STUDIES ON THE PROPERTIES AND STRUCTURE OF THE β-LYTIC PROTEASE OF *MYXOBACTER 495*

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ABSTRACT

β-Lytic protease is one of several proteolytic enzymes produced by the soil bacterium, Myxobacter 495, an organism which first attracted attention by its ability to lyse various species of soil nematodes and soil bacteria. Previous investigations had established that the enzyme was a metallo-proteinase, with one gram-atom of zinc per mole of protein, and that it was responsible for much of the lytic activity of the organism.

The specificity of β-lytic protease was investigated using glucagon, a peptide of known sequence, and the five peptides, A, B, C, D, and E, produced by cleavage of β-lytic protease with cyanogen bromide. The analyses and sequence determinations of the hydrolysis products isolated from β-digests of these peptides indicated that, with one exception, the β-enzyme cleaved peptide linkages with glycyl residues as donors of the carbonyl group of the linkage and/or with hydrophobic amino acids as donors of the imino group of the linkage. The exception was cleavage of a threonyl-lysyl linkage in Peptide D. Cleavage of linkages in front of hydrophobic residues is a common attribute of neutral proteases such as thermolysin and B. subtilis neutral protease. These enzymes have little or no affinity for glycyl residues and, in this respect, the β-enzyme appears to be unique.

As well as providing an extensive set of data on the specificity of the β-enzyme, these studies made the

following contributions to a determination of the amino acid sequence of β-lytic protease. Previous investigations had assigned a sequence to residues 1-8 and 12-24 of Peptide A (32 residues), had assigned a sequence to residues 1-8 of Peptide B (12 residues), and had determined a complete sequence for Peptide C (5 residues). The data from the studies with β-lytic protease confirmed the assigned sequences for Peptides A and B and completed the sequence determination for both peptides. Peptide C was not cleaved by the β-enzyme. The isolation, analysis and sequencing of the products from digestion of Peptides D and E with the β-enzyme were carried out concurrently with similar studies by Damoglou and Whitaker using a variety of other enzymes - trypsin, chymotrypsin, pepsin, α-lytic protease, and a protease from Staphylococcus aureus. The β-enzyme provided an extensive set of cleavage products from Peptide D (82 residues) and this data, in conjunction with that from the other enzyme digests, determined the sequence of Peptide D with the exceptions of the assignment for five residues in one tryptic peptide and the identification of one residue which is either glutamine or glutamic acid. Peptide E (46 residues) is an extremely hydrophobic peptide and the β-enzyme was the only enzyme which, by itself, gave a nearly complete set of peptides separable by high voltage electrophoresis. This data, in conjunction with that from the other enzyme digests, determined the complete sequence of Peptide E. In addition, a digest of the aminoethylated β-

enzyme with trypsin and β-lytic protease provided two of the peptides required to establish the order of these peptides in the native enzyme; the order is A.C.E.B.D. In short, it was demonstrated that β-lytic protease is a valuable enzyme for the determination of amino acid sequences.

As part of the evidence classifying the β-enzyme as a metallo-proteinase, it had been shown by other investigators that treatment of the β-enzyme with phenanthroline to remove zinc led to a loss of bacteriolytic activity. This evidence was made less equivocal by establishing that activity could be restored by treatment of apo-enzyme with solutions containing zinc ions.

The course of attack by β-lytic protease on the parasitic nematode, Ascaris suum, was investigated by electron microscopy of cuticle from ascarids which had been incubated with the β-enzyme for periods of up to 24 hours. Scanning electron microscopy revealed the presence of localized pits or tears in the cuticle. These lesions were absent from the controls, absent from ascarids which had been incubated with enzyme for periods of 8 hours or less, and evident only when the cuticle was prepared for scanning electron microscopy by freeze-drying after rapid freezing; they were not detectable when the cuticle was dehydrated with ethanol. They are interpreted as indicative of regions of cuticle which had been so weakened by enzyme that they were unable to withstand the stresses from rapid freezing.

One of the major components of the cuticle is an unusual form of collagen. Viscometric assays of the activity of the β-enzyme toward this collagen indicated that the β-enzyme had little collagenase activity.

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GENERAL INTRODUCTION

(a) Isolation of Myxobacter 495 and Initial Observations on its Lytic Activity. (Institute of Microbiology, Canada Department of Agriculture, Ottawa)

In 1964, Katznelson, Gillespie and Cook (1) reported the isolation from Ottawa soil of bacteria with lytic activity towards several species of soil nematodes. The lytic activity was not dependent on the presence of living bacteria, but was equally evident in cell-free culture solutions, suggesting that the lytic principle was an extracellular enzyme (or a group of enzymes) secreted into the culture medium. Nematode larvae were particularly susceptible to attack, and, in some instances, were completely digested. The lytic bacteria were all members of the order Myxobacterales. The isolate designated Myxobacter 495 had particularly high lytic activity and was maintained in the culture collection of the Institute.

Gillespie and Cook (2) extended these observations in a report published the following year. They demonstrated that the culture solutions of Myxobacter 495 had substantial proteolytic activity toward casein and hemoglobin, were capable of completely lysing cells of various Staphylococci and Bacilli, and of the soil bacterium, Arthrobacter globiformis, and were capable of partially lysing cells of other bacteria such as the soil bacterium, Micrococcus lysodeikticus. They attempted to fractionate the enzymes of the culture solution

by passing the filtrate through a column of hydroxylapatite and then displacing the adsorbed enzymes with alkaline Tris and phosphate buffers of increasing ionic strength. The fractionation was not clear-cut but among the fractions obtained, one showed strong proteolytic activity but no bacteriolytic activity, and another showed substantial bacteriolytic activity but no proteolytic activity. Gillespie and Cook concluded that at least two extracellular enzymes were produced by the organism.

These authors described Myxobacter 495 as belonging to the genus, Sorangium. Subsequent investigations in Dr. F. D. Cook's laboratory at the University of Alberta have shown this identification to be untenable (3). Christensen and Cook have concluded that a new genus of Myxobacteria must be established for Myxobacter 495 and two related species. They propose to name this new genus Lysobacter and to give Myxobacter 495 the name, Lysobacter enzymogenes (4).

(b) Isolation and Comparison of α-Lytic Protease and β-Lytic Protease (National Research Council of Canada, Ottawa)

The lytic enzymes of Myxobacter 495 were subsequently studied in detail by Whitaker and co-workers. The first report, published in 1965 by Whitaker, Cook and Gillespie (5), described the course of enzyme production when the organism was grown at 25° in shake cultures and in a 120-liter fermentor. The medium was a solution of mineral salts, glucose and amino

acids from an acid hydrolysate of casein. The pH was initially 7.0, but increased to values as high as pH 8.5 during growth of the organism. The course of glucose uptake, amino acid uptake, bacterial growth, production of lytic activity toward Arthrobacter globiformis cells and production of proteolytic activity toward casein were measured. Peak enzyme production occurred in late log phase, and maximum yields were obtained after about 48 hours of growth.

The isolation of enzymes with lytic activity was described in the next report by Whitaker (6). The major steps were as follows. (1) The medium was freed of cells with a continuous-flow centrifuge and freed of di- and tri-valent ions by passage through a mixed bed of Amberlite IR 45 in the acetate form and Amberlite IR 120 in the ammonium form. (2) The solution was then stirred with the cation exchanger, Amberlite CG 50 at pH 5.0. This operation removed from the solution all lytic activity toward A. globiformis cells, and most of the proteolytic activity toward casein. (3) The resin was titrated to pH 6.25, washed with dilute citrate buffer of pH 6.25, and packed in a column. The bacteriolytic enzymes were displaced with a gradient of sodium citrate buffers of pH 6.25 and increasing ionic strength. Lytic activity appeared in two peaks. The first peak contained enzyme which migrated more slowly than lysozyme on electrophoresis at pH 8 in cellulose acetate; this enzyme was called the β-enzyme. The second peak contained an enzyme which migrated ahead of

lysozyme; this enzyme was called the α-enzyme. (4) The enzymes were precipitated with ammonium sulfate and then freed of ammonium sulfate by dialysis and by elution from a column of Sephadex G-25 equilibrated with sodium acetate buffer. (5) Both enzymes were then refractionated by ion exchange chromatography on Amberlite CG 50, precipitated with ammonium sulfate, and dialyzed. Both enzymes had lytic activity toward A. globiformis cells and proteolytic activity toward casein, and hence they were renamed "α-lytic protease" and "β-lytic protease". Together they accounted for virtually all the lytic activity of the culture solution.

The third report of Jurášek and Whitaker (7) described evidence relating to the homogeneity of these proteases. The main points which emerged were as follows. (1) Both enzymes were homogeneous in the ultracentrifuge. The molecular weights, estimated by the Archibald method with an assumed value of 0.72 for the partial specific volume, were 19,000-19,300 for the α-enzyme, and 19,000 for the β-enzyme. The sedimentation coefficients, estimated from sedimentation rates in acetate buffer, were 2.2 Svedberg units. (2) Both enzymes were homogeneous on starch gel electrophoresis at pH 8, and, with the exception of an electrophoresis in one buffer, were homogeneous on electrophoresis in cellulose acetate with buffers ranging from pH 3 to 9. The exception was electrophoresis in acetate-7M urea buffer of pH 5. In this buffer, the α-enzyme was homogeneous, but the β-enzyme showed two zones. (The explanation

is discussed later). (3) Both enzymes were homogeneous on chromatography on columns of Sephadex G-75 and homogeneous on chromatography on columns of hydroxylapatite. In all these separations, proteolytic activity and bacteriolytic activity were inseparable. It will be recalled that Gillespie and Cook (2) used hydroxylapatite in their fractionation procedure and obtained a fraction with bacteriolytic activity but no proteolytic activity. However, their procedures gave enzymes in extremely dilute solutions. Such solutions were adequate for assays of bacteriolytic activity, but were much too dilute for satisfactory assays of proteolytic activity toward casein, as this assay is at least one order of magnitude less sensitive than the bacteriolytic assay.

The fourth report, by Whitaker, Roy, Tsai and Jurášek (8), compared the proteolytic activity of these enzymes by comparing their cleavage patterns of the A and B chains of performate-oxidized insulin. The cleavage pattern of the α-enzyme was very much like the pattern reported by Naughton and Sanger (9) for porcine elastase. The α-lytic protease cleaved the A chain rapidly at three sites - the Ser_9-Val_{10} linkage, the Val_{10}-$Cys(SO_3H)_{11}$ linkage, and the Ser_{12}-Leu_{13} linkage - and also cleaved the B chain rapidly at three sites - the Val_{12}-Glu_{13} linkage, the Ala_{14}-Leu_{15} linkage, and the Val_{18}-$Cys(SO_3H)_{19}$ linkage. The properties of the β-enzyme were quite different. Under the conditions tested, the enzyme had no appreciable activity toward the A chain, and cleaved the

B chain rapidly at only one site, the Gly_{23}-Phe_{24} linkage.

In the fifth and last paper of this series, cleavage of the cell-wall mucopeptides of Arthrobacter globiformis and Micrococcus lysodeikticus by the α- and β-enzymes were reported by Tsai, Whitaker, Jurášek and Gillespie (10). The β-enzyme was shown to cleave rapidly the linkage between N-acetylmuramic acid and the N-terminal alanyl residue of the peptide chain of the M. lysodeikticus mucopeptide; the α-enzyme cleaved this linkage slowly. Both enzymes hydrolyzed cross-linkages involving the ε-amino group of lysine residues in the peptide chain.

In 1967, Jurášek and Whitaker reported the amino acid and metal composition of these proteases (11). The amino acid compositions of the two enzymes were completely different and assigned a minimum molecular weight of 19,000 to the α-enzyme and 19,800 to the β-enzyme. The α-enzyme contained no divalent metal ions, but the β-enzyme contained one gram-atom of zinc per mole of protein.

The evidence that the β-enzyme contained zinc suggested an explanation for the heterogeneity observed on electrophoresis in acetate-7M urea buffer of pH 5.0. This was reported in 1967 by Whitaker (12). The apparent electrophoretic heterogeneity depended on the period of exposure to the buffer. If the period of exposure was brief, the electropherogram showed a single component of high mobility; if the period of exposure was prolonged, the electropherogram showed

a single component of low mobility; if the period of exposure was of intermediate duration, the electropherogram showed both components. The mobility of the slower component was the same as that of apo-enzyme produced by treatment of the native enzyme with o-phenanthroline. Dialysis of the apo-enzyme against buffer containing Zn^{++} ions led to the reformation of the component of high mobility. In short, the heterogeneity in 7M urea buffer of pH 5.0 was due to a slow conversion of native enzyme to apo-enzyme in this buffer.

(c) Subsequent Characterization of α-Lytic Protease

Subsequent studies with the α-enzyme were briefly as follows. (i) The enzyme was shown to be a serine protease with the same sequence, Asp.Ser.Gly, around its active serine residue as the pancreatic serine proteases (13). (ii) Its kinetic properties were investigated with esters of N-acyl-substituted derivatives of alanine and valine as substrates and were found to be essentially the same as those of purified porcine elastase (14). As α-lytic protease has only one histidine residue (10), this finding threw considerable doubt on the twin-histidine reaction mechanism proposed for serine proteinases by Bender and Kézdy (15). (iii) The sequence about the single histidine was shown to be homologous with that around Histidine-57 of chymotrypsin, trypsin and elastase (16). These findings prompted a determination of the complete amino acid sequence of the α-enzyme by Olson et al. (17).

Dayhoff (18) assessed the homology with the pancreatic enzymes by a computer program which considered all the residues in the sequence and not merely those in regions around the active site. She concluded that the homology was sufficient to indicate that α-lytic protease and the pancreatic enzymes had evolved from a common ancestral enzyme. McLachlan and Shotton (19) also demonstrated by model-building that the sequence of the α-enzyme was compatible with the enzyme having a three-dimensional structure similar to that of chymotrypsin. Work in progress on the X-ray structure and the NMR spectra of the α-enzyme may shortly provide more direct evidence on the homology in three-dimensional structure.

(d) Subsequent Investigation of β-Lytic Protease

Whitaker, Jurášek and Roy briefly discussed the nature of the β-lytic protease in their publication on the nature of α-lytic protease (13). They were unable to assign it to any of the four major groups of proteinases. They pointed out that, unlike the α-enzyme, the β-enzyme was completely unaffected by inhibitors such as diisopropyl phosphorofluoridate (DFP) or isopropyl methylphosphonofluoridate (Sarin), and hence could not be classified as a serine proteinase. Further, as the enzyme has no free sulfhydryl groups and is active at neutral or weakly alkaline pH's, it could not be classified as a "thiol proteinase" (such as papain) or an "acid proteinase" (such as pepsin). The remaining major group

of proteinases is the "metallo-proteinases", i.e., enzymes whose catalytic mechanism is dependent on the presence of a chelated metal ion, usually zinc. Whitaker and Roy (20) converted native β-enzyme with 1.0 g-atom of Zn^{++} per mole, to an essentially zinc-free apo-enzyme (0.04 g-atom of Zn^{++} per mole) by treatment of the enzyme with phenanthroline in acetate buffer followed by ultrafiltration to remove the Zn^{++}-phenanthroline complex. Assays of the bacteriolytic activity of the apo-enzyme showed no loss of activity from the conversion. According to this evidence, the Zn^{++} ion is not essential for activity and the β-enzyme could not be classified as a metallo-proteinase.

Oza and Whitaker (21) re-examined the conclusion in the course of a subsequent survey of potential inhibitors and substrates of the β-enzyme. They found that metal-chelating agents such as phenanthroline and ethylenediaminetetraacetic acid inhibited the bacteriolytic activity and the peptidase activity of the β-enzyme. Furthermore, they were unable to confirm Whitaker, Jurášek and Roy's findings of retention of bacteriolytic activity by the apo-enzyme: in their experience, the conversion of native enzyme to apo-enzyme was invariably accompanied by a loss of activity, and the residual activity was consistent with the amount of residual native enzyme present. They concluded that the β-enzyme was a metallo-proteinase, and attributed the earlier result to a contamination by trace amounts of divalent ions which led to the reformation of metallo-enzyme during storage or dilution of the enzyme.

The most useful substrate detected by Oza and Whitaker's survey was 3(2-furylacryloyl)-glycyl-L-leucine amide (FAGLA). The enzyme cleaved this substrate at the linkage between the glycine and L-leucine amide residues. The pH optimum for this hydrolysis was pH 6.5; the K_m and V_{max} at pH 7.22 were 5.22 mM and 3.67×10^{-6} Ml^{-1}, sec^{-1}, respectively. N-Carbobenzoxylglycyl-L-phenylalanine amide was also a substrate, but it was hydrolyzed too slowly to be of practical value in an assay procedure. None of the esters tested - p-nitrophenyl acetate, and methyl and ethyl esters of various N-acetyl- and N-benzoyl-amino acids - provided any evidence of the β-enzyme having esterase activity.

Matsubara and Feder have designated as "metal chelator-sensitive neutral proteinases", some microbial proteases from various bacteria, e.g., from species of Bacillus and Pseudomonas; from species of Streptomyces, and from the mould, Aspergillus oryzae (22). Characteristic properties of these enzymes are a content of 1 gram-atom of divalent metal ion (usually zinc) per mole of enzyme, inhibition by chelating agents, a pH optimum near neutrality, activity toward FAGLA, and lack of esterase activity. The most thoroughly characterized enzyme in this group is thermolysin, a proteinase from Bacillus thermoproteolyticus. Its amino acid sequence and three-dimensional structure were reported in 1972 (23,24,25). The properties of β-lytic protease described above indicate that it is also a member of this group of enzymes.

(e) Objectives

As evident from the above review, knowledge of the specificity of the β-enzyme was extremely limited. The only proven facts were that the enzyme cleaved the glycyl-phenylalanyl linkage of the insulin B chain, the glycyl-leucyl linkage of FAGLA, the glycyl-phenylalanyl linkage of N-carbobenzoxyglycyl-L-phenylalanine amide, and the linkage between N-acetylmuramic acid and alanine in the cell-wall mucopeptide of Micrococcus lysodeikticus.

The first major objective of this investigation was to extend knowledge of the specificity of the β-enzyme. To this end, the manner in which the enzyme cleaved the polypeptide glucagon was determined. A second major objective was to contribute to a determination of the enzyme's amino acid sequence. The first phase of the program for sequencing the enzyme - the separation of the peptides produced by cleavage of the enzyme with cyanogen bromide - was still under investigation when the studies presented in this thesis were initiated. It was anticipated that cleavages by several enzymes would be necessary to determine the complete amino acid sequence of these peptides. The problem undertaken by the author was to use the β-enzyme as a cleavage agent, and, so far as possible, to characterize the cleavage products. It was felt that this undertaking would not only contribute to the sequence determination but would also provide a more definitive survey of the enzyme's specificity.

Two minor projects were also undertaken - an investigation of the action of the β-enzyme on the cuticle of Ascaris suum, and a further examination of the validity of classifying the β-enzyme as a metallo-proteinase. It will be recalled that study of the lytic enzymes was initiated by the observation of the lytic activity of Myxobacter 495 toward soil nematodes. Whitaker had extended these observations by comparing the action of the α- and β-enzymes on Ascaris suum (26). He noted that, while incubation with the α-enzyme had no apparent effect on the parasite, incubation with β-lytic protease led to a swelling of the cuticle of the parasite and ultimately to the death of the organism. The nature of the initial attack on the Ascaris cuticle was investigated using techniques of electron microscopy.

PART A

THE β-ENZYME AS A METALLO-PROTEINASE

(a) Introduction

Oza and Whitaker's classification of β-lytic protease as a metallo-proteinase was based on the following evidence (21): (1) the presence of 1.0 gram-atom of zinc per mole of native enzyme, (2) inhibition of activity by chelating agents such as o-phenanthroline and ethylenediaminetetraacetate, and (3) loss of activity on conversion of native enzyme to apo-enzyme with o-phenanthroline.

The third point is equivocal in that the loss of activity could be a secondary effect of the removal of zinc- for example, a consequence of an irreversible change in conformation following the loss of zinc, or a consequence of enhanced susceptibility to proteolysis by residual native enzyme. Whitaker had shown that the electrophoretic mobility of the apo-enzyme in cellulose acetate reverted to that of the native enzyme when the apo-enzyme was exposed to zinc ions (12), but mobilities in cellulose acetate are not sensitive indicators of the conformation of a protein. It was desirable therefore to determine whether the loss in activity from the treatment with phenanthroline was restored by exposure to zinc ions.

(b) Materials

Phenanthroline was a Baker Analyzed Reagent. Diisopropylphosphorofluoridate (DFP) was purchased from the Aldrich Chemical Company. Arthrobacter globiformis cells had been prepared according to the method of Gillespie and Cook (2) and were stored at -30°. The β-enzyme used in this experiment and in all other investigations was from a batch of enzyme which had been prepared in 1967 and stored at -30°.

(c) Preparation of Apo-β-Lytic Protease and Regenerated β-Lytic Protease

The enzyme used in this preparation was first treated with DFP, to inactivate any trace of serine protease in the enzyme preparation. A solution of enzyme (60 mg in 6 ml of water) was titrated to pH 7.5 with NaOH, mixed with 60 µl of a 5% solution of DFP in isopropanol, and left at 25° for 45 minutes. One ml of the solution was designated "Native β-lytic protease" and set aside for reference. The remainder was mixed with 150 ml of 0.01M NaOH-acetate buffer (pH 5.5), containing 10^{-3}M phenanthroline, in a 400-ml Amicon ultrafilter fitted with an Amicon UM-2 membrane. The solution was concentrated to a volume of approximately 10 ml under a nitrogen pressure of about 25 psi. The treatment with phenanthroline and the concentration step were repeated. The residual concentrate was diluted with 150 ml of acetate buffer (without phenanthroline) and again concentrated to a volume of about 10 ml. This dilution and concentration procedure was repeated twice

more with 100-ml volumes of water as the diluent. The final concentrate was 15 ml of solution with an enzyme concentration estimated from the absorbance at 280 nm to be 2.8 mg/ml. This solution was designated "Apo-β-lytic protease".

Five ml of this solution was mixed in the ultrafilter with 100 ml of 0.01M NaOH-acetate buffer (pH 5.9), containing 2×10^{-4}M $ZnCl_2$. The solution was concentrated to a volume of approximately 10 ml, diluted with 100 ml of buffer (without zinc), reconcentrated, diluted with 100 ml of water and reconcentrated to give 6.8 ml of solution with an estimated enzyme concentration of 1.6 mg/ml. This solution was designated "Regenerated β-lytic protease".

(d) Assay of Bacteriolytic Activity

The bacteriolytic activity of the enzyme preparations were assayed by the procedure described by Whitaker, Cook and Gillespie (5). The substrate was a dispersion of 30 mg of A. globiformis cells in 100 ml of 0.025M Tris-0.004M KCl-HCl buffer of pH 8.0. At zero time, 200 µl of enzyme solution containing 2 to 5 µg of protein, was mixed with 5 ml of substrate solution maintained at 25.0° in a thermostat. The absorbance at 660 nm was measured at 6 minute intervals for 24 minutes.

(e) Results and Discussion

Table 1 summarizes the data from these assays. It is evident from this data that the loss of activity following

treatment of native β-lytic protease with phenanthroline is restored by treatment of the apo-enzyme with zinc chloride. Point (3) of Oza and Whitaker's evidence is therefore valid evidence for support of their conclusion that β-lytic protease is a metallo-enzyme.

TABLE 1

LYTIC ASSAY WITH NATIVE β-LYTIC PROTEASE, APO-β-LYTIC PROTEASE AND REGENERATED β-LYTIC PROTEASE

Enzyme Preparation	Final Enzyme Concentration (μg/ml)	$-\Delta A_{660}$*
Native β-Lytic Protease	0.4	0.057
	1.0	0.179
Apo-β-Lytic Protease	0.4	0.003
	1.0	0.004
Regenerated β-Lytic Protease	0.4	0.059
	1.0	0.218

*Decrease in absorbance at 660 nm over the interval t = 6 min. to t = 24 min; average of duplicate assays.

PART B

INITIAL INVESTIGATION OF THE SPECIFICITY OF β-LYTIC PROTEASE

I. CLEAVAGE OF GLUCAGON

(a) Introduction

Glucagon and the A and B chains of insulin are favored substrates for assessing the specificity of proteinases. As discussed earlier, the A and B chains of insulin provided little information on the specificity of the β-enzyme. Investigation of the cleavage of glucagon was undertaken to extend this information.

(b) Materials

Glucagon was purchased from Calbiochem. Thioglycolic acid was obtained from the Pierce Chemical Company. All other chemicals were of reagent grade. Carboxypeptidase A (DFP-treated) was from Sigma.

(c) Methods

(i) High Voltage Electrophoresis

Electrophoreses were carried out in Lucite tanks with varsol as the coolant for electrophoresis at pH 2.1 or 3.5, or toluene as the coolant for electrophoresis at pH 6.5 (27). The paper was Schleicher and Schuell 2043B Chromatography and Electrophoresis Paper or Whatman 3MM Chromatography Paper. The buffers were pyridine-acetic acid water (80:2.4:720)

for electrophoresis at pH 6.5; pyridine-acetic acid-water (4:40:760) for electrophoresis at pH 3.5; and 85% formic acid-acetic acid-water (16:64:720) for electrophoresis at pH 2.1. The voltage gradient was 40-50 volts per cm.

Marker strips from dried electropherograms were dipped in cadmium-ninhydrin (28) to locate the position of peptides. The reagent was prepared by mixing 100 ml of a 1% solution of ninhydrin in acetone with 15 ml of a 0.6% solution of cadmium acetate in 33% aqueous acetic acid. The paper was allowed to air-dry until the colour developed (spots appear as red, orange or yellow) and was stored in a plastic bag in the dark. The Ehrlich reagent (29) was used as a supplementary indicator to locate peptides containing tryptophan. A side-strip was dipped in 0.2% dimethylaminobenzaldehyde in acetone, followed by a dip in 10% hydrochloric acid in acetone. Peptides containing tryptophan gave a strong purple colour.

If a peptide required no further fractionation, it was eluted from the paper and collected in a weighed tube. If it required refractionation, the zone was cut from the paper and stitched on to a fresh sheet of paper for re-electrophoresis or partition chromatography.

(ii) Amino Acid Analysis

Samples of eluate were hydrolyzed routinely for 22 hours at 105° in sealed, evacuated tubes with 6N HCl -

0.05% phenol (30). Some peptides were also hydrolyzed in 6N HCl-5% thioglycolic acid (31) for analysis of tryptophan. Norleucine was used as an internal standard. The amino acid analyzer was a Beckman Model 120C or Model 121 analyzer.

(d) Production, Fractionation, and Analysis of Peptides from Digestion with β-Lytic Protease

Glucagon (7.2 mg) was dispersed in 6 ml of 0.025M ammonium bicarbonate containing 137 μg of β-enzyme. One half was digested for 20 minutes, and the other half, for 4 hours, at 25°. The reaction was stopped by adding two volumes of hot ethanol and heating the solution in a boiling water bath until most of the alcohol had evaporated. The residual solution was freeze-dried.

The digests were fractionated initially by high voltage electrophoresis at pH 2.1 on Whatman 3MM paper. Cadmium-ninhydrin indicated 4 zones for the 20 minute digest: 1-20 (μ_{ser} = 1.38), 2-20 (μ_{ser} = 1.00), 3-20 (μ_{ser} = 0.92) and 4-20 (μ_{ser} = 0.72). These zones were refractionated by electrophoresis at pH 3.5. This refractionation removed minor contaminants from the major peptide of zones 2-20 and 3-20. Peptides 3-20 and 4-20 fluoresced in ultra-violet light and gave a positive Ehrlich test for tryptophan. Each peptide was eluted and analyzed. Cadmium-ninhydrin indicated 6 zones after electrophoresis of the 4-hour digest at pH 2.1: 1-4 (μ_{ser} = 1.36), 2-4 (μ_{ser} = 1.19), 3-4 (μ_{ser} = 0.95), 4-4 (μ_{ser} = 0.84)

5-4 (μ_{ser} = 0.74) and 6-4 (μ_{ser} = 0.64). Refractionation of zone 2-4 at pH 3.5 gave two components, 2A-4 and 2B-4; the peptides in the other zones remained homogeneous on refractionation. Peptides 3-4 and 6-4 fluoresced under ultraviolet light and showed a positive Ehrlich test for tryptophan. The peptides were eluted and analyzed. The presence of tryptophan in Peptides 2-20 and 3-4, and its absence in Peptides 4-20 and 5-4 was subsequently verified by amino acid analysis of peptides hydrolyzed in 6N HCl-5% thioglycolic acid.

The composition, yield and identification of the peptides from the two digests are given in Tables 2 and 3, and the cleavage pattern is shown in Fig. 1. The mobilities recorded for these peptides are consistent with mobilities calculated from Offord's data (32) relating mobilities to molecular weight and net electric charge.

(e) Discussion

For reasons which will become more evident later, cleavages by the β-enzyme will be classified into 4 groups:

(1) Gly-HPB cleavages, i.e., cleavages between a glycyl residue and a residue with a hydrophobic side-chain

(2) X-HPB cleavages, i.e., cleavages between a residue other than glycine and a residue with a hydrophobic side-chain

(3) Gly-Y cleavages, i.e., cleavages between a glycyl residue and a residue with a non-hydrophobic side-chain

TABLE 2

PEPTIDES FROM DIGESTION OF GLUCAGON WITH β-LYTIC PROTEASE FOR 20 MINUTES

Peptide	Yield (%)	Amino Acid Composition	Origin
1-20	25	His(0.8)Ser(0.9)Glx(1.0)Gly(1.0)	His_1 -Gly_4
2-20	9	Lys(1.2)His(0.9)Arg(1.8)Asx(3.1)Thr(2.3)Ser(4.2) Glx(3.0)Gly(1.2)Ala(1.0)Val(0.8)Leu(0.9)Tyr(2.1) Phe(2.0)Trp(0.6)	His_1 -Trp_{25}
3-20	14	Lys(0.8)Arg(1.8)Asx(2.9)Thr(1.5)Ser(2.6)Glx(2.1) Ala(1.0)Val(0.9)Leu(1.1)Tyr(1.5)Phe(1.7) (Trp present)	Thr_5 -Trp_{25}
4-20	34	Asx(1.0)Thr(0.8)Met(0.7)Leu(1.0)	Leu_{26}-Thr_{29}

TABLE 3

PEPTIDES FROM DIGESTION OF GLUCAGON WITH β-LYTIC PROTEASE FOR 4 HOURS

Peptide	Yield (%)	Amino Acid Composition	Origin
1-4	38	His(0.9)Ser(0.9)Glx(1.0)Gly(1.0)	His_1 -Glu_4
2A-4	19	Arg(2.0)Asx(1.9)Ser(0.9)Glx(1.0)Ala(1.0)Leu(1.0)	Leu_{14}-Asp_{21}
2B-4	8	His(1.0)Thr(0.9)Ser(0.9)Glx(1.0)Gly(1.0)	His_1 -Thr_5
3-4	26	Arg(1.9)Asx(2.1)Ser(1.0)Glx(2.0)Ala(1.0)Val(1.0) Leu(1.0)Phe(1.0)Trp(0.7)	Leu_{14}-Trp_{25}
4-4	30	Lys(1.1)Asx(1.0)Thr(1.8)Ser(1.9)Tyr(1.7)Phe(1.0)	Thr_5 -Tyr_{13}
5-4	71	Asx(1.0)Thr(1.0)Met(1.0)Leu(1.0)	Leu_{26}-Thr_{29}
6-4	18	Glx(1.0)Val(0.9)Phe(0.9)(Trp present)	Phe_{22}-Trp_{25}

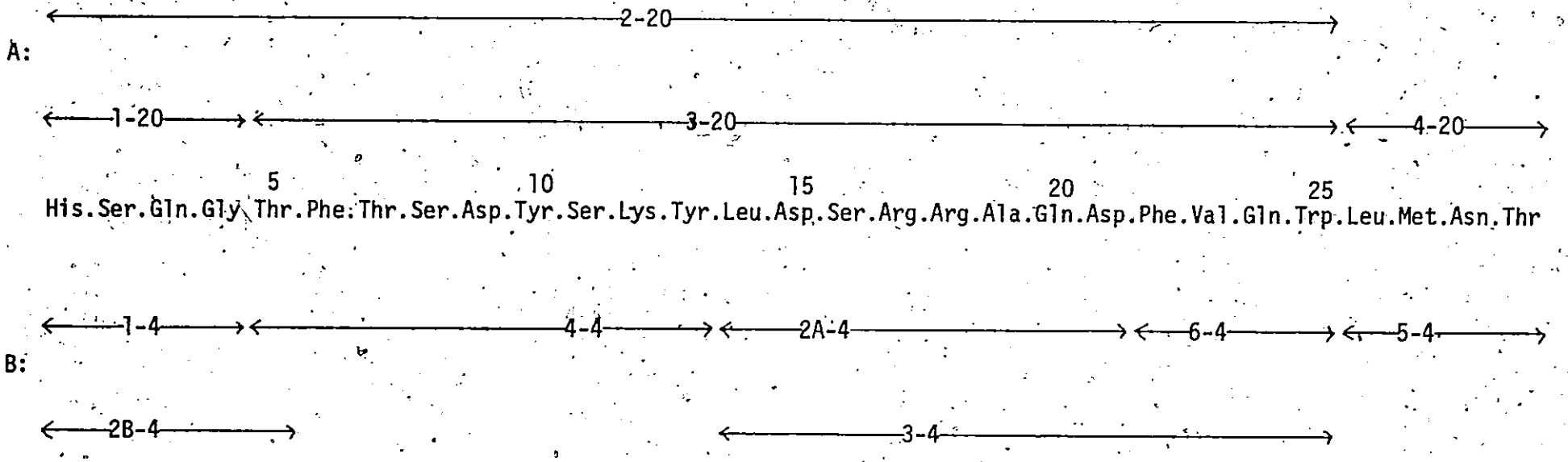


FIGURE 1: CLEAVAGE OF GLUCAGON BY β-LYTIC PROTEASE

A: 20 minute digestion

B: 4 hour digestion

and (4) X-Y cleavages, i.e., cleavages between a residue other than glycine and a residue with a non-hydrophobic side-chain.

The following residues will be classed as hydrophobic: alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan and histidine.

The cleavages demonstrated for glucagon can then be classed as follows:

Fast cleavages	Gly_4-Thr_5	Gly-HPB
	Trp_{25}-Leu_{26}	X-HPB
Slow cleavages	Thr_5-Phe_6	X-HPB
	Tyr_{13}-Leu_{14}	X-HPB
	Asp_{21}-Phe_{22}	X-HPB

It should be noted that glucagon has only one glycyl residue.

II. REACTION OF THE β-ENZYME WITH CBZ-Tyr-Ala AND CBZ-His-Phe-Phe-OEt

(a) Introduction

N-Carbobenzoxyl-tyrosyl-alanine (Cbz-Tyr-Ala) and N-carbobenzoxyl-histidyl-phenylalanyl-phenylalanine ethyl ester (Cbz-His-Phe-Phe-OEt) have proved to be useful substrates in studies with human gastricsin, an acid protease which tends to hydrolyze linkages between hydrophobic amino acids (33). This investigation was undertaken to determine whether they were potentially useful substrates for the β-enzyme.

(b) Materials

N-Carbobenzoxyl-L-tyrosyl-L-alanine and N-carbobenzoxyl-L-histidyl-L-phenylalanyl-L-phenylalanine ethyl ester were gifts rrom Michael Hunkapillar, California Institute of Technology.

(c) Methods

A combination of ninhydrin and a chlorination procedure were used to detect the substrates and any hydrolysis products separated by high voltage electrophoresis. The cadmium-ninhydrin reagent previously described cannot be used prior to chlorination. It was replaced by a ninhydrin reagent consisting of 100 ml of ninhydrin (1% in acetone) and 15 ml of 0.05M sodium acetate. Sheets were dipped in this solution and dried in a fume-hood.

The procedure for chlorination of peptide bonds was that of Rydon and Smith (34). The paper was dipped in 50% ethanol-acetone and was briefly blotted dry. It was placed in a chlorination chamber on glass rod supports over a mixture of 100 ml of $KMnO_4$ (8 g/l) and 100 ml of 1N HCl for 20 minutes, and then was allowed to dechlorinate in the air for 8-10 minutes. The sheet was sprayed with a freshly prepared solution of 1% starch - 1% potassium iodide. Amino acids and peptides give a purple colour with this reagent.

(d) Reaction with β-Lytic Protease and Treatment of the Digest

Cbz-Tyr-Ala (1.1 mg) was dissolved in a solution of

30 µl of pyridine in 100 µl of pyridine-acetate buffer of pH 6.5 (final pH = 6.8); Cbz-His-Phe-Phe-OEt (0.25 mg) was dissolved in a similar solution to which 5 µl of acrylonitrile had been added.

One half of each solution was used as the control, and the other half was digested with β-lytic protease (7.5 µg). After 2 hours at 25°, the controls and digests were fractionated by electrophoresis at pH 2.1 and 6.5. The sheets were treated with ninhydrin and were chlorinated.

(e) Results and Discussion

Ninhydrin-positive spots were not observed with either compound, and chlorination revealed that the controls and digests had identical electrophoretic patterns. Thus these peptides are not substrates of the β-enzyme.

PART C

APPLICATION OF THE β-ENZYME TO THE DETERMINATION OF ITS AMINO ACID SEQUENCE, AND THE RESULTING EXTENSION OF THE SPECIFICITY DATA

I. INTRODUCTION

β-Lytic protease consists of a single chain of amino acids with 4 methionine residues and two disulfide bridges. Cleavage of the methionine residues with cyanogen bromide, followed, if necessary, by reduction and alkylation of the four half-cystine residues should, therefore, yield five peptides. Fig. 2 outlines Damoglou and Whitaker's procedure (35) for the production and isolation of these peptides. Table 4 gives the amino acid composition of β-lytic protease reported by Jurášek and Whitaker in 1967 (11), and the corrections subsequently made by Damoglou and Whitaker (35). Table 5 gives the amino acid composition of Peptides A, B, C, A-C, D and E. The N-terminal sequences of these peptides and of the aminoethylated β-enzyme (35,36), as determined by the Dansyl-Edman procedure, are given in Table 6. The C-terminal residue of the β-enzyme was determined by the method of Matsuo, Fujimoto and Fatsuo (37) to be asparagine. As Peptide A has the same N-terminal sequence as β-lytic protease, and as both Peptide D and the β-enzyme have Asn as their C-terminal residue, the parent peptides are

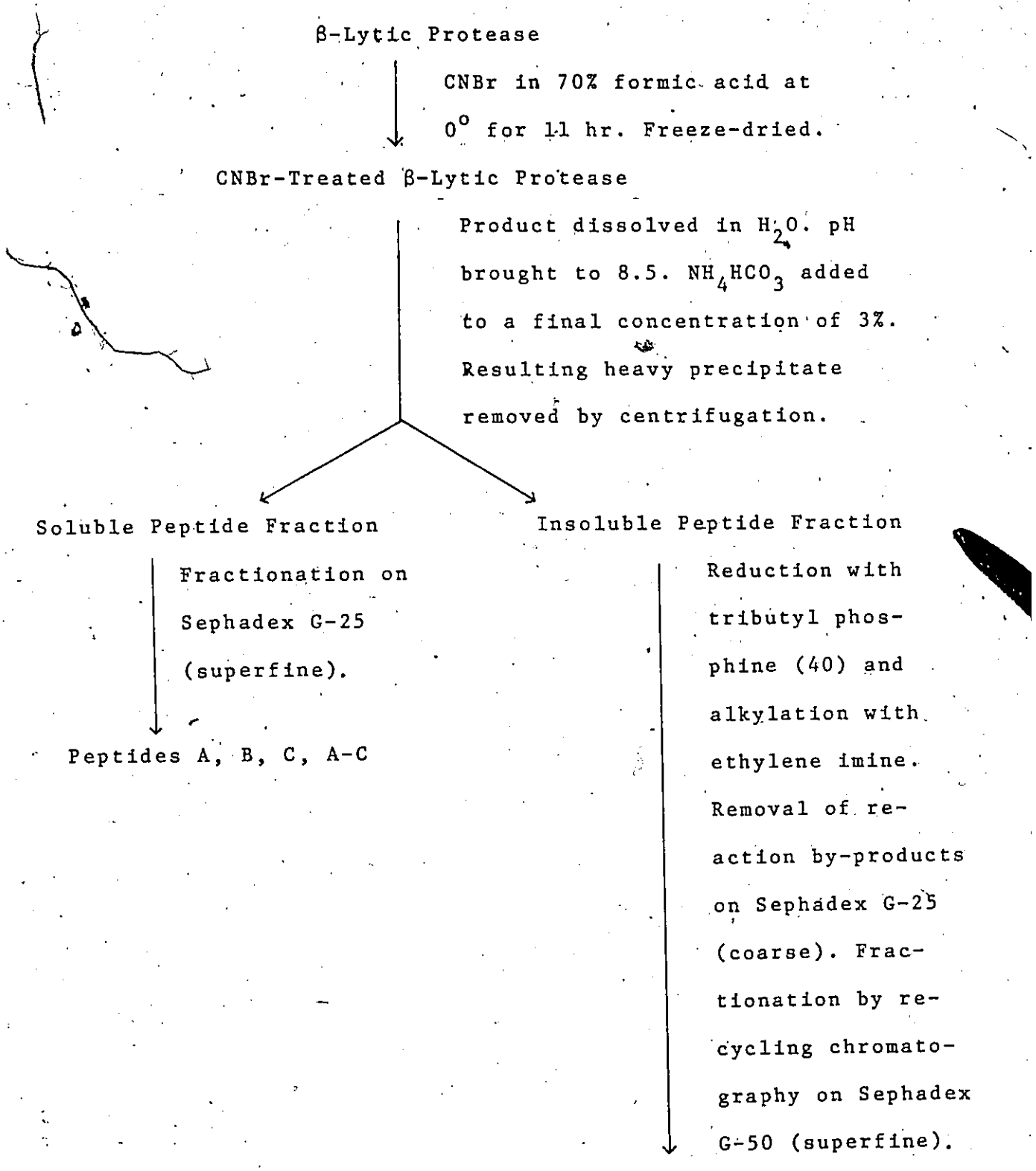


FIGURE 2: PROCEDURE FOR PRODUCTION AND ISOLATION OF CYANOGEN BROMIDE PEPTIDES OF β-LYTIC PROTEASE

TABLE 4

AMINO ACID COMPOSITION OF β-LYTIC PROTEASE

AMINO ACID	NUMBER OF RESIDUES	
	ORIGINAL ANALYSIS (JURÁŠEK & WHITAKER) (11)	CALCULATED FROM SEQUENCE (DAMOGLOU & WHITAKER) (35)
Aspartic acid	21-24	22
Threonine	13-14	14
Serine	19-21	21
Glutamic acid	10	
Proline	8-9	8
Glycine	25-26	25
Alanine	13	
Valine	5	
Methionine	4	
Isoleucine	4	
Leucine	9	
Tyrosine	13	
Phenylalanine	6	
Lysine	3	
Histidine	8	
Arginine	5	
Half-Cystine	4	
Tryptophan	5	4

TABLE 5

AMINO ACID COMPOSITION OF PEPTIDES A, B, C, A-C, D AND E

AMINO ACID	NUMBER OF RESIDUES IN PEPTIDE					
	A	B	C	A-C	D	E
Asx	3	3	1	4	12	3
Thr	2	1		2	8	3
Ser	3	1	2	5	7	8
Glx	1	1		1	6	2
Pro	4			4	4	
Gly	6	1		6	10	8
Ala	2	1		2	6	4
Val	1	1		1	1	2
Ile		1			2	1
Leu	2		1	3	5	1
Tyr	1	1		1	8	2
Phe	2			2	2	2
Lys					2	1
His	2			2	3	3
Arg	1			1	2	2
AE-Cys					3	1
Trp	1				1	2
Hse	1	1	1	1		1
Total Residues	32	12	5	37	82	46

TABLE 6

N-TERMINAL SEQUENCE OF AMINOETHYLATED β-LYTIC PROTEASE AND PEPTIDES A, B, C, A-C, D, AND E

PEPTIDE	N-TERMINAL SEQUENCE
AE-β	Ser.Pro.Asx
A	Ser.Pro.Asx.Gly.Leu.Leu.Glx.Phe
B	Asx.Ile.Glx.Tyr.Asx.Thr.Gly.Ala
C	Ser.Ser.Leu.Asx.Hse
A-C	Ser.Pro
D	Asx.Thr.Ala.Ile.Ala
E	Ser.Arg.Gly.Gly.Gly

in the order A(BCE)D in β-lytic protease. Peptide A-C, a minor product from incomplete cleavage of the linkage between the parent Peptides A and C, indicates that the order is A.C(BE)D. As discussed later, the methionine diagonal technique of Tang and Hartley was used to determine the complete order of these peptides (38). The cystine diagonal technique of Brown and Hartley was used in the determination of sequences around the cystine residues (39).

II. EXPERIMENTAL

(a) Materials

Phenylisothiocyanate, from Fluka AG, was distilled before use. Trifluoroacetic acid was purchased from Matheson, Coleman and Bell. Dimethylaminonaphthalene-5-sulfonyl chloride was obtained from Calbiochem. Pyridine was distilled before use. All other solvents and chemicals were reagent grade.

The polyamide layer sheets for chromatographic identification of dansyl amino acids were a product of the Cheng Chin Trading Company, Ltd., Taiwan.

(b) Methods

(i) Partition Chromatography

The solvent for descending paper chromatography was n-butanol-acetic acid-water-pyridine(30:6:24:20). The sheet was equilibrated with solvent in the chromatography chamber for at least one hour before the inflow of solvent to the paper.

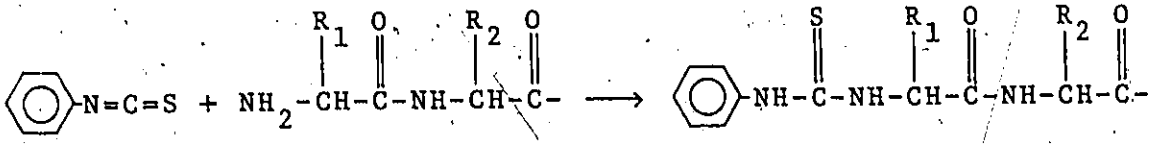
(ii) Amino Acid Analysis

Hydrolysates containing homoserine were evaporated to dryness and were treated with pyridine-acetate buffer of pH 6.5 at 105° for 1 hour, to convert homoserine lactone to free homoserine (41). The solution was evaporated to dryness and the residue was dissolved in water immediately before amino acid analysis.

(iii) Dansyl-Edman Procedure

Peptides were sequenced by the Dansyl-Edman procedure of Gray (42); the particular method used was a modification of that described by Gray and Smith (43). The peptide was added in graded amounts to a series of 6x50 mm tubes - roughly 2 nM of peptide to tube 1 (for determination of the N-terminal residue), roughly 4-8 nM to tubes 2 and 3 (for determination of the 2nd and 3rd residues) and roughly 10-20 nM to tubes 4 and 5 (for determination of the 4th and 5th residues). Samples were dried under vacuum in a heated desiccator with an aluminium base plate with holes into which the tubes could be inserted; the base plate was maintained at a temperature of 45°. The first tube was set aside for determination of the N-terminal residue of the peptide by direct dansylation; the second tube was subjected to one Edman step for determination of the second residue; the third was subjected to two steps, and so on.

Step 1. Reaction with phenylisothiocyanate Twenty-five µl of water and 25 µl of 5% phenylisothiocyanate (PITC) in pyridine were added to each tube; the tubes were flushed with

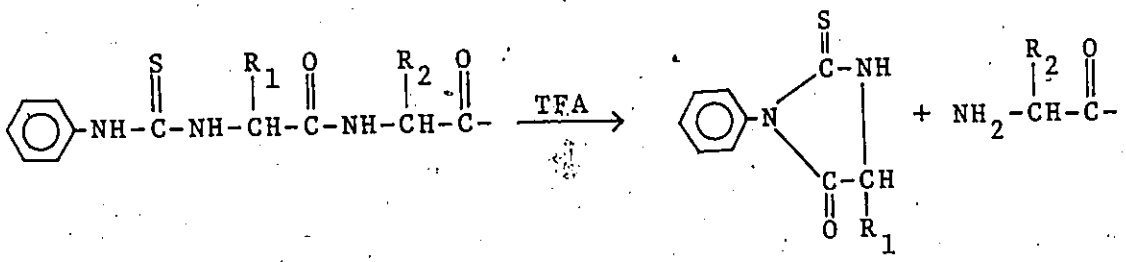


PITC

Phenylthiocarbamyl-peptide (Ptc-peptide)

nitrogen, sealed with parafilm and incubated at 45° for 1.5 hours. The mixtures were centrifuged and dried in vacuo at 45°.

Step 2. Cleavage with trifluoroacetic acid Fifty µl of trifluoroacetic acid was added to each tube. The tubes were flushed with nitrogen, sealed with parafilm, and incubated at 45° for 30 minutes. The bulk of the trifluoroacetic acid



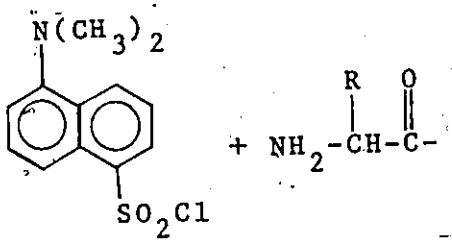
Ptc-peptide

Amino acid phenylthiohydantoin

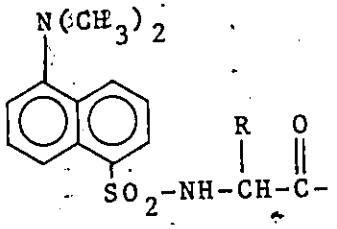
was removed with a fine stream of nitrogen and the final traces were removed over NaOH in a vacuum desiccator at 45°. The second tube was set aside, while the remaining tubes were subjected to the number of Edman steps required, one tube being removed at the end of each step.

Step 3. Extraction with butyl acetate Samples were extracted before the dansyl reaction, and they were also extracted after three to four Edman steps if the samples were not drying readily after treatment with PITC or trifluoroacetic acid. Fifty µl of water and 200 µl of butyl acetate were added to each tube. The tubes were covered with parafilm, and the contents were mixed and centrifuged. The upper layer was removed with a Pasteur pipette. The extraction with butyl acetate was repeated three times, and the samples were then dried under vacuum.

Step 4. Dansylation To each tube was added 10 µl of 0.01M sodium bicarbonate and 10 µl of a 0.5% solution of dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) in acetone. The tubes were covered with parafilm and the contents were mixed, centrifuged, incubated at 45° for 30 minutes,



Dns-Cl



Dns-peptide

recentrifuged and dried. The dansyl peptides were hydrolyzed in 25 µl of 6N HCl in sealed tubes for 16 hours at 105°. The hydrolysates were centrifuged, the tubes were opened, and the

samples were dried. In some instances, tubes were evacuated before they were sealed for the acid hydrolysis.

Step 5. Identification of dansyl amino acids The dansyl amino acids were identified by chromatography on polyamide thin-layer sheets. Five µl of 50% pyridine was mixed with the dried hydrolysate, and the solution was centrifuged to collect it at the bottom of the tube. Two µl of solution was applied on the front and back of a polyamide sheet; one of these applications was on top of a spot containing a standard mixture consisting of the dansyl derivatives of Ile, Pro, Phe, Ala, Thr, and Glu. Sheets were developed with solvent 1 (water-formic acid 100:1.5) in one direction, dried, and developed with solvent 2 (benzene-acetic acid 9:1) at right angles to the first direction. Certain dansyl amino acids could be identified at this stage by examination under ultraviolet light. The sheets were developed with solvent 3 (ethyl acetate-methanol-acetic acid 20:1:1) in the same direction as solvent 2, to separate Dns-Asp from Dns-Glu, Dns-Ser from Dns-Thr, and Dns-NH_2 from Dns-Ala. (Dansyl amine and dansyl hydroxide were always present, as a result of breakdown of dansyl amino acids and dansyl chloride). The patterns of Dns-amino acids on chromatography are shown in Fig. 3. Two other solvents were required to separate the basic Dns-amino acids. The phosphate solvent (0.05M trisodium phosphate-ethanol 3:1) separated Dns-Arg from the pair, Dns-ε-Lys and Dns-His, while the ammonia solvent (1M NH_3-EtOH 1:1) separated

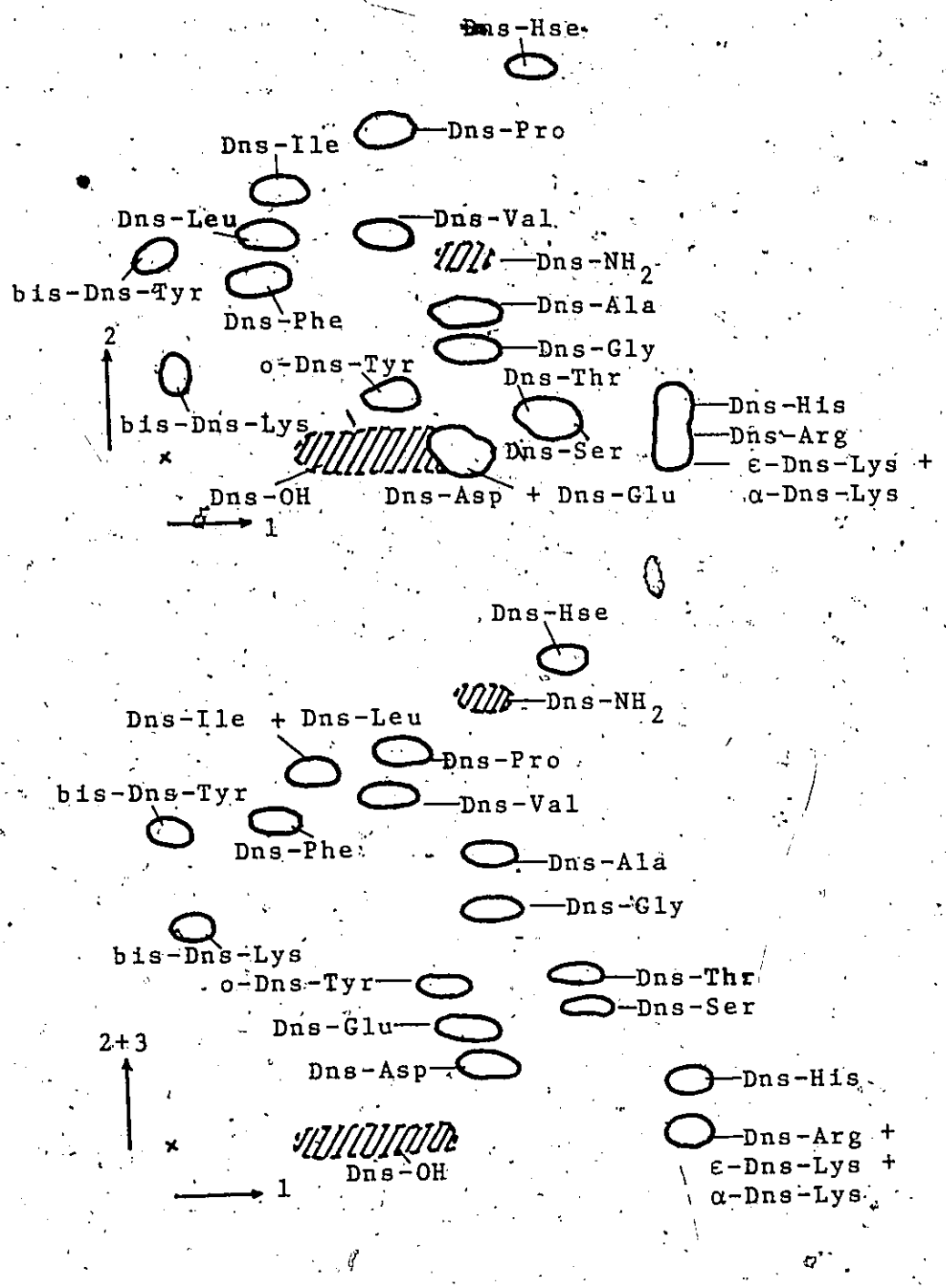


FIGURE 3: CHROMATOGRAPHY OF DNS-AMINO ACIDS ON POLYAMIDE THIN LAYER PLATES.

Solvent 1 (water-formic acid 100:1.5); Solvent 2 (benzene-acetic acid 9:1); Solvent 3 (ethyl acetate-methanol-acetic acid 20:1:1).

Dns-His from the pair, Dns-ε-Lys and Dns-Arg. The position of tryptophan was confirmed by using carboxypeptidase A (44) to determine the C-terminal residues of certain peptides obtained by cleavage with chymotrypsin or β-lytic protease.

If aminoethylcysteine or lysine were not at the N-terminal end of a peptide, the side-chain amino group was converted to the Ptc-derivative by the first Edman step. The α-Dns-ε-Ptc-Lys derivative could be identified, but the α-Dns-ω-Ptc-AEC derivative breaks down on acid hydrolysis.

(iv) Determination of C-Terminal Residues With Carboxypeptidase A (44)

Carboxypeptidase A (25 µl) was suspended in 500 µl of water in a 6x50 mm tube, centrifuged, resuspended in water and recentrifuged. It was dissolved in 100 µl of 2M ammonium bicarbonate buffer and diluted to 500 µl with water, to give a final enzyme concentration of 1 µg/µl. The peptide to be sequenced was dried, dissolved in 250 µl of 0.05M N-ethylmorpholine-acetate buffer of pH 8.5, mixed with 50 µl of carboxypeptidase solution, and incubated at 37° for appropriate time intervals. The solution was then acidified with acetic acid, dried and analyzed.

(v) Calculation of Yield

The amount of each peptide was calculated from the amino acid analysis of the solution eluted from the paper; the ratio of this amount (in nM) to the amount (in nM) of the

substrate used in the digestion is reported as the yield of the peptide. No corrections were made for losses during freeze-drying, or for peptide on the side-strips excised for treatment with ninhydrin or other indicators. Apart from these losses which in principle are determinable, substantial losses are to be expected from the fractionation and elution steps. The reported yields are therefore minimum estimates of the amount of peptide produced during the enzyme hydrolysis.

(vi) Correlation of Electrophoretic Mobility and Net Electric Charge of Peptides

Estimates of the net electric charge on a peptide are based on Offord's graphs (32) relating the electrophoretic mobility of peptides to their molecular weight and net electric charge.

Other methods are the same as those described in Part B.

III. CLEAVAGE OF PEPTIDE A

(a) Production and Fractionation of Peptides

Peptide A (5.4 mg ≈ 1.8 μM) was digested with β-lytic protease (66 μg) in 3.5 ml of 0.05M N-ethylmorpholine-acetic acid buffer of pH 7.5, for 1.5 hours at 37°. The mixture was acidified, freeze-dried, and fractionated by electrophoresis at pH 6.5. Cadmium-ninhydrin detected three basic zones, Aβ-1, Aβ-2 and Aβ-3 (μ_{lys} = 0.47, 0.36 and 0.25 respectively), and one neutral zone, Aβ-4. Zone Aβ-2 was fluorescent. The peptide in zone Aβ-1 was eluted without further fractionation. Peptides in zone Aβ-2 were refractionated by electrophoresis at pH 2.1, to give two peptides, Aβ-2-1 and Aβ-2-2 (μ_{ser} = 1.20 and 1.13 respectively); each peptide was refractionated at pH 3.5 and eluted. The basic peptide in zone Aβ-3 was eluted after refractionation at pH 2.1 (μ_{ser} = 0.90). Refractionation of the neutral peptides of zone Aβ-4 at pH 2.1 yielded 5 zones, Aβ-4-1 to Aβ-4-5, with mobilities (μ_{ser}) of 1.19, 1.11, 0.84, 0.68, and 0.61, respectively. The peptides in these zones were eluted and analyzed. On analysis, the peptide from zone Aβ-4-5 was impure and was refractionated by partition chromatography.

(b) Amino Acid Composition and Sequence Data

Table 7 gives the yields and amino acid compositions of these peptides, their net charge at pH 6.5, their sequences as determined by the Dansyl-Edman procedure, and

TABLE 7

PEPTIDES FROM DIGESTION OF PEPTIDE A WITH β-LYTIC PROTEASE

Peptide	Yield (%)	Composition	Net Charge pH 6.5	Sequence	Assignment of Residues
Aβ-1	33	$Asx_{1.0}Hse_{0.9}Pro_{1.1}Tyr_{1.0}$	+1	Asn, Tyr, Pro, Hse*	29-32
Aβ-2-1	11	$Asx_{1.0}Thr_{1.8}Gly_{2.0}Ala_{1.1}His_{1.1}$	+	Gly, Ala, His, Thr, Asn, Thr, Gly,	20-26
Aβ-2-2	11	$His_{1.7}Ser_{1.0}Gly_{2.0}Ala_{1.5}Val_{1.0}$ $Trp_{1.1}Asx_{0.5}Thr_{0.9}$	+	Ala, Ser, Trp, His, Val, Gly,	14-20
				+ Ala, Ser, Trp, His, Val, Gly, Gly, (Ala His Thr Asn Thr Gly)	14-26
Aβ-3	37	$Arg_{0.9}Glx_{1.0}Pro_{2.1}Gly_{1.0}Leu_{1.8}Phe_{1.8}$	+1	Leu, Leu, Gln, Phe, Pro, Phe, Pro, Arg, Gly,	5-13
Aβ-4-1	8	$Ser_{0.8}Gly_{1.0}$	0	Ser, Gly,	27-28
Aβ-4-2	8	$Ser_{1.0}Ala_{1.0}$	0	Ala, Ser,	14-15
Aβ-4-3	32	$Asx_{1.0}Ser_{0.9}Pro_{1.0}Gly_{1.0}$	0	Ser, Pro, Asn, Gly,	1-4
Aβ-4-4	14	$Asx_{1.2}Pro_{0.9}Tyr_{1.0}Hse_{0.8}$	0	Asn, Tyr, Pro, Hse**	29-32
Aβ-4-5	2	$Asx_{1.0}Ser_{1.1}Pro_{0.5}Gly_{1.4}Tyr_{0.8}Hse_{0.9}$	0	Ser, Gly, Asn, Tyr, (Pro Hse)**	27-32

*C-Terminal residue homoserine lactone

**C-Terminal residue homoserine

their assignment in the entire sequence of Peptide A. Amino acid analysis does not distinguish aspartic acid (Asp) from asparagine (Asn) residues, nor glutamic acid (Glu) from glutamine (Gln) residues, and aspartic acid/asparagine residues and glutamic acid/glutamine residues are indicated as Asx and Glx residues in the amino acid composition. The assignment of Asp, Asn, Glu and Gln residues in the sequence is based on the following considerations. The Asx residue of peptide Aβ-1 cannot be Asp, as the positive charge of this peptide at pH 6.5 is consistent only with the Asx residue being asparagine, and the homoserine residue being homoserine lactone; all other combinations of Asx and Hse give neutral or acidic peptides. The Asx residue of Peptides Aβ-4-4 and Aβ-4-5 is identified as Asn because the Asx residue of these peptides is clearly the same as that in Peptide Aβ-1. Similarly, as Peptides Aβ-2-1 and Aβ-3 are basic peptides with only one basic amino acid residue, their respective Asx and Glx residues must be Asn and Gln residues. Peptide Aβ-4-3 is a neutral peptide with no basic amino acids; its Asx residue is therefore an Asn residue.

Two peptides require comment. Peptide Aβ-4-5 was obtained in very low yield. Its analysis is marginal but the peptide sequenced cleanly. The analysis of Peptide Aβ-2-2 indicated that this peptide is a mixture of at least two peptides. The analysis is consistent with it being a mixture of the peptides Ala.Ser.Trp.His.Val.Gly and Ala.Ser.Trp.His.Val.

Gly.Gly.(Ala,His,Thr,Asn,Thr,Gly). This assignment is consistent with the sequence determination, which proceeded cleanly, and with the failure to separate the components of Aβ-2-2 by electrophoresis, as the two peptides indicated above could be expected to have similar charge/mass ratios.

(c) Application to the Sequence of Peptide A

The following features of the sequence had been determined previously by Whitaker and Roy (36):

1. Peptide A had the amino acid composition ($Asx_3Thr_2Ser_3Glx_1Pro_4Gly_6Ala_2Val_1Leu_2Tyr_1Phe_2His_2Arg_1Trp_1Hse_1$) (Table 5) and its first eight residues were Ser.Pro.Asx.Gly.Leu.Leu.Glx.Phe. (Table 6).

2. Digestion of Peptide A with trypsin gave a peptide, T-1, with the composition (Asx Ser Glx Pro_2 Gly Leu_2 Phe_2 Arg) and Ser.Pro.Asx as its N-terminal residues, and a peptide, T-2.

3. Digestion of the peptide, T-2, with chymotrypsin gave two peptides. Peptide T-2-Ch-1 had the composition (Gly Ala Ser Trp) and N-terminal sequence Gly.Ala.Ser.Trp, and Peptide T-2-Ch-2 had the composition (Asx_2 Thr_2 Ser Pro Gly_4 Ala Val His_2 Hse) and His.Val.Gly as its N-terminal sequence.

4. Digestion of the peptide, T-2, with thermolysin gave a peptide, T-2-Th-1 with composition (Asx_2 Thr_2 Ser Pro Gly_5 Ala Val Tyr His Hse) and Val.Gly.Gly.Ala.His.Thr.Asx as its N-terminal sequence.

As shown in Fig. 4, these data in conjunction with the data from the β-digest of Peptide A assign a unique amino acid sequence to Peptide A. The data obtained by Whitaker and Roy had yielded the sequence of Peptide A except for the order of residues 9-11 (Pro_2 Phe) and the order of residues 25-31. The β-peptides established the sequence of these unknown areas and confirmed the remainder of the structure. Peptide Aβ-3 showed the sequence of residues 9-11 to be Pro. Phe.Pro. Peptide Aβ-4-5, together with peptides Aβ-4-1, Aβ-1 and Aβ-4-4, determined the order of the C-terminal residues, while Aβ-2-1 showed that residues 25-26 were Thr.Gly.

(d) Relevance to the Specificity of β-Lytic Protease

The cleavages by β-lytic protease are shown below, and they are classed as described for glucagon.

Gly_4 -Leu_5	Gly-HPB
Gly_{13}-Ala_{14}	Gly-HPB
Ser_{15}-Trp_{16}	X-HPB
Gly_{19}-Gly_{20}	Gly-Y
Gly_{26}-Ser_{27}	Gly-Y
Gly_{28}-Asn_{29}	Gly-Y

There is evidence for cleavage after every glycyl residue except the second Gly of the sequence Gly.Gly.Ala.

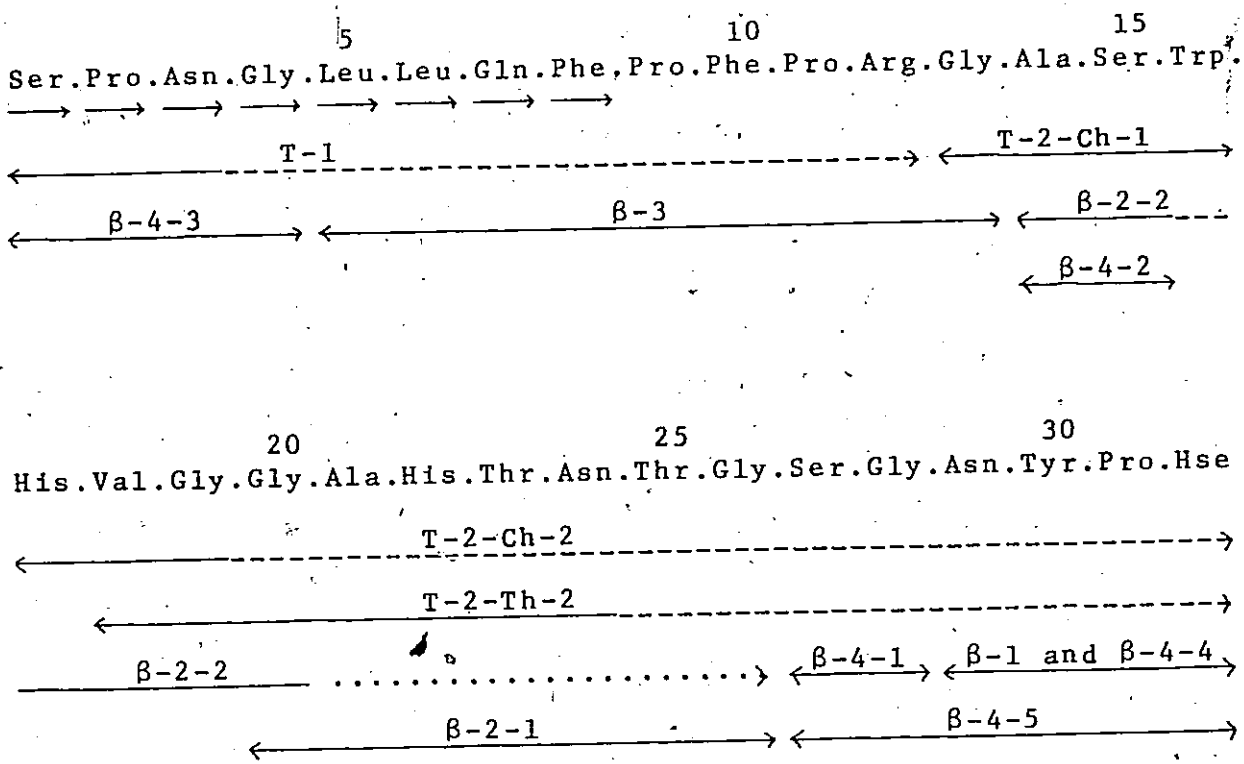


FIGURE 4: AMINO ACID SEQUENCE OF PEPTIDE A

(----- = not sequenced; ..... - see text; peptides labelled "T", "Ch", "Th" and "β" are from digests with trypsin, chymotrypsin, thermolysin and β-lytic protease, respectively).

IV. CLEAVAGE OF PEPTIDE B

(a) Production, Separation, Analysis and Sequencing of Cleavage Products

Peptide B (1.6 mg = 1.2 μM) was digested with β-lytic protease (165 μg) in 4 ml of 0.05M N-ethylmorpholine-acetic acid buffer of pH 7.5, for 19 hours at 37°. The digest was freeze-dried and the peptides were fractionated by electrophoresis at pH 2.1. Cadmium-ninhydrin showed three major peptides, Bβ-1 (μ_{ser} = 1.11), Bβ-2 (μ_{ser} = 0.92) and Bβ-3 (μ_{ser} = 0.61). The first and last of these were eluted, while peptide Bβ-2 was separated from a minor contaminant by electrophoresis at pH 3.5. The amino acid compositions, yield, net charge at pH 6.5, sequences and assignment of these peptides are given in Table 8A.

(b) Relevance of Cleavage Products to the Sequence of Peptide B

As indicated in Tables 5 and 6, the amino acid composition and Dansyl-Edman determination had established the partial sequence

Asx.Ile.Glx.Tyr.Asx.Thr.Gly.Ala(Asx Ser Val)Hse

The data in Table 8A completed the sequence determination (Fig. 4), and established that all the Asx residues are Asn residues, and that the single Glx residue is a Gln residue.

(c) Relevance of Cleavage Products to the Specificity of β-Lytic Protease

The two cleavages detected in Peptide B can be classed as follows:

Gly_7-Ala_8	Gly-HPB
Asn_9-Val_{10}	X-HPB

TABLE 8

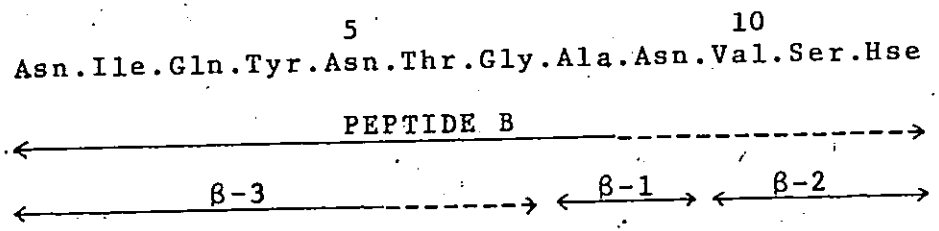
A: PEPTIDES FROM DIGESTION OF PEPTIDE B WITH β-LYTIC PROTEASE

Peptide	Yield (%)	Composition	Net Charge pH 6.5	Sequence	Assignment of Residues
Bβ-1	15	$Asx_{1.0}Ala_{1.0}$	0	Ala→ Asn→	8- 9
Bβ-2	12	$Ser_{1.0}Val_{1.0}Hse_{1.0}$	0	Val→ Ser→ Hse→	10-12
Bβ-3	13	$Asx_{2.1}Thr_{1.1}Glx_{1.0}Ile_{1.0}Tyr_{0.7}$	0	Asn→ Ile→ Gln→ Tyr→ Asn→ (Thr Gly)	1- 7

B: PEPTIDE C AFTER DIGESTION WITH β-LYTIC PROTEASE

Peptide	Yield(%)	Composition	Assignment of Residues
Cβ-1	38	$Asx_{0.7}Ser_{2.0}Leu_{1.0}Hse_{0.9}$	Peptide C

AMINO ACID SEQUENCE OF PEPTIDE B



AMINO ACID SEQUENCE OF PEPTIDE C

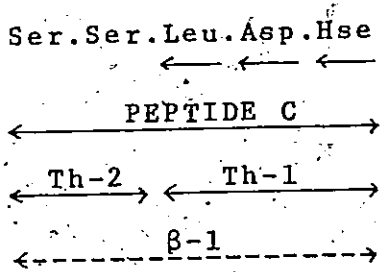


FIGURE 5: AMINO ACID SEQUENCES OF PEPTIDES B AND C

(----- = not sequenced; ⟵ = sequenced using carboxypeptidase A at pH 5; peptides labelled "β" and "Th" are from digests with β-lytic protease and thermolysin, respectively).

V. ATTEMPTED CLEAVAGE OF PEPTIDE C

(a). Digestion and Fractionation Procedure

Peptide C (0.5 mg = 0.9 μM) was treated with hot pydridine-acetate buffer, freeze-dried, and digested with β-lytic protease (5 μg) in 1 ml of 0.05M N-ethylmorpholine-acetic acid buffer of pH 7.5 for 17 hours at 37°. The digest was acidified, dried and fractionated by electrophoresis at pH 2.1. Cadmium-ninhydrin showed only trace amounts of peptides other than a peptide with the same mobility and amino acid composition as Peptide C itself (Table 8B). It was concluded that Peptide C is not readily degraded by the β-enzyme.

(b) Discussion

The data in Table 6 combined with data from digestion of Peptide C with carboxypeptidase (35) assigned the sequence Ser.Ser.Leu.Asp.Hse, to Peptide C (Fig. 5). The Ser.Leu linkage is a potential cleavage point for the β-enzyme (an X-HPB cleavage) and is a cleavage point for thermolysin (36), as seen in Fig. 5. However, the β-enzyme does hydrolyze this linkage in the intact aminoethylated protein, as shown by evidence presented in Part C-VIII. Peptide C has only five residues, and end effects may account for the absence of cleavage in Peptide C itself.

VI. CLEAVAGE OF PEPTIDE D

(a) Production and Fractionation of Peptides

A dispersion of Peptide D (5.6 μg ≈ 700 nmoles) in 2 ml of 0.05N N-ethylmorpholine-acetic acid buffer of pH 7.5 was digested with β-lytic protease (60 μg) for 4 hours at 37°. The mixture was acidified, freeze-dried and fractionated by electrophoresis at pH 6.5. Cadmium-ninhydrin indicated 6 major zones, Dβ-1 to Dβ-6; the mobilities (μ_{lys}) of the first five zones were 0.66, 0.56, 0.47, 0.32 and 0.20, respectively, while the sixth band was neutral. These bands were further fractionated as follows:

(1) Peptide Dβ-1 in zone 1 was eluted after refractionation by electrophoresis at pH 2.1 (μ_{ser} = 1.34).

(2) Zone 2 yielded two bands, Dβ-2-1 and Dβ-2-2 (μ_{ser} = 1.41 and 1.24, respectively), on electrophoreses at pH 2.1. Peptide Dβ-2-1 was refractionated at pH 3.5, while Peptide Dβ-2-2 was eluted without further fractionation.

(3) Zone 3 was fractionated by electrophoresis at pH 2.1 to yield 2 major bands, Dβ-3-1 and Dβ-3-2 (μ_{ser} = 1.26 and 1.20, respectively). Each was refractionated at pH 3.5 to remove minor contaminants.

(4) Peptide Dβ-4 in band 4 was refractionated by electrophoresis at pH 2.1 (μ_{ser} = 1.27) and at pH 3.5 to remove minor contaminants.

(5) Peptide Dβ-5 in zone 5 was eluted after refractionation at pH 2.1 (μ_{ser} = 1.16) and at pH 3.5.

(6) Refractionation at pH 2.1 of the neutral peptides of band 6 yielded three zones, Dβ-6-1 to Dβ-6-3, with mobilities (μ_{ser}) of 1.03, 0.83, and 0.62, respectively. The first zone, Dβ-6-1, yielded two major bands, Dβ-6-1-1 and Dβ-6-1-2 on electrophoresis at pH 3.5; the former was eluted and the latter was refractionated by partition chromatography, to give Peptides Dβ-6-1-2-1 and Dβ-6-1-2-2. Peptides Dβ-6-2 and Dβ-6-3 were each refractionated at pH 3.5 and eluted.

(b) Amino Acid Composition and Sequence Data

Table 9 gives the yields, amino acid compositions, net charge at pH 6.5, sequences and assignment of these peptides. The composition of two peptides requires comment. The analysis of the neutral peptide, Dβ-6-1-1, is more consistent with the composition Ala Thr_2 Gly_2 than with that of the indicated tripeptide; however, the sequence data and electrophoretic mobility at pH 2.1 (Offord's data (32) assigns a molecular weight of 210-295 for a net charge of +1) support the indicated assignment. Although Peptide Dβ-6-2 has an anomalously high alanine and proline content and low threonine content, its first four residues sequenced cleanly. Neither of these peptides was required to establish the sequence, but both are relevant to the specificity of the enzyme.

The Asx and Glx residues in these peptides were assigned as aspartic acid or asparagine, and glutamic acid or glutamine, from Offord's correlations between electrophoretic mobilities, molecular weight and net charge (32). The

TABLE 9

PEPTIDES FROM DIGESTION OF PEPTIDE D WITH β-LYTIC PROTEASE

Peptide	Yield (%)	Composition	Net Charge pH 6.5	Sequence	Assignment of Residues
Dβ-1	4	Lys(1.0)AEC(1.0)Asx(2.0)Glx(1.1) Gly(1.2)Tyr(1.1)	+2	Lys, Asn, Gly, Gln, (Asn Tyr AEC)	66-72
Dβ-2-1	7	AEC(1.0)Asx(1.0)Gly(1.3)Leu(0.8)	+1	Leu AEC Asn, Gly,	15-18
Dβ-2-2	5	Lys(1.0)AEC(0.9)Asx(2.0)Glx(1.3) Gly(2.0)Tyr(1.7)	+2	Lys, Asn, Gly, Gln, Asn, Tyr, AEC Tyr, Gly,	66-74
Dβ-3-1	13	Lys(0.8)Asx(1.2)Glx(1.0)Gly(1.2) Leu(0.7)	+1	Leu Lys Gln, Asn, Gly,	30-34
Dβ-3-2	6	Arg(1.0)Thr(0.8)Gly(1.0)Ile(0.7) Tyr(1.1)	+1	Gly, Tyr, Arg, Ile, Thr,	45-49
Dβ-4	17	Arg(1.2)AEC(1.0)Asx(1.8)Thr(1.0) Ser(2.2)Tyr(1.1)	+1	Ser, Ser, (Tyr Asn Thr Asp AEC Ser Arg)	53-61
Dβ-5	13	His(1.7)Thr(1.0)Ser(1.8)Glx(2.0) Pro(1.3)Gly(1.9)(Trp)	+	Gly, Glx, Ser, Thr, Gly, Pro, His, Glx, His, (Trp Ser)	19-29
Dβ-6-1-1	7	Thr(1.1)Gly(1.0)Ala(0.5)	0	Ala, Thr, Gly,	50-52
Dβ-6-1-2-1	7	Thr(1.0)Leu(1.0)	0	Leu, Thr,	64-65
Dβ-6-1-2-2	5	Ser(1.2)Leu(1.0)	0	Leu, Ser,	38-39

TABLE 9 - continued

Peptide	Yield (%)	Composition	Net Charge pH 6.5	Sequence	Assignment of Residue
Dβ-6-2	7	Asx(1.0)Glx(0.9)Pro(1.4)Ala(2.8) Ile(0.6)Thr(0.2)	0	Ile→ Ala→ Asn→ Ala→ (Pro Asn Thr Gln)	4-(11)
Dβ-6-3	7	Asx(2.3)Pro(1.7)Gly(1.1)Val(0.7) Tyr(1.0)	0	Tyr→ Val→ Asn→ Pro→ Gly→ Pro→ Asn→	76-82

mobilities and molecular weights of Peptides Dβ-1 and Dβ-2-2 are consistent with the peptide having a positive charge of +2 at neutral pH, and as they each contain two basic amino acid residues, their one Glx and two Asx residues are identified as glutamine and asparagine residues. Similarly, the mobilities of Peptides Dβ-2-1 and Dβ-3-1 are consistent with a net charge of +1 at neutral pH, and as each has a single basic amino acid residue, the Asx residue of the former must be asparagine, and the Asx and Glx residues of the latter are asparagine and glutamine. Since Peptide Dβ-4 has a positive charge of +1 at pH 6.5, and contains two basic amino acids, one Asx residue is asparagine and the other is aspartic acid. Their assignments as Asn and Asp were made by Damoglou and Whitaker in the course of other work. Also, the first Glx residue of Peptide Dβ-5 was identified as Gln by Damoglou and Whitaker in the course of other work. The second Glx residue of this peptide is flanked by histidine residues and the net charge of about +1 at pH 6.5 could represent the sum of two partial positive charges from partial ionizations of the two histidine residues in the sequence, His.Gln.His, or it could represent a net positive charge from more complete ionization of the histidines in the sequence, $His^{+}.Glu.His^{+}$. Peptides Dβ-6-2 and Dβ-6-3 are neutral peptides and contain no basic residues; the Glx and Asx residues of Dβ-6-2 and the Asx residue of Dβ-6-3 are therefore identified as glutamine and asparagine.

(c) Application to the Sequence of Peptide D

Peptides from digests of Peptide D with trypsin and α-lytic protease, and from a digest of the native β-enzyme with pepsin, were characterized and sequenced by Damoglou and Whitaker (35) concurrently with the sequencing of the peptides derived from β-digestion of Peptide D. This work is summarized briefly in Fig. 6. Peptides labelled "T" in Fig. 6 are from a tryptic digest. Peptides labelled "Z" are from a prolonged tryptic digest which gave a number of additional cleavages. Peptides labelled "α" are from digestion with α-lytic protease. Peptides labelled "P" were isolated after digestion of the native β-enzyme with pepsin by the cystine diagonal procedure described in Part C-IX.

These data in conjunction with the N-terminal sequence of Peptide D (Table 6) and the data from the β-digest of Peptide D, assigned a complete sequence to Peptide D with the exception of assignments within Peptide T-3 and the assignment of Glx_{26} previously discussed.

A major contribution of the β-peptides was the provision of overlaps between tryptic peptides; these are shown below.

Peptide	Overlap between Tryptic Peptides
Dβ-2-1	T-1 and T-2
Dβ-3-1	T-2 and T-3
Dβ-3-2	T-3 and T-4
Dβ-1 and Dβ-2-2	T-6 and T-7
Dβ-2-2	T-7 and T-9

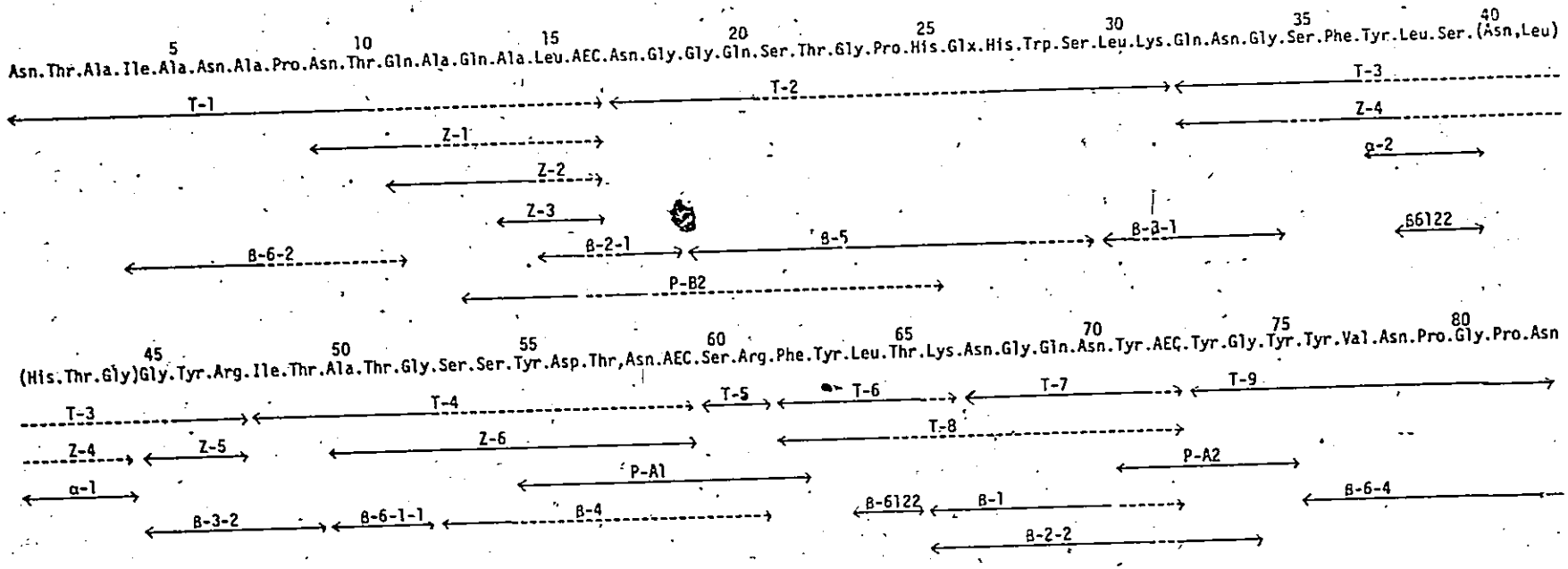


FIGURE 6:, AMINO ACID SEQUENCE OF PEPTIDE D

with the exceptions of the assignments of Glx_{26} and residues 40-44 in Peptide T-3. (----- = not sequenced; peptides labelled "T", "Z", "P", "α" and "β" are from digestion with trypsin, trypsin (prolonged digestion), pepsin, α-lytic protease and β-lytic protease, respectively).

Peptide Dβ-4 was not sequenced completely, but its composition showed it overlapped Peptides T-4 and T-5.

(d) Relevance of Cleavage Products to the Specificity of β-Lytic Protease

The cleavages of Peptide D by β-lytic protease, classed as described for glucagon, are shown below.

X - HPB Cleavages

Ala_3 - Ile_4

Ala_{14} - Leu_{15}

Ser_{29} - Leu_{30}

Tyr_{37} - Leu_{38}

Thr_{49} - Ala_{50}

Tyr_{63} - Leu_{64}

AEC_{72} - Tyr_{73}

Tyr_{75} - Tyr_{76}

Gly - Y Cleavages

Gly_{18} - Gly_{19}

Gly_{34} - Ser_{35}

Gly_{52} - Ser_{53}

Gly - HPB Cleavage

Gly_{74} - Tyr_{75}

X - Y Cleavage

Thr_{65} - Lys_{66}

VII. CLEAVAGE OF PEPTIDE E_a

(a) Production and Fractionation of Peptides

Peptide E (5 mg = 1 μmole) was dispersed in 4.5 ml of 0.05M N-ethylmorpholine-acetic acid buffer of pH 7.5 and digested with β-lytic protease (53 μg) for 4 hours at 37°. The digest was acidified, freeze-dried and fractionated by electrophoresis at pH 6.5. Cadmium-ninhydrin revealed six major bands, designated Eβ-1 to Eβ-6. The acidic peptide in zone Eβ-1 (μ_{asp} = 0.54) was eluted. The neutral peptides in band 2 were refractionated by electrophoresis at pH 2.1. An error in the timing of this electrophoresis resulted in the recovery of only two neutral peptides, Eβ-2-1 and Eβ-2-2 (μ_{ser} = 0.71 and 0.55, respectively); the former was refractionated at pH 3.5, while the latter was eluted. Fractionation at pH 2.1 of the basic peptides in zone Eβ-3 (μ_{asp} = -0.33) yielded 2 major peptides which were eluted - Eβ-3-1 and Eβ-3-2 (μ_{ser} = 1.27 and 1.20, respectively). The peptides in zone Eβ-4 (μ_{asp} = -0.43) were fractionated by electrophoresis at pH 2.1 to give two peptides, Eβ-4-1 (μ_{ser} = 1.60) and Eβ-4-2 (μ_{ser} = 1.47). The peptides in bands Eβ-5 and Eβ-6 (μ_{asp} = -0.49 and -0.59, respectively) were refractionated at pH 2.1 (μ_{ser} = 1.75 for each).

In order to isolate the other neutral peptides, a second digest of Peptide E was performed under identical conditions. On electrophoresis at pH 2.1, the peptides in the neutral zone, $E\beta_2$-2, yielded 5 bands, designated $E\beta_2$-2-1 to

$E\beta_2$-2-5 (μ_{ser} = 1.06, 1.00, 0.94, 0.82, and 0.57, respectively). The peptide in each of zones $E\beta_2$-2-1 to $E\beta_2$-2-3 was refractionated at pH 3.5 and eluted, while the peptide in each of zones $E\beta_2$-2-4 and $E\beta_2$-2-5 was freed of minor contaminants by partition chromatography. An additional basic peptide, $E\beta_2$-6 (μ_{asp} = -0.69) was also isolated from this digest. It was fractionated by electrophoresis at pH 2.1 (μ_{ser} = 1.78) and by partition chromatography.

(b) Amino Acid Composition and Sequence Data

The yields, amino acid compositions, net charge at pH 6.5, sequences, and assignment of these peptides are shown in Table 10. The C-terminal residues of Peptide Eβ-2-1 were also determined after a 2.5 hour digest of the peptide with carboxypeptidase A. The His and AEC residues of Peptide Eβ-5 were not determined, but this peptide is obviously the same as Peptide T-3, described below. Since Peptide Eβ-1 was acidic, its Glx residue was glutamic acid. Peptides Eβ-2-2 and $E\beta_2$-2-5 were neutral and contained no basic amino acids, and thus their two Asx and one Glx residues were asparagine and glutamine.

(c) Application of Cleavage Products to the Sequence of Peptide E

Damoglou and Whitaker (35) characterized and sequenced peptides from digests of Peptide E with a Staphylococcal enzyme, chymotrypsin, trypsin, and α-lytic

TABLE 10

PEPTIDES FROM DIGESTION OF PEPTIDE E WITH β-LYTIC PROTEASE

Peptide	Yield (%)	Composition	Net Charge pH 6.5	Sequence	Assignment of Residues
Eβ-1	29	Glx(1.1)Ala(1.0)Phe(0.9)	-1	Phe Ala Glu	29-31
Eβ-2-1	30	Thr(2.0)Ser(1.0)Gly(1.0)Tyr(1.1) Trp(0.7)	0	Gly Trp Ser Thr Thr Tyr	37-42
Eβ-2-2	11	Asx(3.0)Ser(2.4)Glx(0.9)Gly(2.2) Ala(2.9)Val(1.0)(Trp)	0	Ser Asn Gln Asn Gly Asn (Trp ValSerAlaSerAlaAlaGly)	6-19
Eβ-3-1	21	His(0.9)Thr(1.0)Gly(1.0)Val(0.7) Ile(0.6)	+	Ile Val His Thr Gly	32-36
Eβ-3-2	7	Tyr(2.0)His(1.3)	+	Tyr Tyr His	42-44
Eβ-4-1	15	Arg(0.9)Ser(1.0)Gly(1.2)	+1	Ser Arg Gly	1-3
Eβ-4-2	5	His(1.0)Tyr(1.0)	+	Tyr His	43-44
Eβ-5	5	His(1.0)AEC(1.0)Ser(1.8)	++	His Ser Ser (AEC)	25-28
Eβ-6	8	Lys(1.1)His(0.9)Arg(0.7)AEC(0.9) Ser(2.5)Phe(1.0)	++++	Ser Phe Lys Arg His Ser Ser (AEC)	21-28
$Eβ_2$-2-1	6	Ser(1.1)Ala(1.0)	0	Ala Ser	15-16
$Eβ_2$-2-2	6	Gly(1.9)Ala(2.0)Ser(0.9)	0	Ala Ala Gly Gly (Ser)	17-20(21)

TABLE 10 - continued

Peptide	Yield (%)	Composition	Net Charge pH 6.5	Sequence	Assignment of Residues
$E\beta_2$-2-3	21	Leu(1.0)Hse(1.0)	0	Leu Hse*	45-46
$E\beta_2$-2-4	13	Ser(1.5)Gly(2.0)Ala(3.0)	0	Ala Ser Ala Ala Gly (Gly Ser)	15-20(21)
$E\beta_2$-2-5	15	Asx(2.7)Ser(1.0)Glx(1.2)Gly(0.7) Trp(1.0)	0	Ser Asn Gln Asn Gly Asn Trp	6-12
$E\beta_2$-6	3	Lys(1.0)Arg(1.3)Ser(1.0)Phe(1.0)	+2	Ser Phe Lys Arg	21-24

*C-Terminal residue homoserine

protease; their results are briefly summarized as follows:

1. Peptide E was cleaved after its single glutamic acid residue using an enzyme from Staphylococcus aureus (a gift of Dr. G. Drapeau, Department of Microbiology, University of Montreal). Digestion of the two resulting peptides, S-1 and S-2, with chymotrypsin yielded the peptides labelled S-1C-1, S-2C-1, S-2C-2, and S-2C-3 in Fig. 7. Peptide S-1 was also digested with α-lytic protease and yielded Peptide S-1α-1.

2. The cysteic acid peptide, P-B1, was isolated from a peptic digest of the native enzyme, as described in Part C-IX, and helped to confirm the overlap between AEC_{28} and Phe_{29} of Peptide E. Peptide P-1, also isolated from a peptic digest of the native enzyme, was located by staining marker bands with Ehrlich's reagent (29).

3. A tryptic digest followed by electrophoresis at pH 6.5 yielded 3 basic peptides, T-1 to T-3 (Arg, Ser.Arg, and His. Ser.Ser.AEC, respectively), and a mixture of peptides which remained at the base-line; digestion of these adsorbed peptides with α-lytic protease gave an acidic peptide, Tα-1, which provided the overlap between the two peptides from the digest of Peptide E with the Staphylococcal enzyme.

4. A chymotryptic digest of Peptide E yielded one peptide, labelled C-1.

These data combined with the data from the β-digest of Peptide E and the N-terminal sequence of Peptide E (Table 6)

gives the amino acid sequence shown in Fig. 7. The sequence is noteworthy for its extended runs of neutral amino acids. These were a source of much frustration in early attempts to sequence the peptide via cleavages with commonly-used enzymes.

(d) Relevance of Cleavage Products to the Specificity of β-Lytic Protease

The cleavages of Peptide E and the β-enzyme have been classified below as described for glucagon:

X - HPB Cleavages	Gly - Y Cleavages
Trp_{12} - Val_{13}	Gly_3 - Gly_4
Ser_{14} - Ala_{15}	Gly_5 - Ser_6
Ser_{16} - Ala_{17}	Gly_{20} - Ser_{21}
Arg_{24} - His_{25}	Gly_{36} - Gly_{37}
AEC_{28} - Phe_{29}	
Glu_{31} - Ile_{32}	
Thr_{41} - Tyr_{42}	
Tyr_{42} - Tyr_{43}	
His_{44} - Leu_{45}	

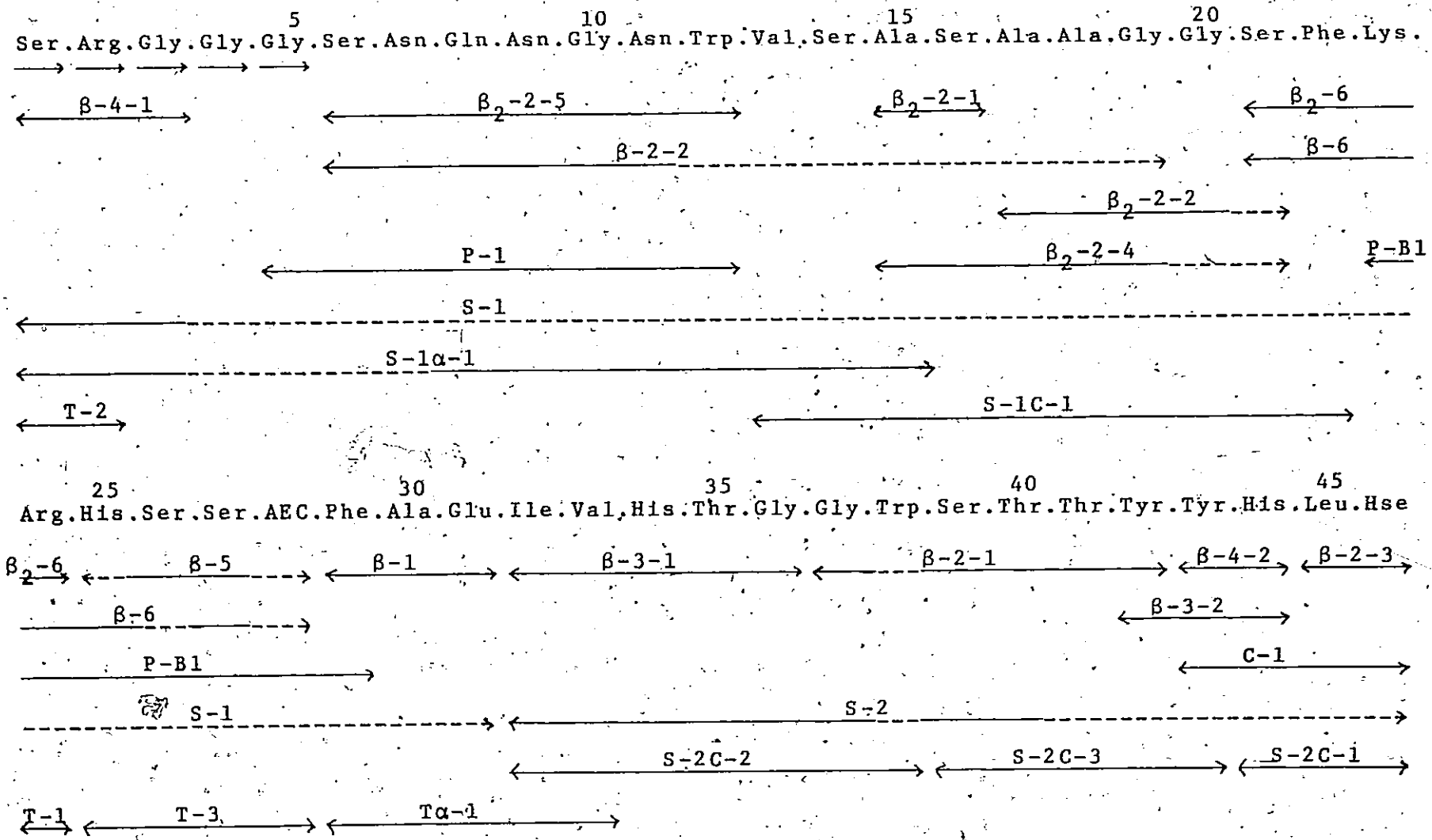


FIGURE 7: AMINO ACID SEQUENCE OF PEPTIDE E

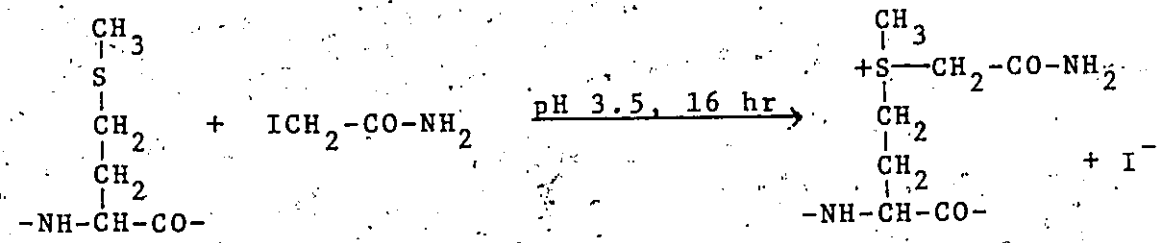
(Peptides labelled "T", "C", "P", "S", "α", and "β" are from digestion with trypsin, chymotrypsin, pepsin, a protease from Staphylococcus aureus, α-lytic protease and β-lytic protease, respectively).

VIII. THE ORDER OF THE CYANOGEN BROMIDE PEPTIDES OF β-LYTIC PROTEASE

(a) Diagonal Technique

The methionine diagonal technique of Tang and Hartley (38) provides a method for ordering peptides obtained from cyanogen bromide cleavage of a protein. Peptides containing methionine were selectively purified from an enzyme digest of the entire protein by the following procedure.

Step 1. Fractionation of digest The peptides were fractionated by electrophoresis on Whatman 3MM paper at pH 6.5, and their positions were located by staining two side-strips with cadmium-ninhydrin. A third side-strip 1 cm wide was cut from the paper, and dye markers were applied at 3 cm intervals to correct for migration of peptides along the strip. The strip was held horizontally between two clamps, sprayed evenly in a fume hood with a solution of 0.1M iodoacetamide in pyridine-acetate buffer of pH 3.5 until the strip was wet but not running, and incubated overnight at 25° in a desiccator containing a tray of the same buffer. This reaction converts methionine to S-carbamoylmethylmethionine (CM-methionine), conferring an additional positive charge on the peptides.

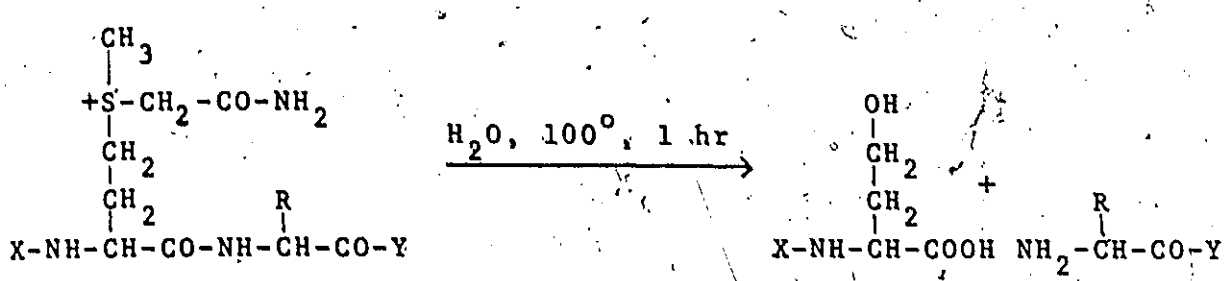


The strip was dried in a fume hood and washed several times with acetone to remove excess iodoacetamide.

Step 2: Diagonal Electrophoresis The third side-strip was stitched on to a fresh sheet of paper and was submitted to electrophoresis at pH 6.5 at right angles to the original direction. The entire sheet was stained with cadmium-ninhydrin to reveal the position of the peptides. Peptides whose electrophoretic mobility had not been altered by the treatment with iodoacetamide lay on a diagonal. Peptides whose electrophoretic mobility had been altered lay off the diagonal toward the cathode.

Step 3. Isolation of Peptides Containing Methionine The bands from the fractionated digest which corresponded in position to the off-diagonal peptides were cut out, modified by the reaction with iodoacetamide as described above, and stitched on to fresh sheets of paper for preparative electrophoresis at pH 6.5 Cadmium-ninhydrin revealed the positions of the CM-peptides, and these were eluted with dilute acetic acid.

Step 4. Amino Acid Analysis CM-Methionine, which is degraded by acid hydrolysis, was converted to homoserine and the peptide was cleaved after the homoserine residue by heating the sample with water, as follows. An aliquot of the eluate was mixed with norleucine, the internal standard, in a hydrolysis tube, and the mixture was dried. After addition



of 100 µl of H_2O, the tube was placed in a larger tube fitted with a ground glass stopper and containing about 1 ml of water. The sample was heated at 100° for 1 hour and dried. It was hydrolyzed under vacuum in 200 µl of 6N HCl-0.05% phenol for 20 hours at 105°, and was treated with pyridine-acetic acid buffer of pH 6.5 for 1 hour at 105° prior to analysis, to convert homoserine lactone to free homoserine.

(b) Enzyme Digestion of AE-β-Lytic Protease

(i) Materials

Aminoethylated β-lytic protease was prepared by Damoglou and Whitaker (35) by inactivating β-lytic protease in 7M urea-ethylenediaminetetraacetic acid at pH 4, reducing the disulfide bridges with tributyl phosphine at pH 9.5 (40) and alkylating the resulting sulfhydryl residues with ethylene imine. The aminoethylated protein was freed of low-molecular weight solutes by passage through a column of Sephadex G-25 with 10% acetic acid as solvent and freeze-dried. Amino acid analysis indicated that all its sulfhydryl groups had been alkylated.

(ii) Digestion with Trypsin and β-Lytic Protease

Aminoethylated β-lytic protease (20 mg) was dispersed in 10 ml of 0.05M N-ethylmorpholine-acetic acid buffer of pH 7.5 and digested with trypsin (600 µg) and β-lytic protease (200 µg) at 37° for 4 hours. The reaction mixture was acidified with acetic acid and freeze-dried. The diagonal technique showed that two major peptides, both derived from the neutral zone, lay off the diagonal. These peptides, AEβ-Tr-β-1 and AEβ-Tr-β-2, were isolated from the neutral zone and subjected to amino acid analysis as described above. The amino acid composition and sequences of these peptides are given in Table 11.

(iii) Digestion with Thermolysin and Trypsin

Aminoethylated-β-lytic protease (21 mg) was dispersed in 10 ml of 0.05M N-ethylmorpholine-acetic acid buffer of pH 6.5. After digestion with thermolysin (210 µg) for 3 hours at 37°, trypsin (400 µg) was added and the digestion was allowed to proceed 7 hours longer. The mixture was acidified with acetic acid and freeze-dried. The diagonal technique showed that 2 major peptides, derived from the neutral zone, lay off the diagonal. These peptides, AEβ-Th-Tr-1 and -2, were isolated from the neutral zone and subjected to amino acid analysis as described above. The amino acid compositions and sequences of these peptides are given in Table 11.

TABLE 11

PEPTIDES ISOLATED FROM AE-β BY METHIONINE DIAGONAL TECHNIQUE

Peptide	Yield (%)	Composition	Sequence	Overlap Between Cyanogen Bromide Peptides
AEβ-Tr-β-1	4	Arg(0.8)Asx(1.0)Ser(1.0)Leu(0.8) Hse(0.5)	Leu→ Asp→ Hse→ (Ser Arg)	C - E
AEβ-Tr-β-2	8	Asx(0.7)Ser(2.0)Pro(1.0)Tyr(0.8) Hse(0.5)	Asn→ Tyr→ Pro→ Hse→ (Ser Ser)	A - C
AEβ-Th-Tr-1	20	Asx(1.0)Hse(0.8)Leu(1.3)	Leu→ Hse→ Asn→	E - B
AEβ-Th-Tr-2	8	Asx(1.8)Thr(1.0)Ser(1.1)Val(1.0) Hse(0.9)	Val→ Ser→ Hse→ Asn→ Thr→	B - D

(c) Discussion

From considerations described in Part C-I, the order of the peptides in the β-enzyme was known to be A.C(BE)D. Peptide AEβ-Tr-β-2 provided the overlap between Peptide A and Peptide C, thus confirming the order of Peptides A and C, while Peptide AEβ-Tr-β-1 gave the overlap between the C-terminus of Peptide C and the N-terminus of Peptide E, establishing the order as A.C.E.B.D. It is noteworthy that Peptides AEβ-Tr-β-1 and -2 indicate that the Ser-Leu linkage, of Peptide C was cleaved by the β-enzyme in the intact protein although it was not cleaved in Peptide C itself.

The first thermolysin-trypsin peptide, AEβ-Th-Tr-1, confirmed the above assignment by an overlap between Peptides E and B. The second thermolysin-trypsin peptide, Peptide AEβ-Th-Tr-2, analyzed for a peptide with two Asn residues but only one was assigned to the peptide by the sequence data. The sequence overlapped Peptides B and D.

IX. THE STRUCTURE OF β-LYTIC PROTEASE

The positions of the disulfide bridges of β-lytic protease were determined by Damoglou and Whitaker (35), using the cystine diagonal technique of Brown and Hartley (39). Two pairs of cysteic acid peptides, P-A1 and P-A2, and P-B1 and P-B2, were isolated from a peptic digest of the native enzyme. The sequences of these peptides and the positions of the disulfide bridges are shown in Fig. 8. The first disulfide bridge is internal to Peptide D, while the second is between Peptides D and E.

The data from the sequences of Peptides A to E, the order of these peptides and the positions of the disulfide bridges specifies the complete sequence of β-lytic protease with the exceptions of the Glx assignment and the short sequence already mentioned in the discussion of Peptide D. The sequence is shown in Fig. 9.

The sequences around the histidine and the glutamic acid residues are of particular interest as a glutamic acid residue and two histidine residues provide the ligands for zinc in both thermolysin (25) and carboxypeptidase A (45). The residues in question are:

for carboxypeptidase A (His_{69}, Glu_{72} and His_{196})

Leu.Gly.Ile.$\underline{His}_{69}$.Ser.Arg.$\underline{Glu}_{72}$.Trp.Ile.Thr

and Leu.Ser.Ile.$\underline{His}_{196}$.Ser.Tyr.Gln

PEPTIDE

P-A2 Tyr.Asp.Thr.Asn.Cys.Ser.Arg.Phe

P-A1 Tyr.Cys.Tyr.Gly.Tyr

P-B2 Gln.Ala.Leu.Cys.Asn(His Thr Ser Gln Pro Gly)

P-B1 Lys.Arg.His.Ser.Ser.Cys.Phe

FIGURE 8: DISULFIDE BRIDGES OF β-LYTIC PROTEASE

The first disulfide bridge is internal to Peptide D, while the second is between Peptides D and E. (Cys was sequenced as cysteic acid.)

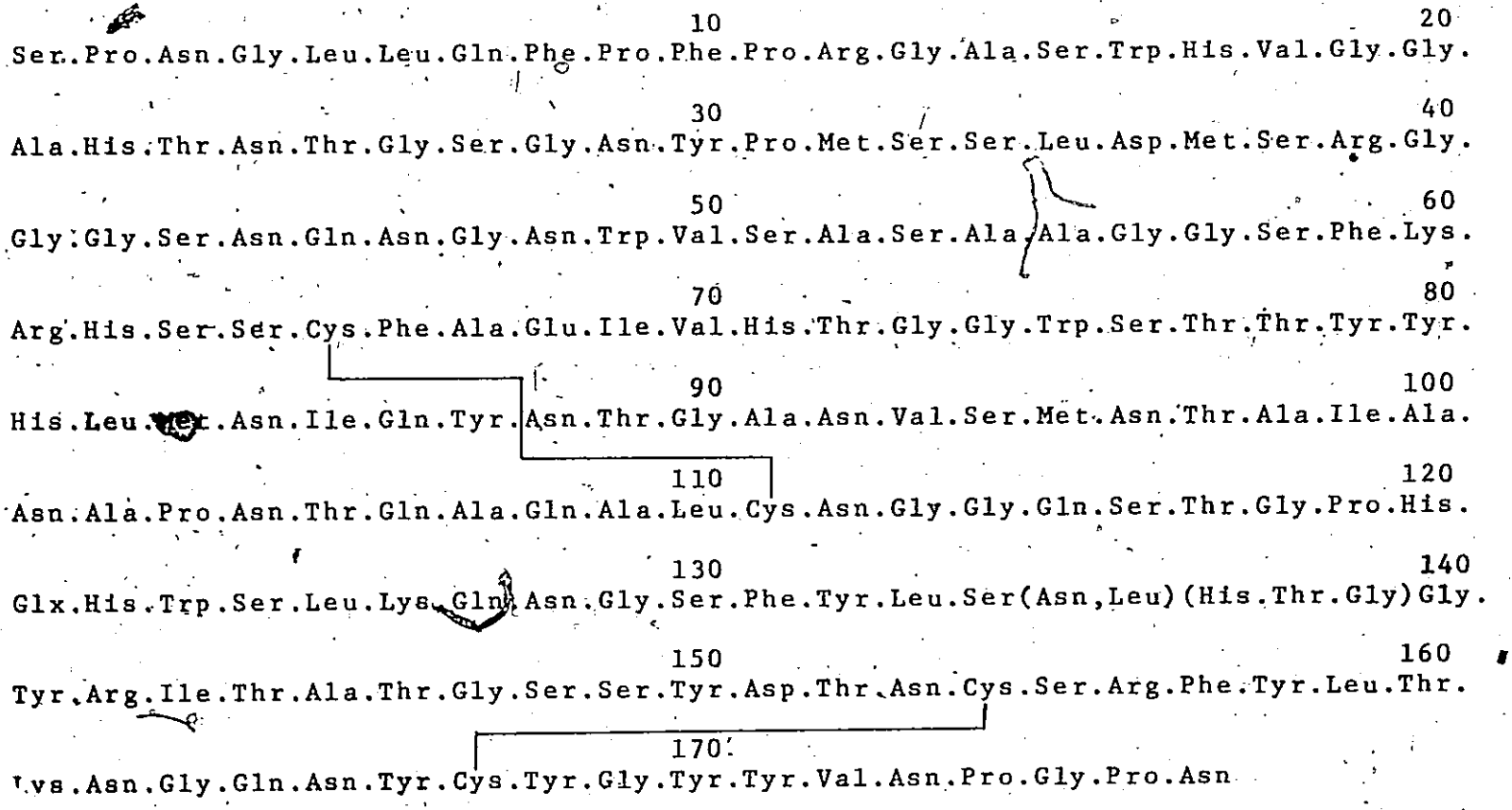


FIGURE 9: AMINO ACID SEQUENCE OF β-LYTIC PROTEASE

with the exceptions of the assignments of Glx_{121} and residues 135-139.

and for thermolysin (His_{142}, His_{146} and Glu_{166})

Val.Val.Ala.$\underline{His}_{142}$.Glu.Leu.Thr.$\underline{His}_{146}$.Ala.Val.Thr

and Ala.Ile.Asn.$\underline{Glu}_{166}$.Ala.Ile.Ser

The sequences around the histidine residues and the glutamic acid residue of the β-enzyme are not homologous with these sequences. A more general, preliminary comparison of the entire sequence has also been made by grouping amino acid residues into the categories defined by Olson et al. (17) and then comparing every trimer sequence of the β-enzyme (residues 1-3, 2-4, etc.) with every trimer sequence of carboxypeptidase A and thermolysin. This comparison also showed no evidence of significant homologies. It is probable, therefore, that all three enzymes are products of different evolutionary pathways.

The amino acid composition of the β-enzyme was compared with the compositions of the neutral protease (22) of B. thermoproteolyticus, B. subtilis, B. subtilis NRRLB3411, B. subtilis var. amylosaccharitus, P. aeruginosa (elastolytic and non-elastolytic enzymes) and T. granulosum. The β-enzyme differed from these neutral proteases in general in two respects: one is that it is about one-half the size of the neutral proteases; the other is that the β-enzyme contains 4 residues of half-cystine, whereas, with the exception of the elastolytic enzyme of P. aeruginosa, the neutral proteases have no half-cystine residues.

X. CONCLUSIONS REGARDING THE SPECIFICITY OF β-LYTIC PROTEASE

The specificity of the β-enzyme has been thoroughly investigated in the course of determining the cleavage pattern of glucagon and the sequences of the cyanogen bromide peptides of the β-enzyme. The cleavages of insulin, glucagon and the cyanogen bromide peptides have been classified into four categories as described in Part B-I-(e); the X-HPB cleavages are summarized in Table 12, the Gly-Y cleavages are given in Table 13, and the Gly-HPB and X-Y cleavages are shown in Table 14. These data show that β-lytic protease exhibits a specificity for cleavage of linkages in which a glycyl residue contributes the carbonyl group, or a hydrophobic residue contributes the imino group.

The X residue of an X-HPB cleavage (Table 12) can be a hydrophobic amino acid, a neutral hydrophilic amino acid, an acidic amino acid on a basic amino acid, for example, Trp and His, Ser and Thr, Glu and Asp, or Arg and AEC. The Y residue of a Gly-Y cleavage (Table 13) can be Gly, Ser, Asn or Thr. The Gly-HPB cleavages, shown in Table 14, satisfy both the criteria for cleavage by the β-enzyme, while the X-Y cleavage, between Thr_{65} - Lys_{66} in Peptide D, satisfies neither and is an exception.

In these cleavages, neither the X nor the Y residue has been observed to be proline. However, one Gly-HPB cleavage (Gly_{13} - Ala_{14} in Peptide A) has a prolyl residue two residues in front of the glycine residue, and one Gly-Y cleavage

TABLE 12

X - HPB CLEAVAGES OF β-LYTIC PROTEASE

Glucagon	Thr_5 - Phe_6	Asp_{21} - Phe_{22}
	Tyr_{13} - Leu_{14}	Trp_{25} - Leu_{26}
Peptide A	Ser_{15} - Trp_{16}	
Peptide B	Asn_9 - Val_{10}	
Peptide D	Ala_3 - Ile_4	Thr_{49} - Ala_{50}
	Ala_{14} - Leu_{15}	Tyr_{63} - Leu_{64}
	Ser_{29} - Leu_{30}	AEC_{72} - Tyr_{73}
	Tyr_{37} - Leu_{38}	Tyr_{75} - Tyr_{76}
Peptide E	Trp_{12} - Val_{13}	Glu_{31} - Ile_{32}
	Ser_{14} - Ala_{15}	Thr_{41} - Tyr_{42}
	Ser_{16} - Ala_{17}	Tyr_{42} - Tyr_{43}
	Arg_{24} - Phe_{25}	His_{44} - Leu_{45}
	AEC_{28} - Phe_{29}	

TABLE 13

GLY - Y CLEAVAGES OF β-LYTIC PROTEASE

Glucagon	Gly_4 - Thr_5
Peptide A	Gly_{19} - Gly_{20}
	Gly_{26} - Ser_{27}
	Gly_{28} - Asn_{29}
Peptide D	Gly_{18} - Gly_{19}
	Gly_{34} - Ser_{35}
	Gly_{52} - Ser_{53}
Peptide E	Gly_3 - Gly_4
	Gly_5 - Gly_6
	Gly_{20} - Ser_{21}
	Gly_{36} - Gly_{37}

TABLE 14

GLY - HPB AND X - Y CLEAVAGES OF β-LYTIC PROTEASE

GLY - HPB CLEAVAGES

Insulin	Gly_{23} - Phe_{24}
Peptide A	Gly_{4} - Leu_{5}
	Gly_{13} - Ala_{14}
Peptide B	Gly_{7} - Ala_{8}
Peptide D	Gly_{74} - Tyr_{75}

X - Y CLEAVAGE

Peptide D	Thr_{65} - Lys_{66}

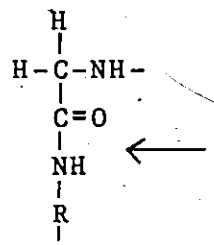
(Gly_{21} - Asn_{22} in Peptide A) has a prolyl residue two residues behind the Y residue. In two X-HPB linkages which were not cleaved by the β-enzyme (Gly_7 - Phe_8 in Peptide A, and Asn_6 - Ala_7 in Peptide D), the HPB residue was followed immediately by proline. Thus proline appears to hinder cleavage only if it is immediately adjacent to residues which form the potential cleavage site.

Thermolysin (46,47) and the neutral proteases of B. subtilis (48,49), S. griseus (49) and P. aeruginosa (50) all have specificities similar to the combined X-HPB and Gly-HPB specificities of the β-enzyme. The first two enzymes also resemble the β-enzyme in that neither cleaves an X-HPB linkage if the hydrophobic residue is followed by a prolyl residue (51,52). However none of these neutral proteases have been demonstrated to have a marked Gly-Y specificity. Cleavage of the Gly_8 - Ser_9 linkage of the insulin B chain has been reported for the neutral proteases, I and II, of B. subtilis var. amylosaccharitus (53), and cleavage of the Gly_{20} - Glu_{21} linkage of the same substrate has been reported for the neutral protease of Proteus mirabilis (54), but all three cleavages were minor cleavages. It should be noted that many of these neutral proteases have not been tested thoroughly against substrates which might reveal a specificity for cleavage after glycine. Insulin and glucagon have been the favored substrates, and these contain few glycyl residues. The β-enzyme's preference for cleavage following glycine only

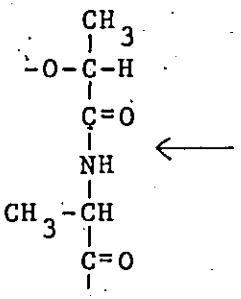
became apparent when its cleavage pattern toward Peptide A was determined. However, thermolysin has been tested with a variety of substrates, for example, with beef heart cytochrome C (51), tobacco mosaic virus coat protein (46), and Pseudomonas azurin (55), and it shows no specificity corresponding to the Gly-Y specificity of the β-enzyme.

The cleavage patterns with the B chain of performate-oxidized insulin show another difference. The neutral proteases of B. thermoproteolyticus (46,47); B. subtilis (48, 49), P. aeruginosa (50), S. griseus (49); A. oryzae (49) and B. megaterium (56) hydrolyze six linkages of this substrate - the His_5-Leu_6, His_{10}-Leu_{11}, Ala_{14}-Leu_{15}, Tyr_{16}-Leu_{17}, Gly_{23}-Phe_{24} and Phe_{24}-Phe_{25} linkages, whereas the β-enzyme cleaves only one - the Gly_{23}-Phe_{24} linkage.

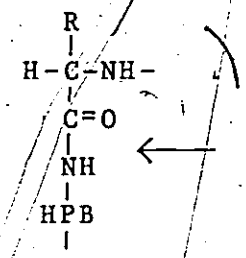
The nature of the group donating the carbonyl bond to the linkages hydrolyzed by β-lytic protease are summarized in Fig. 10. Fig. 10A represents a Gly-Y or Gly-HPB cleavage with glycine donating the carbonyl group. Since glycine has no asymmetric carbon, there can be no absolute requirement of stereospecificity to be fulfilled here. Fig. 10B shows the linkage that the β-enzyme hydrolyzes in the cell wall mucopeptide of the bacterium, M. lysodeikticus. This cleavage is between the lactyl group of N-acetyl muramic acid and L-alanine, the first amino acid of the peptide chain. In this case, the sugar, which contributes the carbonyl group to the hydrolyzed linkage, has the D-configuration (10). Fig. 10C



A: Gly



B: D-Lactyl Group



C: L-Amino Acid

FIGURE 10: CONFIGURATION OF GROUP CONTRIBUTING THE CARBONYL GROUP TO THE HYDROLYZED BOND

shows an X-HPB cleavage between an L-amino acid and a hydrophobic residue; as discussed previously, X can be neutral, hydrophobic, acidic or basic. Clearly, β-lytic protease has no configurational requirement with respect to the residue contributing the carbonyl group to the hydrolyzed linkages, for the enzyme can cleave after glycine, or a group of the D-configuration, or an L-amino acid. It may, however, have a conformational requirement, and possibly the significance of glycine residues in the enzyme's specificity is the minimal restriction on conformation which a glycine residue imposes.

Peptides from digests by the β-enzyme made a major contribution to the determination of the sequences of Peptides A, B, D, and E, and to the ordering of these peptides. In the case of Peptides D and E, β-digests provided extensive information, whereas, particularly with Peptide E, digests with trypsin, chymotrypsin and α-lytic protease provided very limited information. In short, the β-enzyme has clearly proved to be a useful digestive agent in the determination of its own sequence and would undoubtedly be helpful in the determination of the sequences of other proteins.

PART D

STUDIES OF THE ACTION OF β-LYTIC PROTEASE ON ASCARIS SUUM

I. INVESTIGATION BY ELECTRON MICROSCOPY

(a) Introduction

Ascaris suum (Goeze, 1782) is a parasitic nematode in the intestinal tract of pigs. It is closely related to Ascaris lumbricoides (Linnaeus, 1758), a parasite of the human intestinal tract; some authors classify the porcine parasite as a subspecies, var. suum, of A. lumbricoides. The outer layer of Ascaris is a multi-layered cuticle, whose innermost layer is firmly attached to layers of muscle tissue between the cuticle and the body cavity. The attachment gradually weakens when ascarids are exposed to the β-enzyme, and eventually the cuticle peels off very readily. The enzyme must penetrate the cuticle before it can weaken the attachment between the two tissues, and the primary aim of this study was to determine whether electron microscopy could reveal the course of the initial attack.

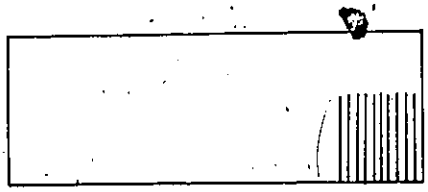
(b) Materials

The ascarids were collected immediately after slaughter of their host at Canada Packers plants in Hull or Toronto. They were washed thoroughly with running tap-water, and were kept overnight in the basal salts medium of Ellison et al. (57) - 2.3 mM KCl, 140 mM NaCl, 1.6 mM $CaCl_2$, 11 mM $MgCl_2 \cdot 6H_2O$, 0.6 mM KH_2PO_4 and 6.1 mM Na_2HPO_4 of pH 7.2.

Glutaraldehyde was biological grade, for use as a histological fixative. All other chemicals were of reagent grade.

(c) Treatment of Ascarids with β-Lytic Protease

Four nematodes, usually two males and two females, were placed in each of a series of 250 ml Erlenmeyer flasks containing 100 ml of the culture medium of Ellison. Five mg of β-enzyme was added to the flasks other than those for the controls. The flasks were gently shaken on a rotary shaker in an incubator at 25°. Nematodes were removed from the flasks after treatment periods of 1, 2, 4, 8, 16 and 24 hours. A section about 4 cm long was then cut from the central region of each worm and opened lengthwise to remove the viscera. A series of cuts, approximately 1 mm apart, were made with a razor blade along one edge of the section, as illustrated below. The entire section was fixed for 1 hour at 25° in a



4% solution of glutaraldehyde in the culture medium, and was stored at 4°. The fixation process involves crosslinkage of amino groups by glutaraldehyde. The thin strips cut along one edge provided the material to be embedded for transmission microscopy.

(d) Transmission Electron Microscopy

Pieces 1x1 mm were cut from the narrow strips, washed twice in Solution A (29 mM sodium barbital, 29 mM sodium acetate, 125 mM sucrose and 1.8 mM calcium chloride) for 15 minutes each time, and fixed in Solution A containing 1% osmium tetraoxide (OsO_4) for 75 minutes. The pieces were dehydrated in ethanol (for 15 minutes in each of 30%, 50%, 70%, 85%, and 95% ethanol and in absolute alcohol changed once over the course of 30 minutes). These pieces were embedded in Spurr Low-Viscosity Embedding Medium (58) - 10 g vinylcyclohexene dioxide, 6 g diglycidyl ether of polypropyleneglycol, 26 g nonenyl succinic anhydride and 0.4 g dimethylaminoethanol. The pieces were treated according to the following schedule:

(1) 15 min. in propylene oxide (PO)

(2) 15 min. in PO (repeat of (1))

(3) 15 min. in PO - Spurr medium (2:1)

(4) 15 min. in PO - Spurr medium (1:1)

(5) 15 min. in PO - Spurr medium (1:2)

(6) 1 hr. in Spurr medium

(7) 3 hr. in Spurr medium

Each piece was set in a block of Spurr medium, and the block was cured by heating overnight at 60°. The block was trimmed with an LKB-Pyramitome and sections were cut with a Reichert ultramicrotome; when floated on water, the sections gave a silvery interference pattern, indicating a thickness of 0.06-0.09 μ. The sections were picked up on grids and were stained

with uranyl acetate (30 minutes in 2.5% aqueous uranyl acetate) and with Reynold's lead for 10 minutes (59). They were examined using an Elmiskop 1 transmission electron microscope at 80 KV.

Ascaris cuticle is not an ideal material for electron microscopy, as it has poor staining properties, and much of it, particularly the layers below the cortical layers, is virtually amorphous. Photography was essential for critical examination of these layers as the viewing screen of the microscope did not provide sufficient contrast. At a 10,000-fold magnification, about 10 photographs were required for an overlapping photographic record of the entire cuticle. Bird and Deutsch (60) identified nine layers in the cuticle of Ascaris suum: an external cortical and an internal cortical layer, a fibrillar layer, a homogeneous layer, a boundary layer, three fiber layers and a basal lamellar layer. All of these layers could be identified, with the exception of the boundary layer, which, as Bird and Deutsch point out, is sometimes difficult to detect.

A detailed examination of five sections from the controls and twelve sections from enzyme-treated ascarids (24-hour exposure) failed to reveal any significant differences between the two sets, and the comparison was not pursued further. It was appreciated that the failure to observe differences may simply have been a consequence of the small number of sections examined.

(e) Scanning Electron Microscopy

Pieces measuring about 1x1 cm were cut from the fixed material, and were washed in distilled water for 6 hours to remove excess glutaraldehyde and buffer. The tissue was dehydrated either by freeze-drying or by treatment with ethanol. In the first technique, the tissue for freeze-drying was rapidly frozen in Freon 12 and freeze-dried overnight in a Speedivac - Pearse Tissue Dryer Model 1. In the second technique, the tissue was dehydrated with alcohol by gentle shaking for 15 minutes in each of the following solutions - 30% ethanol, 50%, 60%, 75%, 85%, and 95% ethanol, and in absolute alcohol which was changed 4 times over the course of 1 hour; the tissue was then air-dried. Each piece of tissue was mounted on a metal stub covered with silver paint; and when the paint had dried, the stub was inserted in a Speedivac Coating Unit Model 12E6/1258. Gold was diffused on to the sample from two angles to a depth of about 300 Å.

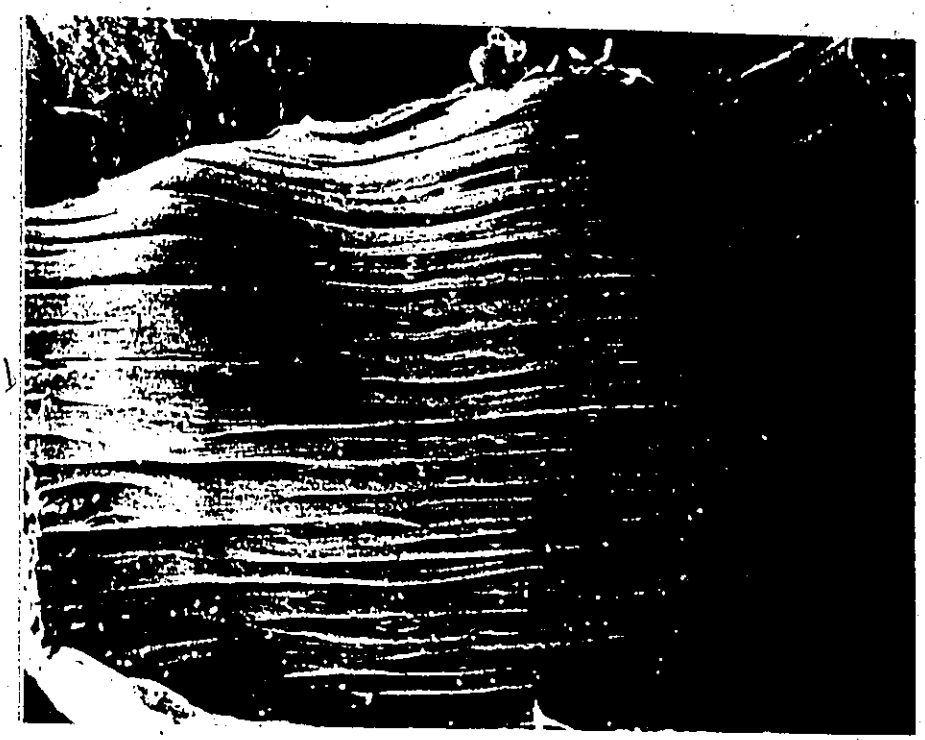
The coated specimens were examined by scanning electron microscopy using a Cambridge Stereoscan Mark IIA electron microscope.

Fig. 11A is a photograph taken at low magnification (14 x) of cuticle from an ascarid which had been exposed to the β-enzyme for 24 hours; the cuticle had been dehydrated by freeze-drying after rapid freezing. It will be noted that the surface has numerous small pits. The pits appeared only in cuticle which had been exposed to the β-enzyme for at least

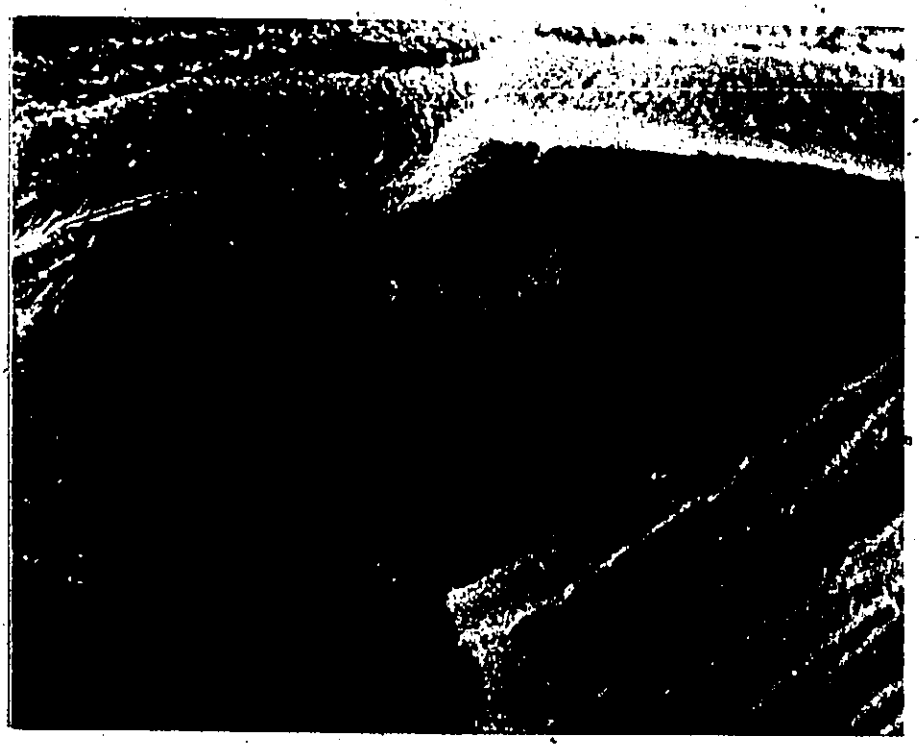
FIGURE 11A: SURFACE OF CUTICLE EXPOSED TO THE β-ENZYME FOR 24 HOURS AND DEHYDRATED BY FREEZE-DRYING (14 x). About 20 pits can be seen. The film was Kodak FX 135-20 Panatomic X.

B: ONE TEAR IN THE SAME SAMPLE (1225 x).

(A)



(B)



16 hours, and which had been dehydrated by the freeze-drying technique. Fig. 12A shows two of the three pits which were observed in a 1x1 cm sample of cuticle from an ascarid which had a 16-hour exposure to enzyme. No fewer than 10 were observed in cuticle from ascarids with a 24-hour exposure. They were absent from the controls and also from cuticle which had been exposed to β-lytic protease for 24 hours and dehydrated with alcohol.

At higher magnification (Figs. 11B and 12B), the pits appear as deep tears extending through several layers of cuticle. These tears varied in length from 50 to about 200 μ. They were never observed on direct examination of the cuticle by optical microscopy, and, as mentioned above, they were never observed when the cuticle was dehydrated with alcohol. The obvious explanation is that they represent regions of cuticle which had been sufficiently weakened by enzyme attack to be unable to withstand the mechanical stresses from rapid freezing.

These lesions appear more frequently in the grooves than in the ridges of the outer surface. Otherwise they showed no obvious distribution pattern. They are hardly consistent with a uniform layer-by-layer attack on the cuticle. Rather they suggest that the attack proceeds by a series of localized penetrations. The weakening of the cuticle is not accompanied by extensive digestion as this would lead to gross changes, detectable by visual examination with the optical microscope,

FIGURE 12A: SURFACE OF CUTICLE EXPOSED TO THE β-ENZYME FOR 16 HOURS AND DEHYDRATED BY FREEZE-DRYING (240 x). Two pits in the surface can be seen.

B: ONE TEAR IN THE SAME SAMPLE (1200 x).

(A)

(B)

and the absence of such digestion suggests that much of the structural protein in the cuticle is not readily degraded to soluble products by the β-enzyme. An example of one such component is discussed in the following section.

II. ACTION OF THE β-ENZYME ON ASCARIS COLLAGEN

(a) Introduction

Most of the fiber layers and roughly 30% of the total dry weight of the Ascaris cuticle are accounted for by a form of collagen which was first described by Chitwood in 1936 (61). As shown by Harrington and his co-workers, its amino acid composition (62) differs from that of vertebrate tropocollagen (63) in several respects: it contains fewer glycine than proline residues, it contains very few hydroxyproline residues, and it contains a substantial number of half-cystine residues (Table 15, cols. 1 and 2). It has a molecular weight of about 900,000. On reduction of the half-cystine residues, the molecule is converted to a mixture of 2 non-identical subunits of 62,000 molecular weight.

As this protein is a major component, and the only well characterized component, of Ascaris cuticle, it was obviously of interest to determine whether the β-enzyme had appreciable activity toward it. A bacterial collagenase, the collagenase of Clostridium histolyticum, and α-lytic protease were used as standards for comparison.

TABLE 15

AMINO ACID COMPOSITION OF RAT SKIN COLLAGEN AND ASCARIS COLLAGEN

	Rat Skin Collagen (Gross & Piez) (63)	Ascaris Collagen (McBride & Harrington) (62)	Ascaris Collagen* (This preparation)
Lys	29	45	45
His	5.1	8.4	9.7
Arg	49	29	28
Asx	47	69	64
Thr	20	19	14
Ser	41	19	18
Glx	74	67	65
Pro	117	296	298
Gly	327	274	282
Ala	106	72	73
1/2-Cys	0	27	33
Val	22	18	16
Met	6.3	5.2	3.6
Ile	10	10	8.5
Leu	25	19	19
Tyr	3.2	3.7	1.9
Phe	13	10	8.8
Hyp	100	16	14
Hyl	5.7	-	-

*Analysis after hydrolysis for 24 hours

(b) Materials

The α-enzyme had been prepared as outlined in the introduction. Collagenase-CLS (2 x crystallized) from Clostridium histolyticum was obtained from Mann Research Laboratories. This enzyme preparation probably contained both collagenase A and B, as its method of preparation antedated the procedure for separating the two enzymes. Collagenase A and B have the same lysine content (64) and the concentration of collagenase was estimated from an amino acid analysis of a stock solution of the enzyme. The ammonium sulfate used was Mann-Schwarz Special Enzyme Grade. All other chemicals were reagent grade.

(c) Preparation of Ascaris Collagen

Cuticle was obtained by clipping off the two extremities of the nematodes, slitting the body wall lengthwise, removing the viscera, and then scraping the muscle off the cuticle. This scraping operation was carried out by placing the nematode on a glass plate with the cuticle side down and scraping off the muscle with a glass slide. The cuticle was washed thoroughly in water, drained, and pressed dry with Kimwipes.

Ascaris collagen was prepared from this cuticle by the method of Harrington and co-workers (62,65). Cuticle (25 g of the pressed-dry material) was ground to a slurry with a mortar and pestle and was extracted overnight at 4° by gentle stirring in 1 liter of 0.5 N NaCl. This extraction

removed most of the soluble non-collagenous protein. The extracted cuticle was collected by centrifugation and homogenized by hand, firstly in a loosely-fitting ground glass homogenizer, and then in a tightly-fitting ground-glass homogenizer. The homogenate was stirred for 40 hours in 150 ml of 0.5 N NaCl at 4°, and then centrifuged at 15,000 X g for 20 minutes. The supernatant fraction, containing the Ascaris collagen, was mixed with one third of its volume of a solution of ammonium sulfate (saturated at 4°); the mixture was left overnight at 4°. The precipitated collagen was collected in a centrifuge, dissolved in 60 ml of 0.5 N NaCl and precipitated by addition of 25 ml of saturated ammonium sulfate. The pellet was redissolved by stirring it for 48 hours with 40 ml of 0.20 N NaCl at 4°. The solution was then dialyzed in Visking dialysis tubing for 3 days against several changes of 0.20 N NaCl at 4°. The dialyzed solution was centrifuged at 78,000 X g for one hour, to yield 46 ml of clear viscous collagen solution.

Table 15 compares its amino acid composition with that reported for Ascaris collagen by McBride and Harrington (62). The concentration of collagen in the solution was estimated from the amino acid analysis to be 1.9 mg/ml.

(d) Enzymic Activities toward Ascaris Collagen

A viscometric assay was used to compare enzyme activities toward Ascaris collagen. The two viscometers were Cannon capillary viscometers with flow times of 63.31 and 66.29

sec when loaded with 5.0 ml of distilled water at 25.0°. The substrate stock solution was a solution of Ascaris collagen (1.9 mg/ml) in 0.20 N NaCl; the enzyme stock solutions were solutions of enzyme in phosphate buffer of pH 7.2 prepared from 0.25 M KH_2PO_4 and 0.25 M Na_2HPO_4. Immediately prior to the assay, both solutions were brought to 25.0° in the thermostat and at zero time, 4.5 ml of substrate solution was mixed with 1.0 ml of enzyme solution; 5.0 ml of the mixture was added to the viscometer. The flow times were measured at intervals over the course of 1 to 2 hours. The time that a measurement was made was taken as the time at the mid-point of the flow time. Flow times were also measured for the solvent (5 ml of a mixture of 4.5 ml of 0.20 N NaCl and 1.0 ml of phosphate buffer).

For the purposes of this simple comparison, the viscosity of a solution can be taken to be given by the equation:

$$\eta = C\rho t$$

where η is the viscosity of the solution, ρ is the density, t is the flow-time, and C is the viscometer constant. The specific viscosity, η_{sp}, of a solution of collagen or its degradation products, is given by the equation:

$$\eta_{sp} = \frac{\eta - \eta_o}{\eta_o} = \frac{\rho t}{\rho_o t_o} - 1$$

where η, ρ and t are the viscosity, density and flow-time of the solution and η_o, ρ_o and t_o are the viscosity, density and

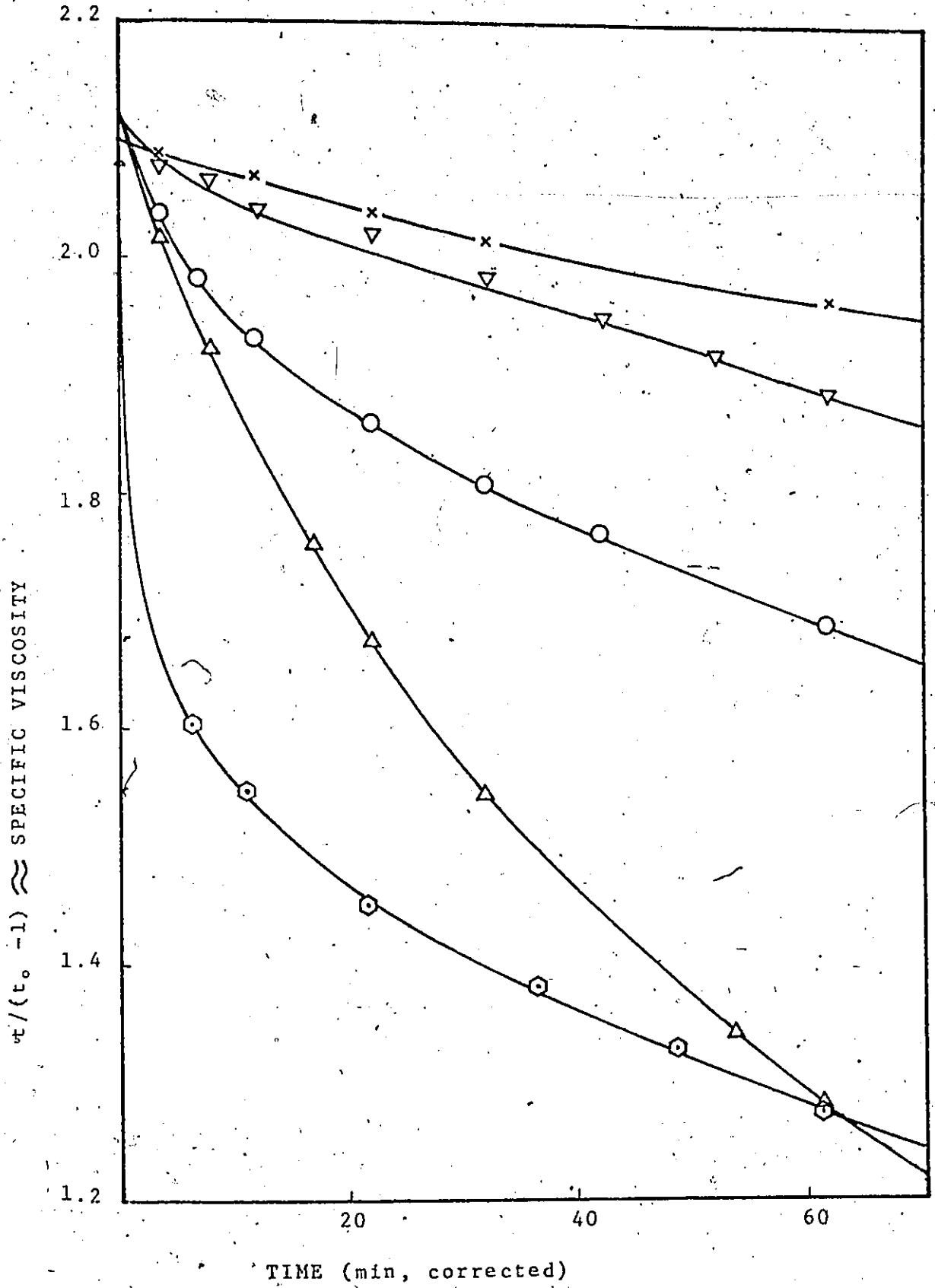
flow-time of the solvent. The densities of the collagen solution and solvent were estimated by the weight of the solution in a 5 ml volumetric flask, and as they were observed to be very nearly identical, their ratio was taken as one.

Fig. 13 shows the change in specific viscosity over the course of 1 hour at various concentrations of α-lytic protease, β-lytic protease and Clostridium histolyticum collagenase. It will be noted that β-lytic protease at a concentration of 146 µg/ml has considerably less collagenase activity in this assay than C. histolyticum collagenase at a concentration of only 3.3 µg/ml. Even α-lytic protease, an enzyme with no lytic activity toward Ascaris, has a much greater activity toward Ascaris collagen than the β-enzyme. According to this comparison, then, the β-enzyme is an enzyme with extremely weak collagenase activity. This finding is consistent with other data on the specificity of the enzyme. Oza and Whitaker had found that glycyl-L-prolyl-glycyl-glycine and N-Cbz-glycyl-L-prolyl-glycyl-glycyl-L-prolyl-L-alanine were not hydrolyzed at an appreciable rate by the β-enzyme (21); these compounds are usually good substrates for enzymes with high activity toward mammalian collagen. It should also be noted that the previously discussed cleavages of the A and B chains of insulin, glucagon, and the cyanogen bromide peptides of the β-enzyme provided not one example of a cleavage in the immediate vicinity of a proline residue.

Clearly, the lytic activity of the β-enzyme toward

FIGURE 13: ACTIVITY OF β-LYTIC PROTEASE, C. HISTOLYTICUM COLLAGENASE AND α-LYTIC PROTEASE TOWARD ASCARIS COLLAGEN; SPECIFIC VISCOSITY VERSUS TIME.

The β-lytic protease concentrations are 14.6µg/ml (×) and 146 µg/ml (○); the C. histolyticum collagenase concentrations are 0.7 µg/ml (▽) and 3.3 µg/ml (△); the α-lytic protease concentration is 80 µg/ml (⊙).


2.2
2.0
1.8
1.6
1.4
1.2
20
40
60
$t/(t_o - 1) \approx$ SPECIFIC VISCOSITY
TIME (min, corrected)

nematodes cannot be attributed to its collagenase activity. On the other hand, an enzyme with an ability to cleave peptide linkages with glycine as a carboxyl donor or with hydrophobic residues as the donor of the amino group, is an enzyme which is very likely to find targets for attack in a great variety of tissues. Further, β-lytic protease, unlike α-lytic protease, has no known counter-part among the pancreatic enzymes which Ascaris must withstand in the intestinal tract of its host. Hence, when attacking Ascaris, the β-enzyme is unlikely to be challenged by inhibitors analagous to the parasite's trypsin inhibitors (66,67) and its chymotrypsin inhibitor (68). These relatively unspecific factors - versatility in attack and freedom from inhibition - are probably the factors which contribute most to the β-enzyme's helmintholytic activity.

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