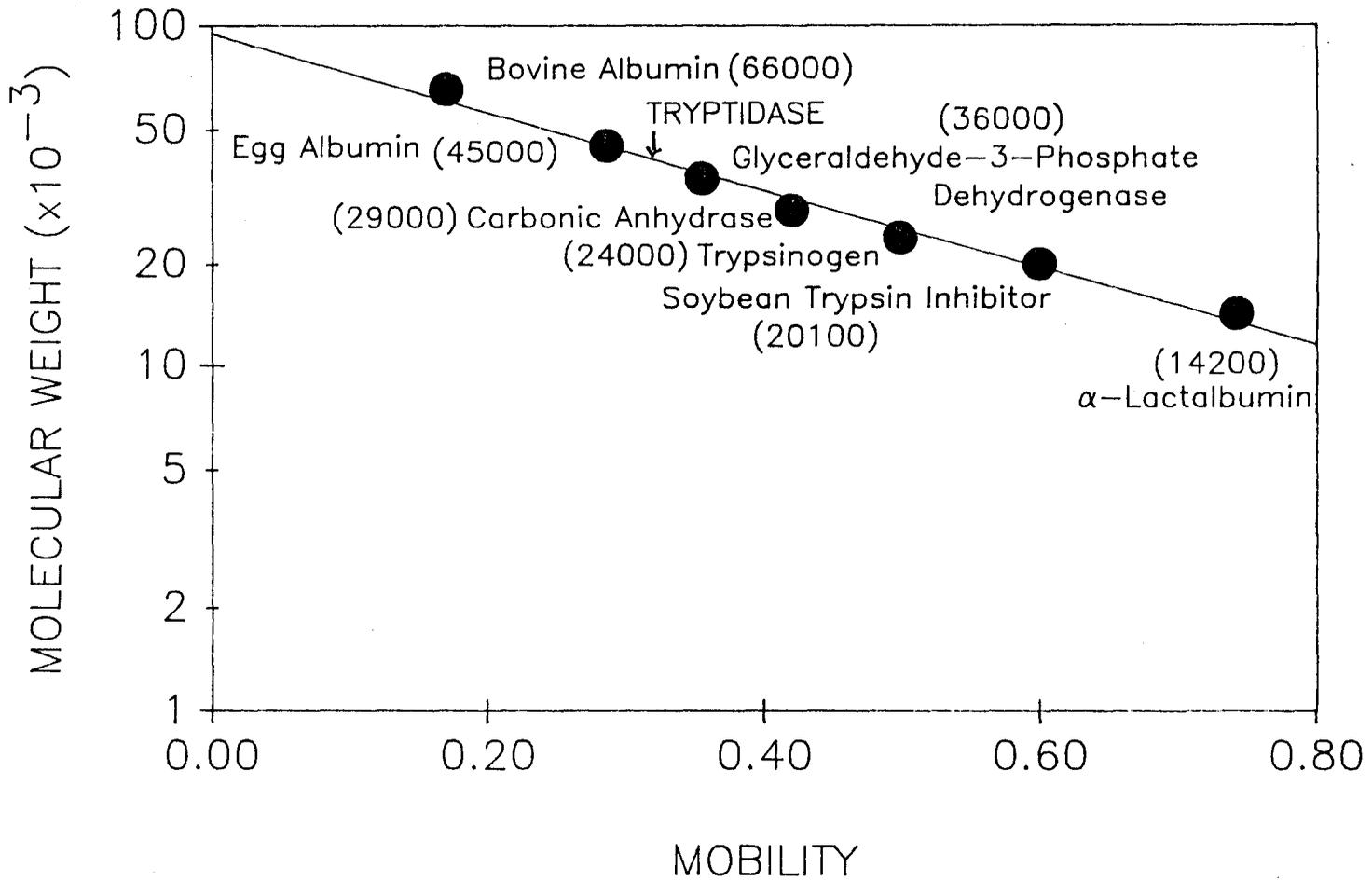


Figure 13. Molecular weight of tryptidase by SDS gel electrophoresis. The enzyme was electrophoresed in SDS polyacrylamide gels under both reducing and non-reducing conditions as described in METHODS. Molecular weight markers were bovine albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, soybean trypsin inhibitor and  $\alpha$ -lactalbumin. The mobility of the markers was calculated as described in METHODS and plotted against their corresponding molecular weight to generate a standard curve.



a different molecular weight band under reducing conditions suggests that the subunits of tryptidase are essentially single polypeptide chains.

### 13. Isoelectric Focusing Of Tryptidase

The pI of tryptidase was determined using precast PAG plates containing ampholytes in the pH range of 3.5-9.5. After completion of focusing, the gels were immediately fixed and then stained with Coomassie brilliant blue. Five bands in close proximity to each other were observed (Figure 14) suggesting microheterogeneity of the enzyme. Such a phenomenon is not surprising in view of the glycoprotein nature of the enzyme. The pI of tryptidase corresponding to these bands ranged from 4.5 to 4.9 with the most prominent band at 4.8. Calculations of pI were based on the standard curve given in Figure 15.

### 14. Substrate Profile of Tryptidase

A number of single amino acid synthetic ester and amide substrates as well as a whole range of tri(tetra)peptide p-NA substrates were assayed in order to determine the substrate specificity of tryptidase.

#### a. Single Amino Acid Substrates

Table XVI contains the list of single amino acid substrates which were tested against the enzyme. Of the trypsin-type substrates, tryptidase cleaves BAEE the best. The enzyme is unable to hydrolyze the chymotrypsin substrate ATEE. The tryptase substrate TAME is cleaved at only 50%

Figure 14. Isoelectric focusing of tryptidase. The experiment was conducted according to the protocol described in detail in METHODS. Coomassie brilliant blue was used to stain the enzyme and pI marker proteins. Lanes A and E contain the marker proteins: a) amyloglucosidase, b) soybean trypsin inhibitor, c)  $\beta$ -lactoglobulin, d) carbonic anhydrase (bovine), e) carbonic anhydrase (human), myoglobin, f) L-lactate dehydrogenase and g) trypsinogen. Lanes B--C and F--G contain only tryptidase from two different preparations.

A B C E F G

a  
b  
c  
d  
e  
f  
g

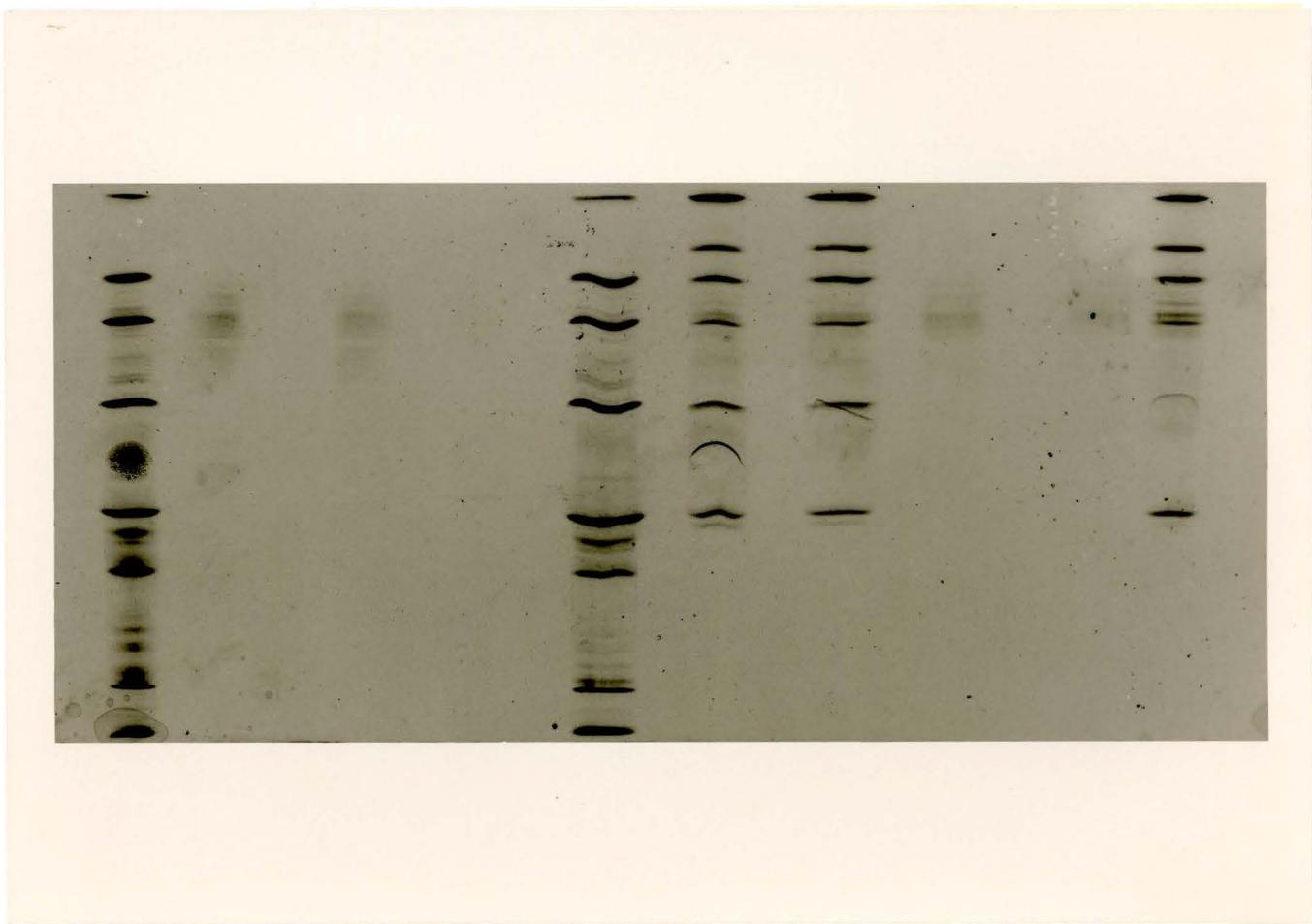


Figure 15. Isoelectric focusing of tryptidase. The experiment was conducted according to the protocol described in detail in METHODS. Coomassie brilliant blue was used to stain the enzyme and the following pI marker proteins: amyloglucosidase, soybean trypsin inhibitor,  $\beta$ -lactoglobulin, carbonic anhydrase (bovine), carbonic anhydrase (human), myoglobin, L-lactic dehydrogenase and trypsinogen. The pI's of the marker proteins were plotted as a function of their distance of migration from the anode.

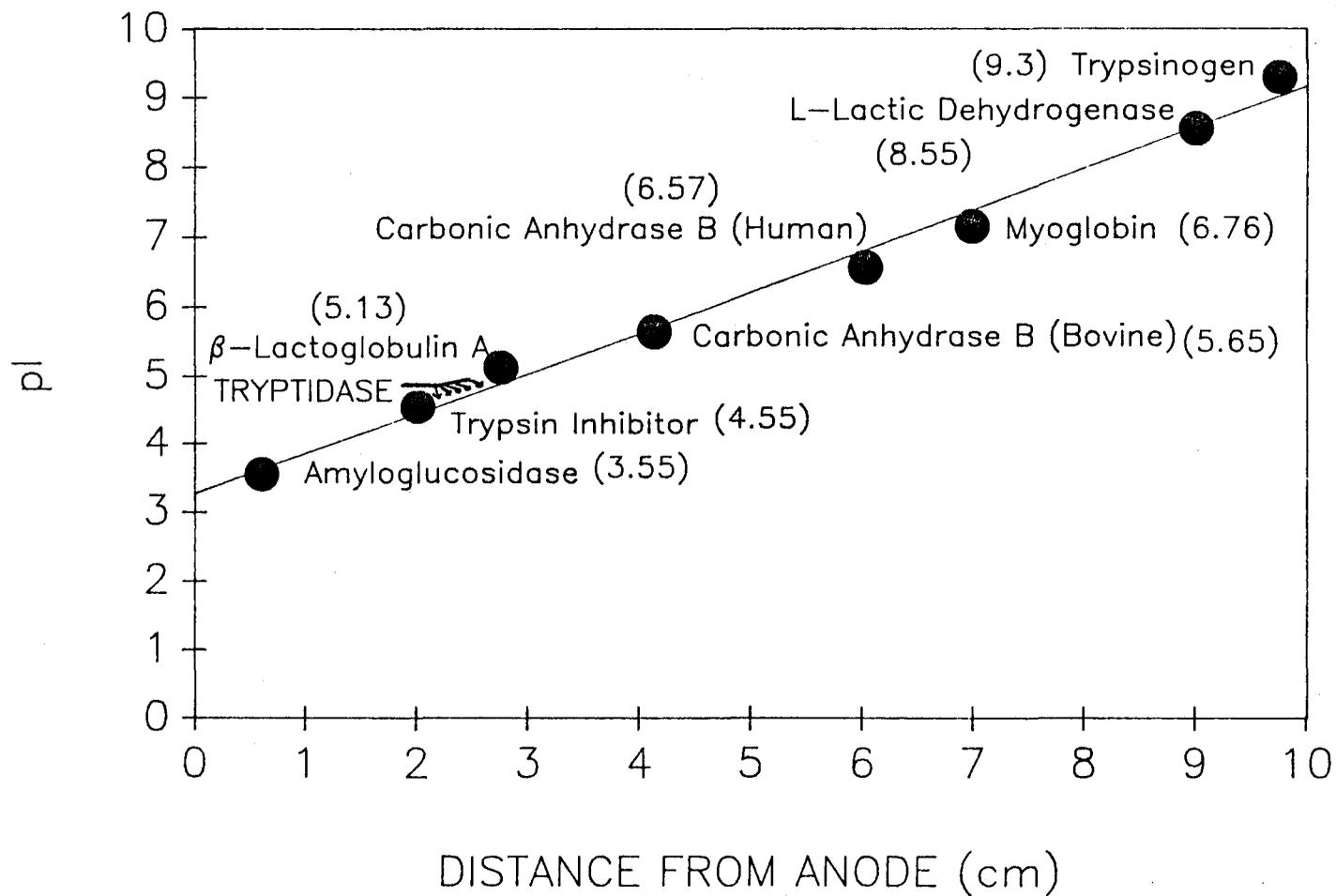


TABLE XVI  
HYDROLYSIS OF SINGLE AMINO ACID SUBSTRATES  
BY PURIFIED TRYPTIDASE

| SUBSTRATE   | ACTIVITY (U/ml) | % MAXIMUM |
|-------------|-----------------|-----------|
| BAEE        | 2.93            | 100       |
| TAME        | 1.28            | 43.7      |
| BAPNA (D,L) | 0.16            | 5.46      |
| ATEE        | 0.0             | 0         |

All assays were conducted in a Gilford Response spectrophotometer at 25<sup>0</sup> in a final volume of 1 ml. Concentrations of all substrates in the final assay was 1 mM. Assays for TAME were done at 246 nm, for BAPNA at 410 nm, and for ATEE at 237 nm.

the BAEE rate. The enzyme also clearly hydrolyzed the ester substrate BAEE faster than it did the amide substrate BAPNA. In assaying BAPNA, two different stock solutions were used. One contained the L-isomer of the amino acid (data not shown) and the other contained a mixture of the D- and L-isomers (Table XVI). Cleavage of the mixture of isomers proceeded at a significantly slower rate than cleavage of the pure L-form suggesting that tryptidase has a stereospecific preference for the L-form. The lower rates seen with the mixture may also be due to inhibition produced by the D-form.

b. Hanes-Woolf Plots for BAEE and BAME

Plots of  $[S]/v$  vs.  $[S]$  for BAEE and BAME as substrates for tryptidase are represented in figure 16 and 17, respectively. The enzyme was assayed with each of these substrates at concentrations ranging from  $0.5 K_m$  to at least  $5 K_m$  using the standard assay described in METHODS. The plots showed that the  $K_m$  for BAEE was 0.0128 mM and the  $V_{max}$  was 11 umoles/min. In the case of BAME the  $K_m$  was 0.0196 mM and the  $V_{max}$  was 16.2 umoles/min.

The data obtained in these experiments were also analyzed by several other graphical methods by means of a computer program. The results are shown in Table XVII. Close agreement between values for the  $K_m$  and  $V_{max}$  was seen irrespective of the analysis used with the exception of the Lineweaver-Burke plot for BAME.

Figure 16. Hanes plot for BAEE. The experiment was performed using the standard assay described in METHODS at different concentrations of substrate and identical aliquots of enzyme. Each point is an average of two values.

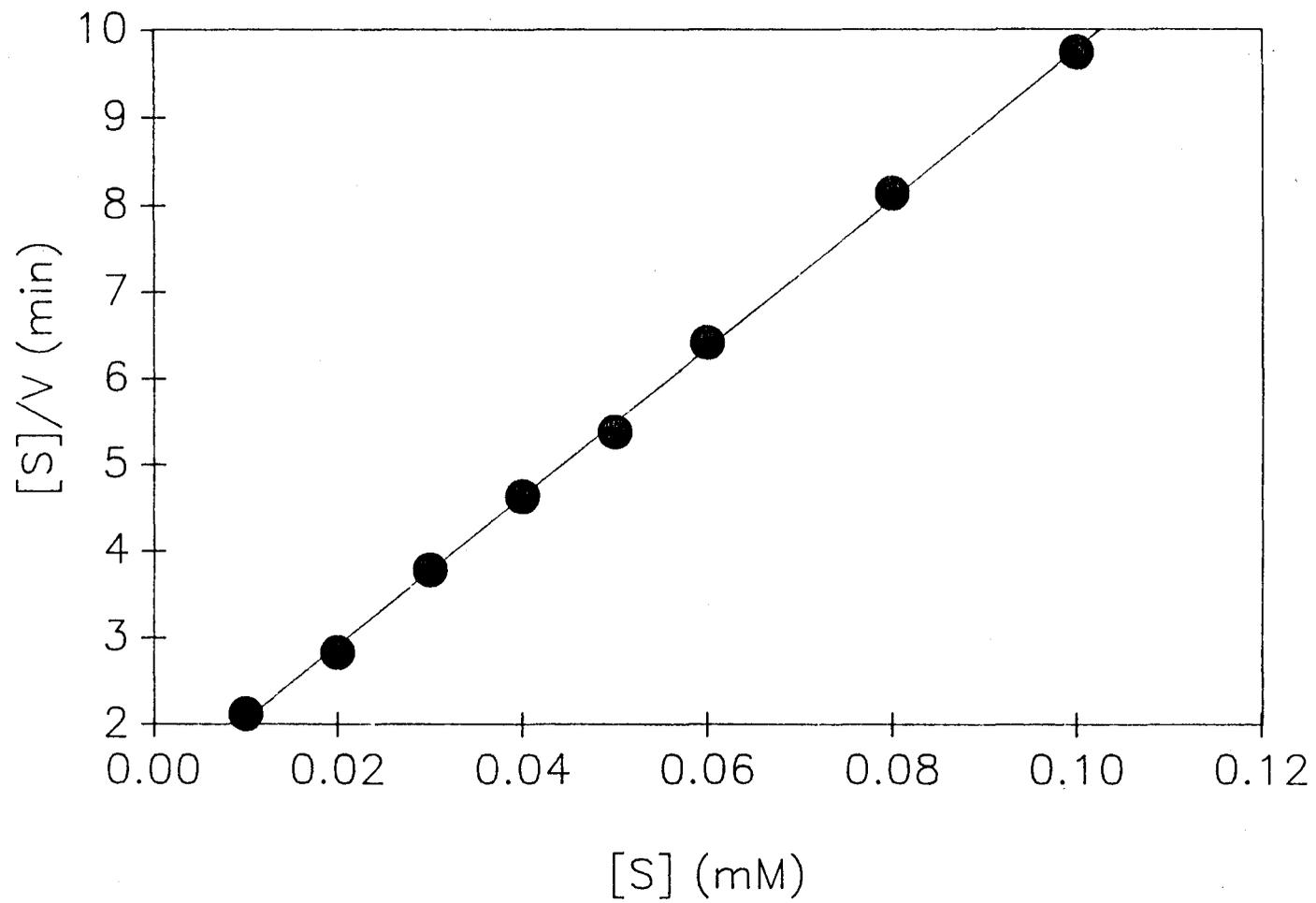


Figure 17. Hanes plot for BAME. The experiment was performed in a manner similar to that described for Figure 16, substituting BAME as the substrate. Each point is an average of two values.

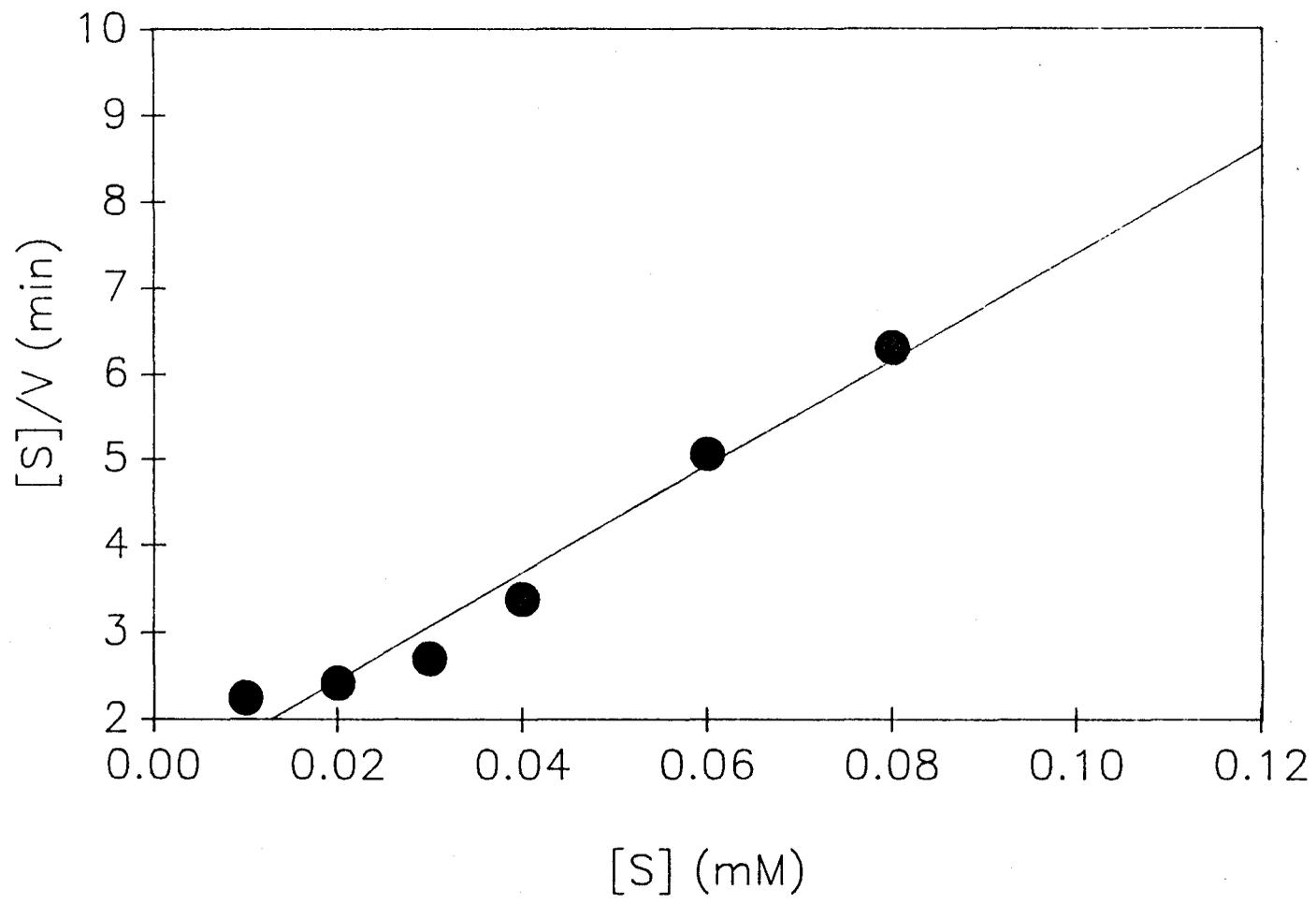


TABLE XVII  
KINETIC PARAMETERS FOR TRYPTIDASE

| SUBSTRATE | PLOT TYPE        | $K_m$<br>(mM) | Vmax<br>(umoles/min) |
|-----------|------------------|---------------|----------------------|
| BAEE      | Direct Plot      | 0.0134        | 11.0                 |
|           | Lineweaver-Burke | 0.0134        | 11.2                 |
|           | Hanes-Woolf      | 0.0128        | 11.0                 |
|           | Eadie-Hofstee    | 0.0128        | 11.0                 |
| BAME      | Direct Plot      | 0.0202        | 15.9                 |
|           | Lineweaver-Burke | 0.0367        | 21.5                 |
|           | Hanes-Woolf      | 0.0196        | 16.2                 |
|           | Eadie-Hofstee    | 0.0222        | 17.0                 |

All assays were conducted at 25<sup>0</sup>, in duplicate, in a Gilford Response spectrophotometer for 5 min and initial velocities obtained through its builtin program. Range of substrate concentrations used spans 0.5  $K_m$  to 10  $K_m$ . Values for  $K_m$  and Vmax were computer calculated using the Enzpack program (Elsevier Publications)

c. Tri- and Tetrapeptide p-Nitroanilide Substrates

The longer p-NA substrates which were used to study the specificity of the enzyme are listed in Table XVIIIa and Table XVIIIb. A clear pattern emerges from the table that suggests that the enzyme in trypsin-like fashion favors a charged basic residue in the P<sub>1</sub> position (cf. Schechter and Berger (1967) for terminology). Furthermore, of the two basic amino acids, arginine seems to be favored over lysine since activity against H-D-Val-Leu-Arg-pNA is about four times higher than against H-D-Val-Leu-Lys-pNA. However, the activity with Tos-Gly-Pro-Lys-pNA is slightly higher than with Tos-Gly-Pro-Arg-pNA. Proline in the P<sub>2</sub> position also seems to be important. The most rapidly cleaved substrate, H-D-Ile-Pro-Arg-pNA, has a proline in that position as does four of the five best substrates. As far as the P<sub>3</sub> site on the substrate is concerned, substrates with a hydrophobic amino acid in that position along with proline and arginine in the P<sub>2</sub> and P<sub>1</sub> positions, respectively, show good activity with the enzyme.

15. Tryptidase and Larger Peptides

Vasopressin (AVP), oxytocin and bradykinin are three of the larger peptides that were assayed for cleavage by tryptidase. Figure 18 shows chromatograms obtained using HPLC which demonstrate that there were no detectable products formed from either bradykinin (Figure 18 A) or oxytocin (Figure 18 B). However, AVP was hydrolyzed at the

TABLE XVIII  
 TRI(TETRA) PEPTIDE pNA SUBSTRATE PROFILE  
 OF PURIFIED TRYPTIDASE

| COM. NAME           | SUBSTRATE SEQUENCE   | ACTIVITY<br>umol/min/ml | % MAXIMUM |
|---------------------|--|-------------------------|-----------|
| S-2288              | H-D-Ile-Pro-Arg-pNA  | 41.1                    | 100       |
| Spectro-<br>zyme Ca | H-D-Pro-Pro-Arg-pNA  | 36.6                    | 88.5      |
| S-2238              | H-D-Phe-pip-Arg-pNA  | 27.3                    | 66.5      |
| Chromo-<br>zyme Try | H-D-Val-Gly-Arg-pNA  | 18                      | 43.8      |
| Chromo-<br>zyme P   | Tos-Gly-Pro-Lys-pNA  | 15.7                    | 38.2      |
| S-2222              | Bz-Ile-Glu-(-O-R <sup>a</sup> )Gly-Arg-pNA                         | 13.8                    | 33.7      |
| Chromo-<br>zyme TH  | Tos or CBZ-Gly-Pro-Arg-pNA   | 11.8                    | 28.7      |
| S-2337              | Bz-Ile-Glu-(-O-R <sup>b</sup> )Glu-Arg-pNA                         | 9.92                    | 24.1      |
| C <sub>1</sub> E-1  | C <sub>2</sub> H <sub>5</sub> O-Lys-( $\epsilon$ -CBO)-Gly-Arg-pNA | 7.99                    | 19.4      |
| S-2266              | H-D-Val-Leu-Arg-pNA  | 5.89                    | 14.3      |
| CBS 34.47           | H-D-CHG-But-Arg-pNA  | 4.53                    | 10.9      |
| S-2160              | Bz-Phe-Val-Arg-pNA   | 3.06                    | 7.4       |
| CBS 31.39           | CH <sub>3</sub> -SO <sub>2</sub> -D-Leu-Gly-Arg-pNA                | 2.30                    | 5.6       |
| S-2251              | H-D-Val-Leu-Lys-pNA  | 1.59                    | 3.9       |
|                     | Bz-Pro-Phe-Arg-pNA   | 1.36                    | 3.3       |
| S-2302              | H-D-Pro-Phe-Arg-pNA  | 1.19                    | 2.9       |

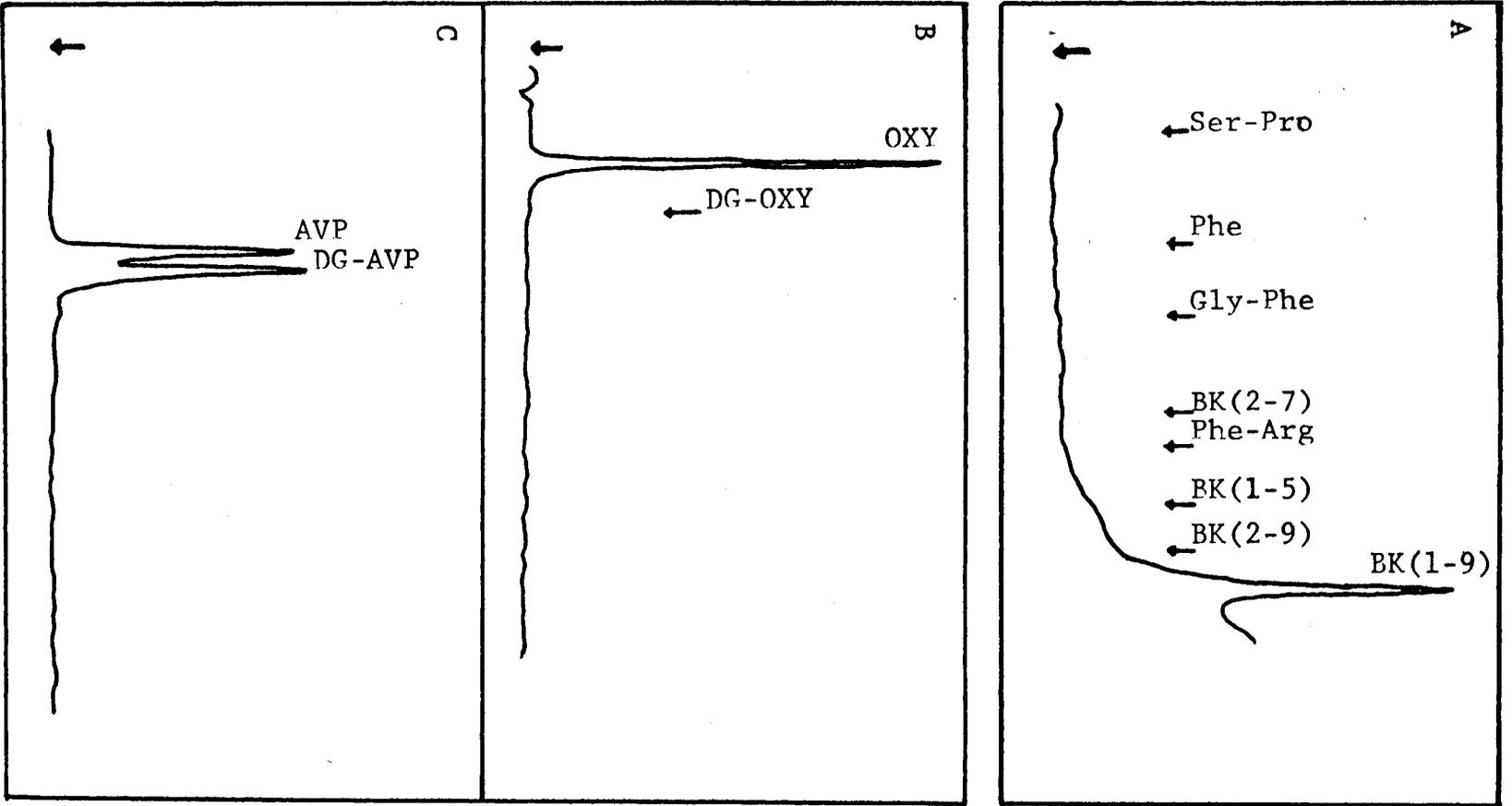
TABLE XVIII b  
 TRI (TETRA) PEPTIDE pNA SUBSTRATE PROFILE  
 OF PURIFIED TRYPTIDASE (contd.)

| COM.                  | SUBSTRATE<br>NAME       | SEQUENCE | ACTIVITY<br>umol/min/ml | % MAXIMUM |
|-----------------------|-------------------------|----------|-------------------------|-----------|
| Spectro-<br>zyme-PL   | H-D-Nleu-CHT-Lys-pNA    |          | 1.02                    | 2.5       |
| S-2586                | MeO-Suc-Arg-Pro-Tyr-pNA |          | 0.014                   | 0.03      |
| S-2484                | <Glu-Pro-Val-pNA        |          | 0                       | 0         |
| Spectro-<br>zyme-PKal | H-D-Pro-HHT-Arg-pNA     |          | 0                       | 0         |
| Chromo-<br>zym GK     | H-D-Val-CHA-Arg-pNA     |          | 0                       | 0         |

Concentrations of all substrates in the assay mixture were 1 mM. Assays were conducted at 30<sup>0</sup>, in a Multistat III F/LS centrifugal analyzer, in a total volume of 0.25 ml.

<sup>a</sup>R = CH<sub>3</sub> or H; <sup>b</sup>R = Piperidyl

Figure 18. Hydrolysis of nonapeptides by tryptidase. Bradykinin (A), Oxytocin (B) and [8-Arginine] Vasopressin (C) were screened for cleavage by the enzyme using procedures described in METHODS. After incubation the enzyme was eliminated using a PM-30 Amicon membrane and the filtrate was chromatographed by HPLC using conditions given in METHODS. Eluent peaks were detected by UV absorbance at 214 nm. The downward arrow indicates the injection point for the filtrate. Time scale for A is 50 min and for B and C is 20 min.



Arg<sup>8</sup>-GlyNH<sub>2</sub> bond to yield (desGly-NH<sub>2</sub>)AVP (Figure 16C). Further verification that Gly-NH<sub>2</sub> was released from AVP by the enzyme was obtained by using AVP which was radioactively labelled in this moiety. When this substrate was incubated with the enzyme, a [<sup>14</sup>C]-Gly-NH<sub>2</sub> peak was detected by HPLC using a flow through radioactive scintillation detector. No such peak was evident in controls that had no tryptidase.

The pH optimum for AVP cleavage was determined and the results are expressed in Figure 17. Tryptidase cleaved AVP optimally at pH 7.5 which is also the optimal pH for BAEE hydrolysis.

#### 16. Tryptidase Assays for Proteinase Activity

In casein-agar plate assays (Figure 18), no zones of lysis were seen which were similar to those seen for trypsin controls. The quantity of enzyme used was equivalent in BAEE activity to the corresponding control used.

Similarly, as shown in Table XIX no proteinase activity for tryptidase was detected when haemoglobin or azocoll (a collagen substrate with an azo dye prepared from cowhide) was used as a substrate. Substantial cleavage of these substrates was observed in controls in which trypsin was substituted for tryptidase. In these cases, tryptidase assays contained twice the BAEE activity of the corresponding trypsin control.

#### 17. Active Site Titration of Tryptidase

Since NPGB and MUGB were shown to inhibit the enzyme,

Figure 19. pH optimum for [8-Arginine] Vasopressin. Buffers (0.1 M) were prepared as described in METHODS. The enzyme was incubated in 113  $\mu$ l of each buffer for 17 h at room temperature at the end of which the reaction was terminated using 10 mM heptane sulfonic acid phosphate, pH 2.5 and the enzyme was removed by filtration through a PM-30 Amicon membrane. Filtrates with their corresponding controls were then chromatographed by HPLC. Incubations containing no enzyme served as controls. The area under the peaks detected for desglycinamide vasopressin (DG-AVP) were calculated and used to determine the % of AVP cleaved which was then plotted as a function of pH.

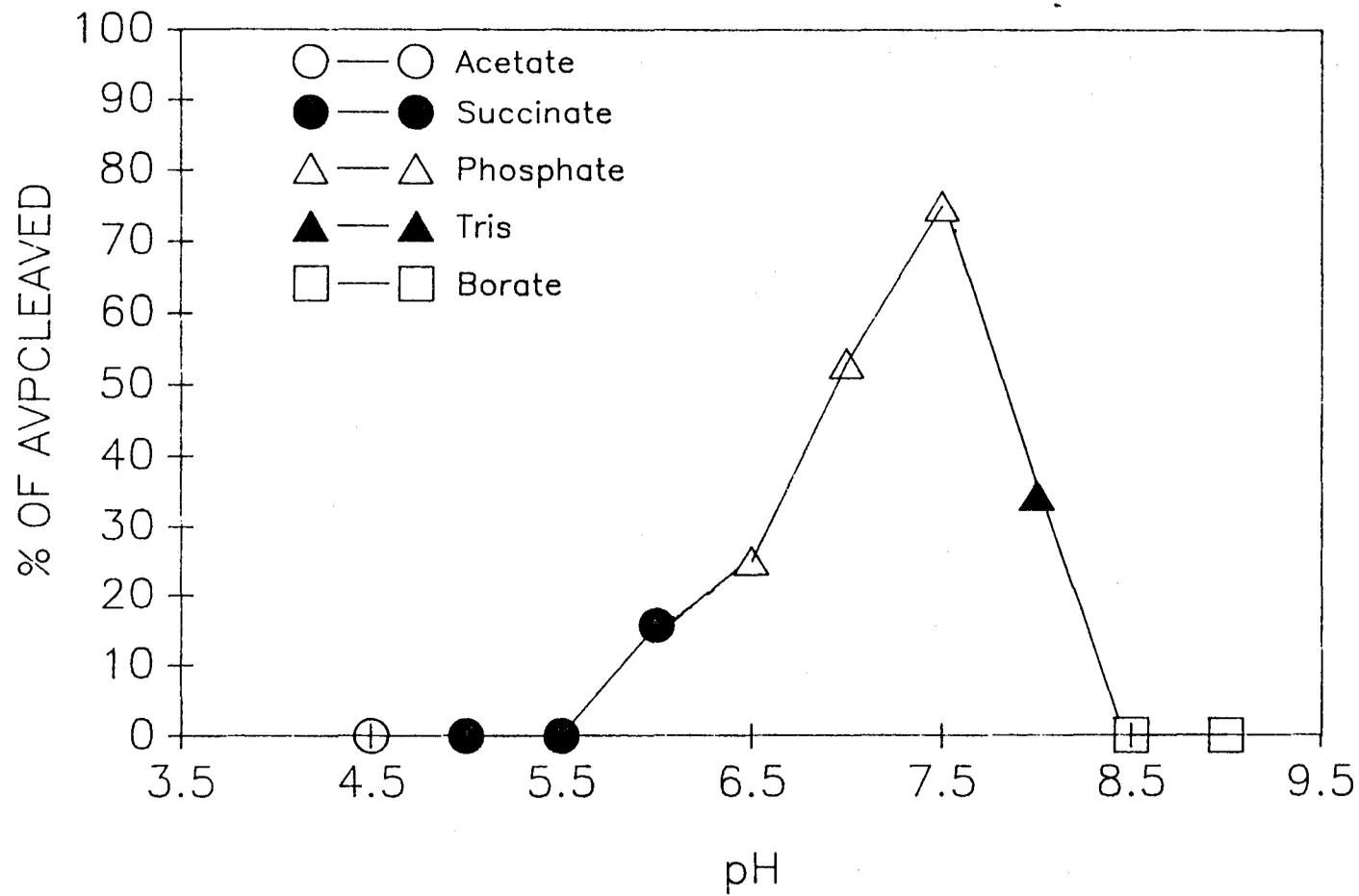


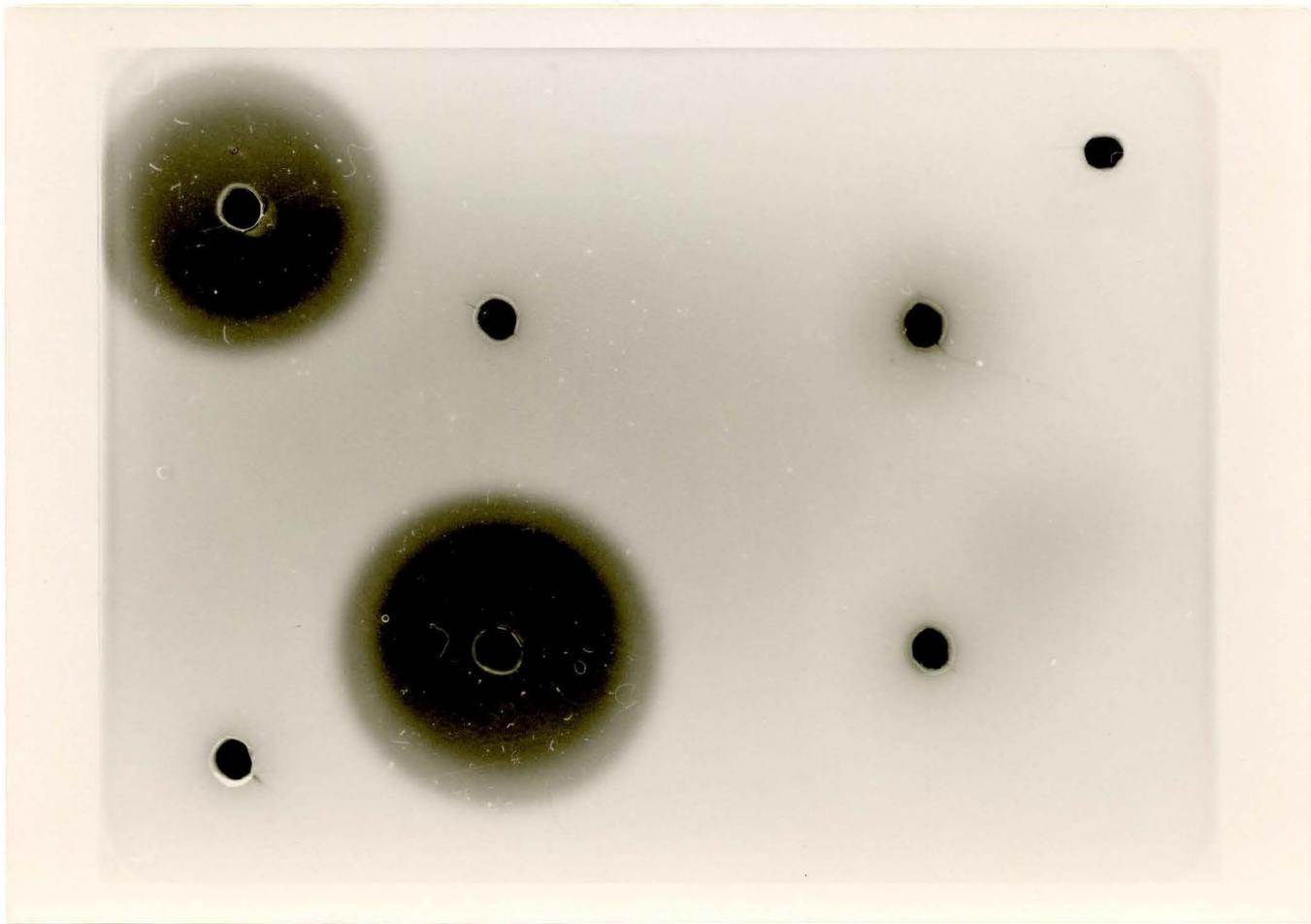
Figure 20. Assay for caseinolytic activity by tryptidase. Details of the protocol used to prepare the casein agar plates are described in METHODS. Trypsin was used as a control and an aliquot of enzyme equivalent to the BAEE activity of trypsin was used. The enzyme buffer and the buffer used to reconstitute trypsin were also run as controls. Clear areas around trypsin controls indicate cleavage. No such cleavage was apparent around wells containing buffer controls or enzyme.

TRYPsin (5 ul)

TRYPtidase (15 ul)

0.001 N HCl ( 10 ul)

PHOS-EDTA, 0.5 M NaCl, pH 6.0 (30 ul)



TRYPsin (10 ul)

TRYPtidase (30 ul)

TABLE XIX  
EFFECT OF TRYPTIDASE ON PROTEIN SUBSTRATES

| PROTEIN     | TYPE OF ASSAY                   | HYDROLYSIS |            |
|-------------|---------------------------------|------------|------------|
|             |                                 | TRYPSIN    | TRYPTIDASE |
| CASEIN      | agar gel                        | +++        | ---        |
| AZOCOLL     | colorimetric<br>(azo dye)       | +++        | ---        |
| HAEMOGLOBIN | Colorimetric<br>(Folin reagent) | +++        | ---        |

The amount of protein in the assay was 18.5 g/l in the case of casein and haemoglobin and 23 g/l in the case of azocoll. All assays were performed at 25<sup>0</sup> and incubation was for 30 min. Absorbances for azocoll were read at 520 nm and for haemoglobin at 578 nm. Further details of the assays are given in METHODS.

it was possible to attempt to use them as active site titrants to stoichiometrically quantitate the enzyme as has been done for trypsin (Jameson et al., 1976). Since NPGB requires the use of a spectrophotometric assay with a limit of detection of 1  $\mu$ M, it was impossible to use the reagent because of the large amounts of enzyme required to make valid measurements. MUGB, which is used in a fluorescent assay with detection possible in the pM range, was therefore used for tryptidase. A 50 nM concentration of MUGB was used in the assays. Figure 21 depicts curves of change in % fluorescence versus time for varying quantities of enzyme. The pattern of the curves indicates an initial rapid undetected "burst" followed by a plateau phase which presumably represents an acylenzyme intermediate that turns over very slowly (see Discussion).

Figure 22 expresses the burst in terms of % fluorescence as a function of the volume of enzyme solution used. The linearity of the curve is proof that extrapolation of the plateau phase in Figure 19 to the y axis is a good measure of the stoichiometric quantity of the enzyme, and is not a substrate depletion phenomenon. Moreover, an aliquot of enzyme assayed at double and triple the MUGB concentrations gave the same quantitative "burst".

The % fluorescence corresponding to the burst of the enzyme was used to determine the molarity of the enzyme from a standard curve of concentration of methyl umbelliferone

Figure 21. Active site titration of tryptidase. The protocol used in this experiment is described in detail in METHODS. The % fluorescence as a function of time was monitored for 5 ul, 10 ul and 20 ul volumes of the enzyme. The burst was extrapolated to the ordinate axis and the size of the burst determined after subtraction of the MUGB blank.

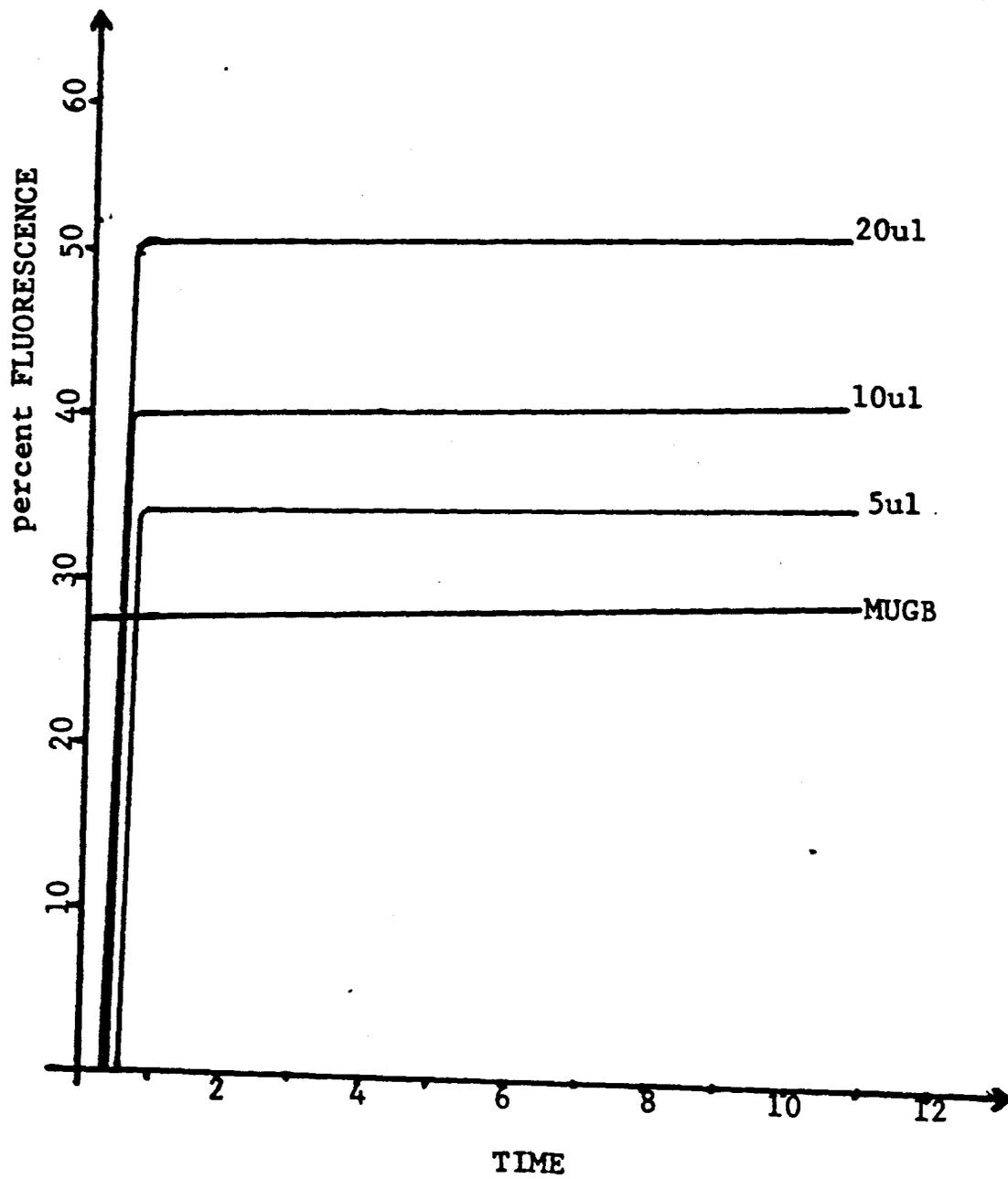
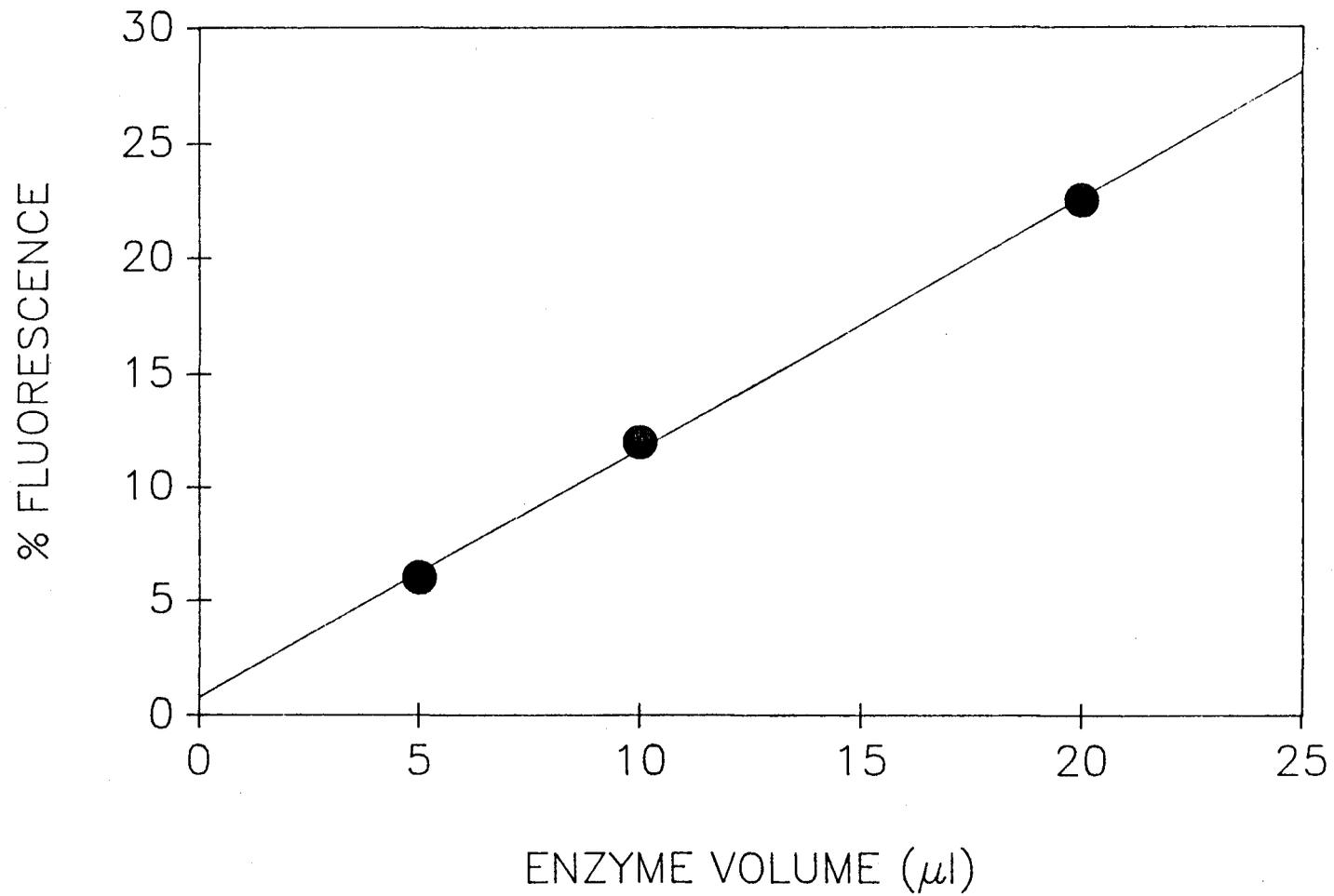


Figure 22. Active site titration of tryptidase. The experiment was performed as described in Fig. 21. The "burst" was expressed as % fluorescence and was plotted as a function of enzyme volume used in the assays. Enzyme aliquots used were from the same preparation.



(MU) vs. % fluorescence. The results obtained are shown in Table XX. The table shows that estimates of the enzyme weight (ng) made on the basis of active site titration and the enzyme molecular weight match very closely the weight calculated on the basis of the Bradford protein assay. This serves as a further proof of the purity of the enzyme, indicating the absence of non-enzymatic protein contamination.

#### 18. Partial N-Terminal Sequence of Tryptidase

The N-terminal sequence of tryptidase has been obtained to the first 25 amino acids (Figure 23). This region shows 40% homology with trypsin. The first four amino acids are identical to that of bovine trypsin. Residue 21 is a putative glycosylation site due to the threonine at position 23. Based on this partial sequence figure 23 shows that tryptidase can be distinguished from other well known serine enzymes.

TABLE XX  
ACTIVE SITE TITRATION-BASED STOICHIOMETRIC  
QUANTITATION OF TRYPTIDASE

| ENZ. VOL.<br>(ul) | ENZ. WT <sup>a</sup><br>(ng) | "BURST" <sup>b</sup> | QUANTITATION        |                          |
|-------------------|------------------------------|----------------------|---------------------|--------------------------|
|                   |                              |                      | pmoles <sup>c</sup> | weight <sup>d</sup> (ng) |
| 5                 | 270                          | 6%                   | 7.1                 | 284                      |
| 10                | 541                          | 12%                  | 15.5                | 620                      |
| 20                | 1080                         | 22.5%                | 30.2                | 1200                     |

<sup>a</sup>Weight of enzyme in corresponding volume based on Bradford microassay estimations

<sup>b</sup>Burst is the % fluorescence obtained on extrapolation of curves shown in Fig. 19 back to the y axis minus inherent substrate fluorescence,

<sup>c</sup>Calculations are based on a standard curve of % fluorescence vs. concentration of methylumbelliferone, for which  $m = 0.714$ ,  $c = 0.948$  and the  $RVal = .998$

<sup>d</sup>Estimates based on a molecular weight of 40,000 determined from SDS polyacrylamide gels.

Figure 23. Partial N-Terminal Sequence of Tryptidase. 25 ng of enzyme were processed as described in METHODS. The amino acid sequencing was a commercial courtesy of Applied Biosystems Incorporated (ABI). Amino acid sequences for the other serine proteases were from a) Mikes et al., (1966), b) Shotton and Hartley (1970), c) Chung et al., (1986), d) Woodbury et al., (1978), e) Woodbury et al., (1978), f) Thomas and Bradshaw (1981), g) Fock-Nuzel et al., (1980), h) Wiman (1977), i) Zwilling et al., (1975), j) Franz (1977), k) Cromlish et al., (1987), l) Lottspeich et al., (1979), m) Cool et al., (1985). Boxed segments indicate homology with tryptidase.

|   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  | 23  | 24  | 25  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| <b>Tryptidase</b><br>(rat)  | Ile | Val | Gly | Gly | Gln | Glu | Ala | Ser | Gly | Asn | Lys | Trp | Pro | Trp | Gln | Val | Ser | Leu | Arg | Val |     | Asp | Thr | Tyr | Trp |
| <b>Trypsin<sup>a</sup></b><br>(bovine)                                      | Ile | Val | Gly | Gly | Tyr | Thr | Cys | Gly | Ala | Asn | Thr | Val | Pro | Tyr | Gln | Val | Ser | Leu | Asn | Ser | Gly | Tyr | His | Phe | Cys |
| <b>Chymotrypsin A<sup>b</sup></b><br>(bovine)                               | Ile | Val | Asn | Gly | Glu | Glu | Ala | Val | Pro | Gly | Ser | Trp | Pro | Trp | Gln | Val | Ser | Leu | Gln | Asp | Lys | Thr | Gly | Phe | His |
| <b>Kallikrein<sup>c</sup></b><br>(human, bovine)<br>(2 chains)              | Ile | Val | Gly | Gly | Thr | Asn | Ser | Ser | Trp | Gly | Glu | Trp | Pro | Trp | Gln | Val | Ser | Leu | Gln | Val | Lys | Leu | Thr | Ala | Gln |
| <b>Elastase<sup>d</sup></b><br>(porcine)                                    | Val | Val | Gly | Gly | Thr | Glu | Ala | Gln | Arg | Asn | Ser | Trp | Pro | Ser | Gln | Ile | Ser | Leu | Gln | Tyr | Arg | Ser | Gly | Ser | Ser |
| <b>RMCP I<sup>d</sup></b><br>(rat)  | Ile | Ile | Gly | Gly | Val | Glu | Ser | Arg | Pro | His | Ser | Arg | Pro | Tyr | Met | Ala | His | Leu | Glu | Ile | Thr | Thr |     |     |     |
| <b>RMCP II<sup>e</sup></b><br>(rat)   | Ile | Ile | Gly | Gly | Val | Glu | Ser | Ile | Pro | His | Ser | Arg | Pro | Tyr | Met | Ala | His | Leu | Asp | Ile | Val | Thr |     |     |     |
| <b>Y-NGF<sup>f</sup></b><br>(mouse)   | Ile | Val | Gly | Gly | Phe | Cys | Glu | Lys |     | Asn | Ser | Gln | Pro | Trp | His | Val | Ala | Val | Tyr | Arg | Tyr | Thr | Gln | Tyr | Leu |
| <b>Acrosin<sup>g</sup></b><br>(boar)  | Val | Val | Gly | Gly | Met | Ser | Ala | Glu | Pro | Gly | Ala | Trp | Pro | Trp | Met | Val | Ser | Leu | Gln | Ile | Phe | Met | Tyr | His | Asn |
| <b>Plasmin<sup>h</sup></b><br>(human)                                       | Val | Val | Gly | Gly | Cys | Val | Ala | His | Pro | His | Ser | Trp | Pro | Trp | Gln | Val | Ser | Leu | Arg | Thr | Arg | Phe | Gly |     |     |
| <b>Thrombin<sup>i</sup></b><br>(pig, bovine)                                | Ile | Val | Glu | Gly | Gln | Asp | Ala | Glu | Val | Gly | Leu | Ser | Pro | Trp | Gln | Val | Met | Leu | Phe | Arg |     |     |     |     |     |
| <b>Factor Xa<sup>i</sup></b><br>(bovine)                                    | Ile | Val | Gly | Gly | Arg | Asp | Cys | Ala | Glu | Gly | Glu | Cys | Pro | Trp | Gln | Ala | Leu | Leu | Val | Asn |     |     |     |     |     |
| <b>B Kallikrein B<sup>j</sup></b><br>(porcine)<br>(pancreatic)<br>(A chain) | Ile | Ile | Gly | Gly | Arg | Glu | Cys | Glu | Lys | Asn | Ser | His | Pro | Trp | Gln | Val | Ala | Ile | Tyr | His | Tyr | Ser | Ser | Phe | Gln |
| <b>Tryptase<sup>k</sup></b><br>(human)<br>(pituitary)                       | Ile | Val | Gly | Gly | Gln | Glu | Ala | Pro |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| <b>Tryptase<sup>k</sup></b><br>(human lung)                                 | Ile | Val | Gly | Gly | Gln | Glu | Ala | Pro | Arg | Ser |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| <b>Kallikrein<sup>l</sup></b><br>(human urine)                              | Ile | Val | Gly | Gly | Trp | Glu | Cys | Glu | Gln | His | Ser | Gln | Pro | Trp | Gln | Ala | Ala | Leu | Tyr | His | Phe | Ser | Thr | Phe | Gln |
| <b>Tissue-PA<sup>m</sup></b><br>(human)                                     | Ile | Lys | Gly | Gly | Leu | Phe | Ala | Asp | Ile | Ala | Ser | His | Pro | Trp | Gln | Ala | Ala | Ile | Phe | Ala | Lys | His | Arg | Arg | Ser |

## CHAPTER V

### DISCUSSION

#### A. Purification Of Tryptidase

There are obviously several BAEE hydrolyzing enzymes present in skin tissue (Fraki et al, 1983). Of these, carboxamidopeptidase (cathepsin A) (Simmons & Walter, 1981) and tryptase (in non-skin tissue) have been purified to apparent homogeneity. The other enzymes have not been extensively purified and hence studies done on them describe the mixed characteristics of more than one enzyme. Tryptidase represents yet another BAEE hydrolyzing enzyme isolated from skin. Having pure tryptidase allows us to identify those characteristics which distinguish it from carboxamidopeptidase and tryptase of the skin as well as from similar enzymes from other tissue sources.

The purification scheme developed for tryptidase has been optimized to yield pure enzyme in the least possible time with an economy of materials. The initial extraction step is the first example of this optimization. Previous workers had frequently used 1 M NaCl to extract skin proteases (Fraki et al., 1983). However in the case of carboxamidopeptidase from toad skin, a 1:1 dilution of the 1 M NaCl extract was required to improve the efficiency of the subsequent ammonium sulfate step resulting in increased

volume (Simmons & Walter, 1981). Since Table I indicated that extraction of rat skin with 1 M NaCl did not yield significantly more BAEE activity than extraction with 0.5 M NaCl the latter concentration was chosen which eliminates the need for dilution. This economized on the use of both NaCl and  $(\text{NH}_4)_2\text{SO}_4$  and reduced purification time by minimizing the volumes of extract that had to be handled. Although the ammonium sulfate step results in only a small increase in purification it does help to concentrate the enzyme to a volume which is more manageable for subsequent chromatography. Furthermore almost 50% of the original starting protein is eliminated in this step. This appears to be important since an attempt to eliminate this step from the purification scheme and to apply the homogenate directly to a specific affinity column was not successful. Lipid and protein contaminants in the homogenate clogged the column. Even the use of filters did not prevent this from occurring.

Initial studies were carried out on the use of ion exchange chromatography for the purification of the enzyme. However, since the procedures did not provide significant advantages and were time consuming, they were subsequently dropped from the preparation scheme. In retrospect, some of the problems encountered with ion exchange chromatography may have related to lack of knowledge at the time about the presence of multiple enzymes which cleave BAEE.

The key step of the purification scheme is the dual

column specific affinity chromatography which utilizes A-Gly-Gly-PAB and con A-agarose in tandem. In developing the PAB step, it was assumed that the enzyme which hydrolyzes BAEE would have an affinity for p-aminobenzamidine which has an amidino group similar to the arginine residue in the substrate. However, the bulk of the BAEE activity applied to the A-Gly-Gly-PAB column passed through it. This phenomenon occurred, although to different degrees, irrespective of whether the application of the sample to the column was done at room temperature or at 4<sup>0</sup>. We have repeatedly shown that the lack of binding of most of the BAEE activity is not an overloading effect. This phenomenon confirms the presence of more than one BAEE hydrolyzing enzyme described in the literature (Fraki et al, 1983). It is possible that the glycyl-glycine spacer arm on the column may have provided additional specificity which allowed for selective adsorption of tryptidase but not other BAEE-hydrolyzing enzymes in the mixture. Indeed a p-aminobenzamidine column with a caproic acid spacer arm adsorbed much more BAEE activity.

Even greater purification is achieved in this step by exploiting the temperature dependence of the binding of proteins to specific affinity matrices. The binding affinity is inversely dependent on the temperature (Lowe & Dean, 1974). While tryptidase has a high enough affinity for the column to remain bound at both 4<sup>0</sup> and room

temperature, much contaminating protein which can bind at 4<sup>0</sup> fails to bind at room temperature. As a result, better purification factors were achieved by running the column at room temperature. Thus, the purification scheme exploits the temperature dependence of the differential affinity of various enzymes and proteins for the A-Gly-Gly-PAB column.

While the affinity for A-Gly-Gly-PAB is active site directed, the affinity of tryptidase for concanavalin A depends on binding to carbohydrate moieties. Since these two affinities occur at different parts of the enzyme molecule, it was possible to elute the enzyme from the first column directly onto the concanavalin A-agarose column. Benzamidine, which is used as a competitive ligand to elute the enzyme from the A-Gly-Gly-PAB column, can be easily diluted out in the process of binding, washing and eluting the enzyme from the Con A column. Thus there was no need to physically subject the enzyme to additional steps to remove the benzamidine and concentrate the enzyme for application to the Con-A column. This results in considerable savings of time and enzyme.

Concanavalin A covalently attached to agarose binds to glycoproteins (Bishayee and Bachhawat, 1974). This lectin is from jack bean (Canavalin ensiformis) and interacts specifically with glucose, fructose, mannose, and glucosamine and other sterically related sugars. Bound glycoproteins can be dissociated from Con-A by  $\alpha$ -methyl-D-

mannoside or  $\alpha$ -methyl-D-glucoside (reviewed in Lowe & Dean, 1974). Thus the binding of tryptidase to the Con A column establishes the glycoprotein nature of the enzyme. Tryptidase elution from the column is achieved with  $\alpha$ -methyl-D-mannoside (100 mg/ml). While the specific activity of tryptidase does not increase much during this step, it is an important part of the purification scheme since a contaminating protein(s?) eluting from the A-Gly-Gly-PAB column is eliminated (cf. Figure 3). Hence the step is essential in the present scheme to obtain the final pure enzyme.

The salient features of the purification scheme for tryptidase are summarized below:

- a) The enzyme is not subjected to gross changes in buffer or pH or salt concentrations.
- b) The entire purification procedure, from tissue preparation (for 12 rat skins) until storage of concentrated final pure enzyme, can be achieved in 4 days.
- c) The procedure yields apparently homogeneous enzyme in essentially two steps. This is a rare achievement for a purification scheme starting with a tissue source.
- d) The presence of 1 mM EDTA (or 0.02%  $\text{NaN}_3$ ) throughout the purification scheme prevents bacterial contamination of the preparation.

e) Both the A-Gly-Gly-PAB and Con-A affinity column used in the scheme can be regenerated and used repeatedly. (Repeated use of these columns through more than 15 preparations to date have yielded eminently reproducible results.)

Tryptidase can be stored for extended periods of time without significant loss of activity in phos-EDTA-0.5 M NaCl-6.0. The presence of salt is important to the long range stability of the enzyme even at 4<sup>0</sup>. In its absence, the enzyme undergoes some form of irreversible denaturation. Whether the ionic components of NaCl prevent this by stabilizing the tetrameric form of the enzyme is not known.

B. Selective Substrates for Monitoring the Purification of Tryptidase.

The trypsin-like substrate BAEE was used to monitor most of the purifications in this project. However, because there are other enzymes beside tryptidase in the preparation which can cleave this substrate, the purification factors and recoveries calculated on the basis of BAEE do not give accurate reflections of these parameters for tryptidase. Calculations based on subtracting non-tryptidase breakthrough activity yielded better results.

Much higher purification factors and recoveries were achieved when Chromozym PL, S-2288 and S-2238 were used to monitor the purification of tryptidase. The breakthrough from the PAB column showed very little activity against the

former two substrates. These facts suggest that Chromozym PL and S-2288 are highly selective for tryptidase in rat skin extract. Both of these substrates are tripeptide p-nitroanilides. Their selectivity for tryptidase is based on two factors: a) other enzymes cleave this substrate poorly relative to BAEE and b) tryptidase has enhanced activity toward these substrates presumably because of the presence of P<sub>2</sub> and P<sub>3</sub> residues in the substrate which interact with an extended binding pocket at the enzyme active site. In fact, the rates for the tripeptides having an arginine residue in the P<sub>1</sub> position are much higher than those seen for BAPNA which has only a single amino acid.

### C. Physical, Chemical, Enzymatic and Structural Characteristics of Tryptidase

Since early evidence collected on the enzyme suggested the likelihood of a novel enzyme, it was our goal to fingerprint the enzyme with respect to its physical, chemical, catalytic and structural properties. This would enable us to compare the enzyme with other enzymes of the skin and similar enzymes from other sources.

#### 1. Chemical Characteristics.

Tryptidase was found to be partially stable over a wide range of pH's (3-8.5) in its purified state. Its maximum activity toward BAEE was at pH 7.5. Its pH optimum for the larger substrate vasopressin was also pH 7.5. The pH studies with BAEE were done at almost 100 times the

substrate  $K_m$  and consequently may be expected to reflect primarily the effect of pH on enzyme turnover ( $k_{cat}$ ). The increased turnover seen above pH 6.0 can be attributed to an amino acid residue in the active site of the enzyme that has a  $pK_a$  of around 6.5. It has been suggested in the case of trypsin, chymotrypsin and other serine proteases that the deprotonation of a histidine residue above pH 6.5 is involved in the generation of catalytic activity (Kraut, 1977; Huber and Bode, 1978). Trypsin is a serine protease on the basis of its inhibition by DFP; hence its activity may be explainable in similar terms by analogy. Since the enzyme is also inhibited by TLCK which acts by alkylating a histidine in the active site of serine proteases, it is reasonable to suggest that a histidine is also present in the active site of trypsin and plays a role in the "charge relay system". At pH's above 8.5, the enzyme loses activity rapidly. This loss of activity can be explained, at least in part, in terms of the instability of the enzyme at these pH's. It is possible that these higher pH's result in the deprotonation of a residue that is important for the stability of the enzyme. It is noteworthy that the pH optimum of the enzyme is in the pH region where it is beginning to show instability.

In the case of trypsin, an Asp-189 has been found in the binding pocket of the enzyme and has been implicated in the binding of substrate arginines or lysines (Huber & Bode,

1978). At pH's close to the pH optimum at which the catalytic histidine is deprotonated this residue has a negative charge and can serve as a cationic binding site for the positively charged arginine or lysine residue. It is likely that a similar cationic binding site is available in tryptidase and confers on it its trypsin-like specificity for basic amino acid residues.

Among the protein proteinase inhibitors tested against tryptidase, Trasylol was the only one that proved effective. SBTI, LBTI, ovom inhibitor, and  $\alpha_1$ -antitrypsin, which are capable of inhibiting trypsin at a 1:1 molar ratio (Kassel, 1970a; 1970b; 1970c), had no effect even though higher molar excesses of these inhibitors were used in these experiments. The ratio of inhibitor to enzyme for Trasylol was one tenth that used for the other inhibitors.

Four of the microbial inhibitors isolated from Actinomyces preparations by Umezawa and coworkers (Umezawa and Aoyagi, 1977), were tested, and only antipain and leupeptin were effective inhibitors of tryptidase. Both antipain (a tetrapeptide) and leupeptin (a tripeptide), which contain a C-terminal argininal and inhibit trypsin, were equally effective against the enzyme. However, chymostatin (a tetrapeptide with a C-terminal phenylalaninal) and elastatinal (a tetrapeptide with a C-terminal alaninal), had no effect on the enzyme at concentrations as high as 200 ug/ml. Chymostatin and

elastatinal likewise do not inhibit trypsin. The aldehyde function of the basic residue in antipain and leupeptin is the likely cause of inhibition of tryptidase. Other microbial inhibitors that had no effect on tryptidase were pepstatin (an inhibitor of acid proteases) and bestatin (an inhibitor of aminopeptidases).

The active site titrants of trypsin, NPGB and MUGB, were highly effective inhibitors of tryptidase. Inhibition by these esters is explained in terms of a rapid acylation step followed by a slow deacylation that results in a stable guanidinobenzoyl-enzyme intermediate (Chase & Shaw, 1970). The initial instantaneous hydrolysis of these substrates followed by slow turnover allows for the quantitation of the stoichiometric reaction between enzyme and substrate (Bender et al., 1970). Although the phenolic product of NPGB was not of practical use in quantitative determinations of tryptidase because of its limited sensitivity, the fluorescent product of MUGB, methylumbelliferone, was successfully used to quantitate tryptidase in the nM range.

DFP inhibits proteinases and peptidases that contain a serine residue in their active sites by forming a phosphate ester with this residue. DFP is considered to be a group-specific inhibitor of serine proteases (Barrett, 1977). Tryptidase was significantly inhibited by 1 mM DFP upon preincubation at pH 7.5 for 10 min, and essentially all of its activity was lost after a preincubation for 30 min at

pH 6.0. On the basis of this inhibition by DFP, tryptidase may be classified as a serine protease (peptidase).

Detailed studies done on serine proteases indicate that they have a "charge relay system" at their active sites comprising a serine residue which is hydrogen bonded to a histidine residue which in turn is hydrogen bonded to an aspartic acid residue (Kraut, 1977). The histidine in this system is susceptible to covalent modification by chloromethyl ketone derivatives of certain amino acids. In the case of trypsin, TLCK alkylates the histidine at its active site (Powers, 1977). Since tryptidase is inhibited by TLCK it is possible that a similar reaction is taking place between the inhibitor and a histidine in its active center. However, in the case of tryptidase this point is a matter of conjecture since chloromethyl ketones are also known to react by alkylating the active site sulfhydryl groups of thiol proteases (Whitaker and Perez-Villasenor, 1968) and there is evidence that the enzyme has a free sulfhydryl group.

p-Chloromercuribenzoate (p-CMB) reacts with free sulfhydryl groups of proteins to form mercaptides (Barrett, 1977). This inhibitor inactivates tryptidase almost completely at concentrations as low as 50  $\mu$ M, implying that the enzyme contains a free sulfhydryl group which could be at its active center or at some position where it can play a critical role in the conformation of the enzyme. If the

sulfhydryl were responsible for catalysis, however, one would expect to see inhibition by E-64, which is not the case with tryptidase. The sulfhydryl could lie uninvolved in the vicinity of the substrate binding site. When p-CMB reacts with the sulfhydryl, the p-CMB group could sterically prevent the binding of substrate (Bai & Hayashi, 1975). The much smaller  $\text{Hg}^{++}$  ion is also able to inhibit the enzyme, but only at mM concentrations (data not shown) suggesting that the relative bulkiness of p-CMB is important for its inhibitory activity.

Throughout most of the purification scheme for tryptidase, the metal chelating agent EDTA is used in the buffer. However, during the concanavalin A-agarose step, EDTA was replaced with a bacteriostatic agent  $\text{NaN}_3$  which has no metal chelating properties. Whether or not EDTA is present has no effect on recoveries or activity of the enzyme, suggesting that the enzyme is not a metalloenzyme. Furthermore, heavy metal ions like magnesium ( $\text{Mg}^{++}$ ), manganese ( $\text{Mn}^{++}$ ), cobalt ( $\text{Co}^{++}$ ) and calcium ( $\text{Ca}^{++}$ ) at 10 mM have no significant activating or inhibitory effect. Of the metal ion studies, only the mercury ion ( $\text{Hg}^{++}$ ) (at 2 mM) inhibited.

Tryptidase, in contrast to tryptase, is not affected by the presence of salt as far as activity is concerned. However, it does require salt for long term stability. It is possible that in the total absence of NaCl, tryptidase

breaks down into inactive subunits. The lowest salt concentration used in the gel filtration experiments was 50 mM which was still sufficient to keep the subunits together (cf Figure 12). Gel filtration on enzyme stored in the absence of salt and run on a column equilibrated in a buffer without salt should be able to prove or disprove this hypothesis.

## 2. Physical Characteristics

In analytical high performance gel permeation chromatography in the presence of 1 M NaCl, the molecular weight of tryptidase was determined to be 158,000. No higher molecular weight aggregates were detected when the experiment was done at 0.5 M NaCl or again at 0.05 M NaCl. However, on sodium dodecyl sulfate (SDS) gel electrophoresis under non-reducing conditions, a single protein band corresponding to a molecular weight of 41,000 was detected. This suggests that tryptidase is a multimeric enzyme made up of at least 4 subunits. When SDS gel electrophoresis was performed under reducing conditions, no new bands were seen and only a single band corresponding to a molecular weight of 40,000 was seen. Thus the subunit of tryptidase is a single polypeptide chain. Experiments in which radio-labelled DFP was bound to the active site showed that the label migrated to the same position in the gel as the stained protein band under both reducing and non-reducing conditions. Calculations of the weight of the enzyme

protein based on the molarity of the enzyme determined by active site titration and a subunit molecular weight of about 40,000 closely approximated to Bradford protein estimates of enzyme protein within experimental error. This suggests that each subunit contains one active site. Whether the single subunit is active by itself is not known. Trypsin in its monomeric form is known to lose activity (Schwartz and Bradford, 1986a).

Unlike trypsin, the pI of tryptidase is in the acidic range. When the enzyme was subjected to isoelectric focusing on PAG-plates up to five bands ranging in pI from 4.5 to 4.9 were observed with the most prominent band at 4.8. This microheterogeneity in pI is not surprising in view of the glycoprotein nature of tryptidase. Since sialic acid or phosphate can contribute to glycoprotein charge heterogeneity, treatment of the enzyme with a sialidase, alkaline phosphatase, or endoglycosidase H might remove these charges and cause all bands to focus together.

The low pI of tryptidase implies that at pH's around 7.5 the enzyme has an overall negative charge. At these pH's the protein inhibitors SBTI, LBTI and ovomucoid would also be negatively charged. Trypsin on the other hand would be positively charged and hence would attract these inhibitors whereas tryptidase would repel them. Hence one possible explanation for the lack of effect of these inhibitors on tryptidase is that they might be unable to

approach the enzyme due to electrostatic repulsion. Trasylol, which is effective against tryptidase, is positively charged and so would be able to approach the enzyme and inhibit it according to this hypothesis.

### C. Enzymatic Characteristics of Tryptidase

The substrate screening suggested that tryptidase has a specificity for basic amino acid residues. It is not possible from the data available in this study to state conclusively that the enzyme has a preference for arginine over lysine. However, since activity against H-D-Val-Leu-Arg-pNA is about four times higher than against H-D-Val-Leu-Lys-pNA (cf Table XVIIIa), this may be an indication of such a preference. No cleavage of the chymotrypsin substrate, ATEE was observed.

On the basis of its relatively high activity toward p-NA substrates having a proline or a proline analog in the P<sub>2</sub> position on the amide side of arginine or lysine, it can be suggested that the enzyme may favor proline in this position.

Tryptidase is further able to act on the larger peptide AVP, removing a glycinamide from the C-terminus of this nonapeptide. Interestingly, AVP has a proline in the P<sub>2</sub> position (----Pro-Arg-GlyNH<sub>2</sub>). The pH optimum for AVP cleavage is the same as for BAEE which suggests that the mechanism of catalysis may have some similarities to that for BAEE.

The rate of cleavage of BAEE is greater than that of BAPNA. In this respect tryptidase behaves in a manner characteristic of serine proteases which hydrolyze ester bonds much faster than amide bonds. BAME and BAEE, two esters with the same acyl groups but different leaving groups are hydrolyzed with similar  $k_{cat}$ 's. This suggests that the deacylation step is rate limiting in ester hydrolysis by tryptidase as it is for the serine protease chymotrypsin (Zerner et al., 1964). A "burst" followed by an extremely low turnover of the acyl-enzyme complex, as seen in active site titration experiments using MUGB, is even more dramatic evidence for the formation of an acyl intermediate and for rate-limiting deacylation.

Three nonspecific protein proteinase substrates, casein, haemoglobin, and azocoll, were resistant to hydrolysis by tryptidase. Hence, there is no evidence at this time to suggest that tryptidase has proteinase activity. The casein-agar assay and azocoll assay were of sufficient sensitivity to detect any significant non-specific proteinase activity if the enzyme has any. The nature of the assays however, precluded detection of any highly selective cleavage of these substrates. Nevertheless, the inability to demonstrate gross cleavage of these protein substrates indicates that the specificity of tryptidase is not as broad as that of trypsin.

#### 4. Structural Characteristics of Tryptidase

The N-terminal sequence of tryptidase (cf Figure 23) shows that it shares several features in common with serine proteases. The first four amino acid residues, Ile-Val-Gly-Gly is homologous to that of trypsin and several other serine proteases. The N-terminal isoleucine has been shown in trypsin and many other serine proteases to form an internal salt bridge upon zymogen activation (Ruhlmann et al., 1973; Young et al., 1978). Hence it is possible that tryptidase has a precursor. Tryptidase also has the glycine at residue 4 and the proline at residue 13 conserved, like all the serine proteases shown in Figure 23. These features corroborate the idea that tryptidase is a serine protease.

The first seven amino acids of tryptidase are identical to those of human pituitary tryptase, and human lung tryptase. However a comparison of the rest of the known sequence of human pituitary tryptase (8 residues) and human lung tryptase (10 residues) with tryptidase shows that the sequences are not homologous, and moreover, do not even share a common type or kind of amino acid. These differences suggest that the tryptidase and tryptase are different enzymes.

#### D. Tryptidase as a Novel Enzyme

The profile of the various chemical, physical and catalytic properties of tryptidase described above suggests that the enzyme has some trypsin-like features. The pH optimum is close to that for trypsin. Inhibitors of trypsin

such as Trasylol, NPGB and MUGB also inhibit tryptidase. The specific ligand, p-aminobenzamidine, used to purify the enzyme, and benzamidine used to elute it from the A-Gly-Gly-PAB column are both trypsin inhibitors. Tryptidase can also hydrolyze the trypsin-like substrates BAEE and BAPNA. Like trypsin, tryptidase is a DFP-sensitive serine protease.

However, a number of other enzymes exist that share similar characteristics with trypsin and yet differ from it. These need to be discussed with reference to tryptidase. The enzyme that shares the greatest similarities in properties with tryptidase is tryptase. Tryptase has been isolated and purified from human skin (Fraki & Hopsu-Havu, 1975), human lung (Schwartz *et al.*, 1981; Smith *et al.*, 1984), human pituitary (Cromlish *et al.*, 1987), human uterine cervix (Ito *et al.*, 1980) and rat mast cells (peritoneal) (Kido *et al.*, 1985a).

The rat mast cell tryptase is inhibited by antipain, leupeptin and aprotinin like tryptidase. However, unlike tryptidase it is also significantly inhibited by SBTI and  $\alpha_1$ -antitrypsin as well as elastatinal at concentrations of 10  $\mu$ M. Another significant difference is that the mast cell enzyme activity doubles in the presence of calcium (10 mM). No such effect was seen with tryptidase. The molecular weight of tryptase determined by gel filtration is 140,000 which is somewhat lower than that of tryptidase which is 160,000. A series of seven tripeptidyl MCA compounds which

were previously tested as substrates for trypsin (Kido et al., 1985) were also assayed with trypsin (data not shown). The specificity pattern for trypsin is markedly different from that for trypsin assayed in the absence of the endogenous inhibitor trypsin.

With regard to trypsinases from the other tissues mentioned above, the human skin trypsin is like trypsin in not being inhibited by SBTI or LBTI. However p-CMB does not affect the enzyme at 100  $\mu$ M whereas trypsin is significantly inhibited at 50  $\mu$ M. The pituitary trypsin is however, inhibited by p-CMB (1 mM). In contrast to trypsin, pituitary trypsin is inhibited 85% by 20 mM  $\text{CaCl}_2$  and is inhibited only 9% by Trasylol at 200  $\mu$ g/ml.

Trypsin from the human tissue sources differs most significantly from trypsin in the effect of NaCl on activity. Whereas there is no significant effect of salts on trypsin at concentrations as high as 1 M, nearly 50% of trypsin activity is lost at a concentration of 0.4 M NaCl. Trypsin from the human source is also different from our enzyme in its significant activity against the synthetic substrate TAME. In comparison with BAEE, the activity toward this substrate is 30% higher for human skin enzyme (Fraki & Hopsu-Havu, 1975). Trypsin activity toward TAME is 50% lower than that toward BAEE. Human trypsin is also inhibited by calcium ions.

Trypsin therefore appears to be different from the

rat mast cell tryptase by several criteria and also has important differences with respect to the human tissue tryptases. Moreover, as discussed above the N-terminal sequence comparisons also show that the two enzymes are different.

The other enzyme purified from skin tissue to apparent homogeneity is carboxamidopeptidase (Simmons & Walter, 1980). Tryptidase is certainly distinct from this enzyme in its lack of chymotrypsin activity, and its insensitivity to chymotrypsin inhibitors.

The substrate profile for tryptidase indicates that the best p-NA substrates are those which were designed for the assay of serine proteases such as thrombin, and plasmin (Fareed et al, 1983). Tryptidase however, differs from thrombin in being inhibited by Trasylol, but not by  $\alpha_1$ -proteinase inhibitor or by the thrombin specific inhibitor, hirudin (Barrett & McDonald, 1980).

Tryptidase is also not inhibited by  $\epsilon$ -aminocaproic acid or SBTI which are inhibitors of plasmin. Moreover, unlike tryptidase which has a single polypeptide chain subunit, plasmin is made up of two chains with the 25,000 dalton light chain having the active site (Barrett & McDonald, 1980). The molecular weight of plasmin is about 85,000 which is much higher than that of tryptidase (subunit of  $M_r = 40,000$ ).

The data from this study on tryptidase, including

inhibitor profile, substrate specificities, chemical and physical characteristics were compared with similar data tabulated in summary form for 59 serine proteases (Barrett and McDonald, 1980). Based on this comparison tryptidase could not be identified with any of them.

Besides, the N-terminal sequence of tryptidase was compared with those of several prominent serine proteases (cf Figure 23). This comparison shows that tryptidase shares several common characteristics of serine proteases with these enzymes as discussed earlier above. However the homology between these enzymes and tryptidase is not significant, and the sequence differences are sufficient to justify the suggestion that tryptidase is not one of them.

A very important significant observation about the known serine proteases is that tryptase and now tryptidase are perhaps the only ones known to have a tetrameric form. It is therefore very likely that tryptidase is a novel trypsin-like skin enzyme, and possibly a novel trypsin-like protease in general.

#### E. Physiological Role of Tryptidase

Tryptidase could be involved in one or more of the physiological functions described for skin proteases (Fraki et al., 1983). If tryptidase is present in mast cells which do abound in skin it could have a role in inflammatory response. Such a role has been postulated for tryptase which is a mast cell enzyme (Kido et al., 1985). Tryptidase

could also be involved in peptide precursor processing, since it does seem to have a preference for a proline residue in the P<sub>2</sub> position next to a basic residue in the P<sub>1</sub> position. "Proline-directed arginyl cleavage" has been recently proposed as a processing mechanism (Schwartz, 1986) for a number of prohormones.

#### F. Future Studies Possible with Pure Tryptidase

As demonstrated above, tryptidase was purified to apparent homogeneity. The availability of the pure enzyme makes it possible to raise antibodies against the enzyme which can then be used to determine the cellular and subcellular localization of the enzyme by immunocytochemical staining. This will be the first step in delineating the physiological role of this enzyme. Tryptidase-specific assays and tryptidase antisera can also be used to monitor enzyme levels in different physiological processes. Furthermore, techniques are being developed to identify the natural substrates of tryptidase. Besides, since the N-terminal sequence of the enzyme is now known it should be possible with techniques common to molecular biology to isolate the precursor of the enzyme and even to identify its gene.

The present study therefore is an important step in laying the groundwork for understanding the role and function of yet another protease that forms a part of the biochemical machinery of mammalian biological systems.

## RESEARCH SUMMARY

This dissertation reports the isolation and characterization of a novel trypsin-like enzyme from rat skin. The enzyme has been provisionally named tryptidase since it is trypsin-like and has been shown to have peptidase activity.

A purification scheme was developed which yields a homogeneous preparation of tryptidase in two main steps. The techniques and procedures were optimized to obtain a high yield of enzyme in minimal time. Minced skin tissue from 12 rats is homogenized at pH 7.0 in buffer containing 0.5 M NaCl. Following  $(\text{NH}_4)_2\text{SO}_4$  fractionation (25-80%) of the extract, the 80% precipitate is dialyzed against pH 6.0 buffer containing 0.5 M NaCl. In the second step, the enzyme is applied to an agarose-glycyl-glycyl-p-aminobenzamide column maintained at room temperature. Tryptidase is eluted from this column with 0.1 M benzamide onto a concanavalin A-agarose (con A) column equilibrated at 4<sup>0</sup>. The enzyme is eluted from the con A column with 100 mg/ml  $\alpha$ -methyl-D-mannoside.

The entire purification scheme can be completed in less than four days with purification factors of over 12,000 and recoveries of approx. 28%. The apparent homogeneity of the enzyme was demonstrated by the presence of only a single band in sodium dodecyl sulfate (SDS) polyacrylamide gel

electrophoresis carried out under either reducing or non-reducing conditions. Enzyme labeled at the active site gave a single labeled band which co-migrated in SDS gels with the single stained band.

The chemical, physical and catalytic properties of tryptidase were studied. High performance gel permeation chromatography gave a  $M_r$  for the enzyme of approx. 160,000. In SDS gels under reducing and non-reducing conditions the enzyme has a molecular weight of approx. 40,000 indicating that the enzyme is a tetramer made up of identical subunits each consisting of a single polypeptide chain. On isoelectric focusing the enzyme displays microheterogeneity with bands occurring in the pI range 4.5 to 4.9 with the band at 4.8 being the most prominent.

Tryptidase is inhibited by the trypsin inhibitors p-nitrophenyl-p'-guanidinobenzoate, p-methylumbelliferyl-p'-guanidinobenzoate, leupeptin, antipain and Trasylol, but unlike trypsin, tryptidase is not inhibited by soybean trypsin inhibitor, lima bean trypsin inhibitor and ovomucoid. Inhibitors like hirudin, pepstatin and bestatin have no effect on the enzyme. The enzyme is inhibited, however, by the serine protease inhibitor diisopropylphosphofluoridate, the chloromethyl ketone derivative of tosyl lysine (Tos-Lys-CH<sub>2</sub>Cl) as well as the sulfhydryl group reagent p-chloromercuribenzoate.

Heavy metal ions like Ca<sup>++</sup>, Co<sup>++</sup>, Mg<sup>++</sup> and Mn<sup>++</sup> have

no activating or inhibiting effect on the enzyme. NaCl is not inhibitory but is necessary for long term stability of the enzyme.

Tryptidase can hydrolyze the trypsin ester substrate benzoyl-arginine ethyl ester and the amide substrate, benzoyl-arginine p-nitroanilide. The enzyme has an extended binding pocket since it can cleave tripeptide substrates more efficiently than single amino acid substrates. Each peptide which has been shown to be cleaved has either an arginine or lysine on the carboxyl side of the scissle bond. Vasopressin can also be cleaved at the Arg<sup>8</sup>-Gly<sup>9</sup>-NH<sub>2</sub> bond with an optimal pH of 7.5.

The N-terminal sequence of the enzyme has been obtained to the first 25 amino acids: Ile<sup>1</sup>-Val<sup>2</sup>-Gly<sup>3</sup>-Gly<sup>4</sup>-Gln<sup>5</sup>-Glu<sup>6</sup>-Ala<sup>7</sup>-Ser<sup>8</sup>-Gly<sup>9</sup>-Asn<sup>10</sup>-Lys<sup>11</sup>-Trp<sup>12</sup>-Pro<sup>13</sup>-Trp<sup>14</sup>-Gln<sup>15</sup>-Val<sup>16</sup>-Ser<sup>17</sup>-Leu<sup>18</sup>-Arg<sup>19</sup>-Val<sup>20</sup>-\_\_<sup>21</sup>-Asp<sup>22</sup>-Thr<sup>23</sup>-Tyr<sup>24</sup>-Trp<sup>25</sup>. This region shows 40% homology with trypsin with the first four amino acids being identical to that of bovine trypsin. Residue 21 is a putative glycosylation site due to the threonine at position 23.

Comparisons of the structural, physical, chemical and catalytic properties of the enzyme with other known serine proteases suggest that tryptidase is a novel trypsin-like enzyme from rat skin.

A role in inflammatory response or prohormone processing has been postulated.

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APPENDIX A

## APPENDIX A

Table AStock Solutions and Reagents  
For SDS Gel ElectrophoresisSolution A

|   |        |
|---|--------|
| Acrylamide                              | 30 g   |
| BIS                                     | 0.8 g  |
| Distilled deionized H <sub>2</sub> O to | 100 ml |

Solution B

|   |        |
|---|--------|
| Tris-OH                                 | 36.6 g |
| Distilled deionized H <sub>2</sub> O to | 100 ml |
| (adjust to pH 8.9 with HCl)             |        |

Solution C

|                                      |        |
|--------------------------------------|--------|
| SDS                                  | 10 g   |
| Distilled deionized H <sub>2</sub> O | 100 ml |

Solution D

|                                      |        |
|--------------------------------------|--------|
| Tris-OH                              | 5.89 g |
| Distilled deionized H <sub>2</sub> O | 100 ml |
| (adjust to pH 6.7 with HCl)          |        |

Table B

## Stock Solutions and Reagents

For SDS Gel Electrophoresis Contd.

|  | <u>Stacking Gel</u><br><u>(12.5%)</u> | <u>Spacer Gel (3%)</u> |
|--|---------------------------------------|------------------------|
| Solution A                                     | 62.55 ml                              | 1.5 ml                 |
| Solution B                                     | 18.75 ml                              | 1.875 ml               |
| Solution C                                     | 1.5 ml                                | 0.15 ml                |
| Dist. deion. H2O                               | 67 ml                                 | 12.225 ml              |
| Ammonium Per-<br>sulfate                       | 150 mg                                | 15 mg                  |
| TEMED, diluted<br>1:10 with dist.<br>deion H2O | 225 ul                                | 75 ul                  |

Table C

## Buffers and Staining Solutions

## For SDS Gel Electrophoresis

Running Buffer (5 X Concentrated)

|                            |        |
|----------------------------|--------|
| Tris-OH                    | 60 g   |
| Glycine                    | 288 g  |
| Solution C                 | 200 ml |
| Distilled deionized H2O to | 4 l    |

Staining Solution

|                            |        |
|----------------------------|--------|
| Coomassie Blue             | 0.5 g  |
| Methanol                   | 250 ml |
| Acetic Acid                | 50 ml  |
| Distilled Deionized H2O to | 500 ml |

Destaining Solution

|                            |         |
|----------------------------|---------|
| Methanol                   | 37.5 ml |
| Acetic Acid                | 37.5 ml |
| Distilled deionized H2O to | 500 ml  |

Table DSolutions for Isoelectric Focusing  
On Polyacrylamide GelsFixing Solution

|                            |         |
|----------------------------|---------|
| Trichloroacetic Acid       | 57.50 g |
| Sulphosalicylic Acid       | 17.25 g |
| Distilled H <sub>2</sub> O | 500 ml  |

Staining Solution

|                            |        |
|----------------------------|--------|
| Coomassie Blue R 250       | 0.46 g |
| Ethanol                    | 100 ml |
| Acetic Acid                | 40 ml  |
| Distilled H <sub>2</sub> O | 260 ml |

Destaining Solution

|                               |        |
|-------------------------------|--------|
| Ethanol                       | 500 ml |
| Acetic Acid                   | 160 ml |
| Distilled H <sub>2</sub> O to | 2 l    |

Preserving Solution

|                            |        |
|----------------------------|--------|
| Glycerol                   | 40 ml  |
| Ethanol                    | 100 ml |
| Acetic Acid                | 40 ml  |
| Distilled H <sub>2</sub> O | 260 ml |

APPROVAL SHEET

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