THE SEQUENCES OF THE TRYPTIC PEPTIDES FROM ACTINIDIN

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ABSTRACT

Actinidin is a plant thiol protease which is isolated from the fruit of *Actinidia chinensis*, the chinese gooseberry. Determination of the primary amino acid sequence of actinidin was undertaken to extend the limited structural information available on this group of enzymes, and therefore enable a better understanding of their physical and chemical properties.

The order of arrangement of the 220 amino acid residues in the primary sequence of actinidin was determined from the sequences of the tryptic peptides. S-carboxy[$^{14}C_2$]methyl actinidin was digested with trypsin, and the twelve tryptic peptides produced were initially separated into seven fractions by gel chromatography on Sephadex G-50. The first four fractions contained tryptic peptides that were purified by DEAE-cellulose chromatography. The last three fractions contained peptides that were sufficiently small to enable purification by paper techniques, and these peptides were sequenced directly by the dansyl-Edman method.

Further degradation of the tryptic peptides purified on DEAE-cellulose with either chymotrypsin, thermolysin, pepsin or *Staphylococcus aureus* V8 protease was necessary to provide smaller peptides that could be sequenced by the dansyl-Edman method. *S. aureus* V8 protease was particularly useful in the determination of amide residues, because of the enzyme specificity for the carboxyl groups of glutamic acid.

The fourth tryptic peptide in the sequence of actinidin could not be located in the tryptic peptide elution profiles of either the Sephadex G-50 or DEAE-cellulose columns. The sequence of this peptide was determined from a tryptic peptide obtained by digestion of maleylated carboxymethyl actinidin.

The N-terminal of actinidin was determined by the dansyl Edman method, and the C-terminal by analysis of cyanogen bromide fragments, and by digestion with carboxypeptidase A.

Radioactively labelling the active site cysteine residue with iodo[$^{14}C_2$]acetic acid, and subsequent purification of the radioactive tryptic digest peptide, enabled the isolation of the tryptic peptide containing the active site cysteine residue. Further digestion of this peptide with chymotrypsin and determination of the sequence of the smaller radioactive peptide, provided the sequence about the active site cysteine residue.

Alignment of the tryptic peptides to reconstruct the primary sequence of actinidin was accomplished with information from cyanogen bromide fragments, information from tryptic peptides of maleylated carboxymethyl actinidin, and information from the three dimensional X-ray crystallographic structure of actinidin determined by Dr E.N. Baker.

The low proportion of basic residues and high proportion of acidic residues in actinidin are in agreement with the enzyme being an acidic protein. Colorimetric analysis of the tryptophan residue content, using 2-nitrophenylsulphenyl chloride, confirmed the presence of six tryptophan residues in the sequence of actinidin.

The amino acid sequences about the seven cysteine residues and the single histidine residue in actinidin were very similar to the analogous sequences in papain and other plant thiol proteases. Furthermore, comparison of the primary sequence of actinidin with that of papain, and the fragments of sequence available for other plant thiol proteases, indicated a considerable homology throughout the sequences of these proteins.

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ABBREVIATIONS

BAW	Butanol/glacial acetic acid/ distilled water.
BAWP	Butanol/glacial acetic acid/distilled water/pyridine.
N-*α*-CBZ-lys-*p*NP	N-*α*-carbobenzoxy-L-lysine-*p*-nitrophenyl ester.
Dansyl, DNS	1-dimethylaminonaphthalene-5-sulphonyl
⇁	Symbol used to indicate direct dansyl-Edman sequencing.
DEAE	Diethylaminoethyl.
DTT	Dithiothreitol.
E.U.	Enzyme unit (μmol substrate transformed.min^{-1}).
FDNB	1-fluoro-2,4-dinitrobenzene.
NpS	2-nitrophenylsulphenyl.
%	All percentages are weight/volume (w/v) unless otherwise stated.
PITC	Phenylisothiocyanate.
POPOP	1,4-di[2-(5-phenyloxazolyl)]benzene.
PPO	2,5-diphenyloxazole.
S. aureus	*Staphylococcus aureus*.
TFA	Trifluoroacetic acid.
TPCK	L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone.
Tris	Tris(hydroxymethyl)amino methane.

Amino acid abbreviations:

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Asx	Aspartyl or Asparaginyl
CMCys	Carboxymethyl cysteine
Cys	Cysteine
$CySO_3H$	Cysteic acid
Gln	Glutamine
Glu	Glutamic acid
Glx	Glutamyl or Glutaminyl
Gly	Glycine

ABBREVIATIONS (contd.)

His	Histidine
Hse	Homoserine
$\overset{\frown}{\text{Hse}}$	Homoserine lactone
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Peptides obtained by enzymic digestion:

T	Tryptic
ChT	Chymotryptic
Th	Thermolytic
P	Peptic
GE	*S. aureus* V8 proteolytic
a	Peptide acidic charged at pH 6.5.
b	Peptide basic charged at pH 6.5.
N	Peptide neutral at pH 6.5

Enzyme EC numbers used are according to:

Recommendations (1972) of I.U.P.A.C. and the International Union of Biochemistry.

Elsevier Scientific Publishing Company, Holland (1975) And Supplement I (corrections and additions (1975)) published in *Biochim. Biophys. Acta*, **429**, 1-45 (1976).

General abbreviations:

Are used according to the "Policy of the Biochemical Journal" *Biochem. J.*, **153**, 1-21 (1976).

CHAPTER I

INTRODUCTION

1.1. Actinidin and its relation to other proteases:

Actinidin, a proteolytic enzyme obtained from the fruit of *Actinidia chinensis*, the chinese gooseberry, was first described by Arcus in 1959. Proteolytic enzymes may be loosely grouped according to the principal residue involved at the active site and to their biological source (Fig. 1.1.) (Dayhoff, 1972).

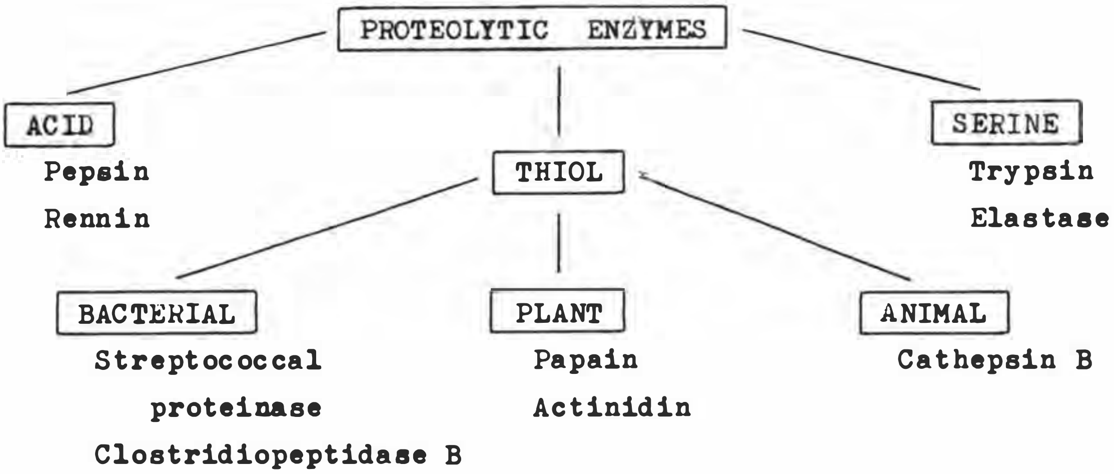


Fig. 1.1. Proteolytic enzyme groups.

Actinidin, having a thiol group essential for its activity, has been classified with the plant thiol proteases. Other enzymes of this group which also require an active thiol group for their activity include ficin (EC 3.4.22.3.), stem bromelain (EC 3.4.22.4.), fruit bromelain (EC 3.4.22.5.), chymopapain (EC 3.4.22.6.), and papain (EC 3.4.22.2.), the latter being the most extensively studied.

The bacterial enzymes, streptococcal proteinase (EC 3.4.22.10.) (Liu and Elliott, 1965) and clostridiopeptidase B (EC 3.4.22.8.) (Mitchell and Harrington, 1970), and the animal protease cathepsin B (EC 3.4.22.1.) (Snellman, 1971), are also thiol proteases, but have been characterised only to a limited extent.

Of the plant thiol proteases, crystalline papain is isolated from the green fruit and latex of Carica papaya, a tropical tree (Balls et al, 1937; Kimmel and Smith, 1954; Blumberg et al, 1970), and chymopapain is isolated from the same source (Ebata and Yasunobu, 1962; Kunimitsu and Yasunobu, 1967). Ficin is derived from the fig tree species Ficus glabrata (Walti, 1938; Williams and Whitaker, 1969) and Ficus carica (Kramer and Whitaker, 1969). The bromelains are obtained from Ananas comosus (L), the pineapple plant of the Bromeliaceae family.

Actinidin exists in nature in the reduced active form, a property it shares with all other plant thiol proteases (Barel and Glazer, 1969; Stein and Liener, 1967). Thus actinidin can be inhibited with thiol reagents such as iodoacetic acid, iodoacetamide and p-chloromercuribenzoate (McDowall, 1970; Shapira and Arnon, 1969; Finkle and Smith, 1958), as well as by heavy metal compounds. The active thiol group can also be reversibly blocked with disodium tetrathionate during preparation (Englund et al, 1968) and the enzyme can be reactivated with a thiol reducing reagent such as dithiothreitol. In contrast to the plant enzymes, streptococcal proteinase, as well as being present in nature in the active enzyme form, was also found to exist as a zymogen with the active thiol group blocked with a volatile mercaptan in the form of a mixed disulphide (Ferdinand et al, 1965).

The plant thiol proteases show considerable stability. Papain retains activity in 8M-urea solutions (Sluyterman, 1967) and is stable to high concentrations of ethanol, methanol and dimethyl sulphoxide during crystallization (Drenth et al, 1968a), but is unstable to pH values below 4 (Lineweaver and Schwimmer, 1941). Actinidin and the other plant thiol proteases demonstrate a similar stability (Englund et al, 1968; Murachi et al, 1964), and both papain and actinidin have a pH optimum of 6. The substrate specificity of actinidin (McDowall, 1973; Boland and Hardman, 1972, 1973) is not unlike that of papain and the other plant thiol proteases (Glazer and Smith, 1971a), showing that these enzymes have kinetic similarities.

In spite of the above broad similarities, the plant thiol proteases show some significant differences. Unlike papain, the fruit and stem bromelains are glycoproteins which have considerably higher molecular weights than papain [33,000 as compared to 23,406 for papain (Mitchel *et al*, 1970)]. Stem bromelain contains one oligosaccharide moiety, of which the structure is known (Yasuda *et al*, 1970), and which is covalently bound to the peptide chain (Murachi *et al*, 1967; Scocca and Lee, 1969). Ficin is also reported to be a glycoprotein (Glazer and Smith, 1971b), but has a lower molecular weight of 23,800 (Englund *et al*, 1968) similar to that of papain. The molecular weight of actinidin, 26,000 (Boland and Hardman, 1972), falls in the middle of this range. The proteins also differ in their isoelectric points. Both papain and ficin are basic proteins with isoelectric points of 8.75 (Smith *et al*, 1954) and greater than 9.0 (Cohen, 1958) respectively, while actinidin is a very acidic protein (isoelectric point of about 3.1) (McDowall, 1970). There are significant differences in amino acid compositions and in particular, because of the variation in cysteine content, the proteins are likely to contain different numbers of disulphide bridges (Mitchel *et al*, 1970; Englund *et al*, 1968; Kunimitsu and Yasunobu, 1967; Ota *et al*, 1964).

The limited structural data available on the thiol proteases have been reviewed (Glazer and Smith, 1971a), and more recently, partial amino acid sequence data for bromelain have been reported (Goto *et al*, 1976).

The complete amino acid primary sequence of papain was obtained by a series of studies on the protein oxidised with performic acid, on S-carboxymethylated protein, and on denatured protein. By using partial acid hydrolysis (McDowall and Smith, 1965), and enzymic digestion (Kimmel *et al*, 1962, 1965), a tentative linear sequence for papain was proposed (Light *et al*, 1964). Determination of the three dimensional structure of crystalline papain by X-ray methods (Drenth *et al*, 1968b), indicated several errors in the tentative primary sequence, which were later corrected by further experiments (Mitchel *et al*, 1970; Husain and Lowe, 1969, 1970a).

Knowledge of the three dimensional structure of papain enabled the determination of the active site geometry, but chemical studies were required to demonstrate the involvement of specific amino acid residues. The active site cysteine residue of papain was identified by reaction of the native protein with iodo[^{14}C] acetic acid and isolation of the radioactive peptide after enzymic digestion of the carboxymethylated protein (Light *et al*, 1964). The amino acid sequences about the active site cysteine residue of other thiol proteases are homologous to the corresponding sequence in papain (Fig. 1.2.).

	15	16	17	18	19	20	21	22	23	24	25	26[f]
papain[a]	Pro-	Val-	Lys-	Asn-	Gln-	Gly-	Ser-	Cys-	Gly-	Ser-	CySH[e]-	Trp
ficin[b]	Pro-	Ile-	Arg-	Gln-	Gln-	Gly-	Gln-	Cys-	Gly-	Ser-	CySH-	Trp
bromelain[c]				Asn-	Gln-	Asp-	Pro-	Cys-	Gly-	Ala-	CySH-	Trp
chymopapain[d]			Lys-	Arg-	Val-	Pro-	Asp-	Ser-	Gly-	Glu-	CySH-	Tyr

Fig. 1.2. Amino acid sequences containing the essential cysteine in some thiol proteolytic enzymes.

[a] Light *et al*, 1964.
[b] Wong and Liener, 1964; Husain and Lowe, 1970b.
[c] Husain and Lowe, 1970c.
[d] Tsunoda and Yasunobu, 1966.
[e] CySH represents the essential cysteine residue.
[f] Numbering of residues as for the sequence of papain.

Further evidence for the plant thiol proteolytic enzymes having a similar active site structure (Fig. 1.3.) was provided when papain, ficin and stem bromelain were all demonstrated, by the use of the bifunctional reagent 1,3-dibromoacetone, to have a histidine residue within 0.5 nm of the active site cysteine residue (Husain and Lowe, 1968a, 1968b, 1970b, 1970c). As well as the cysteine and histidine residues, the involvement in the active site of several other amino acid residues, some of which form a

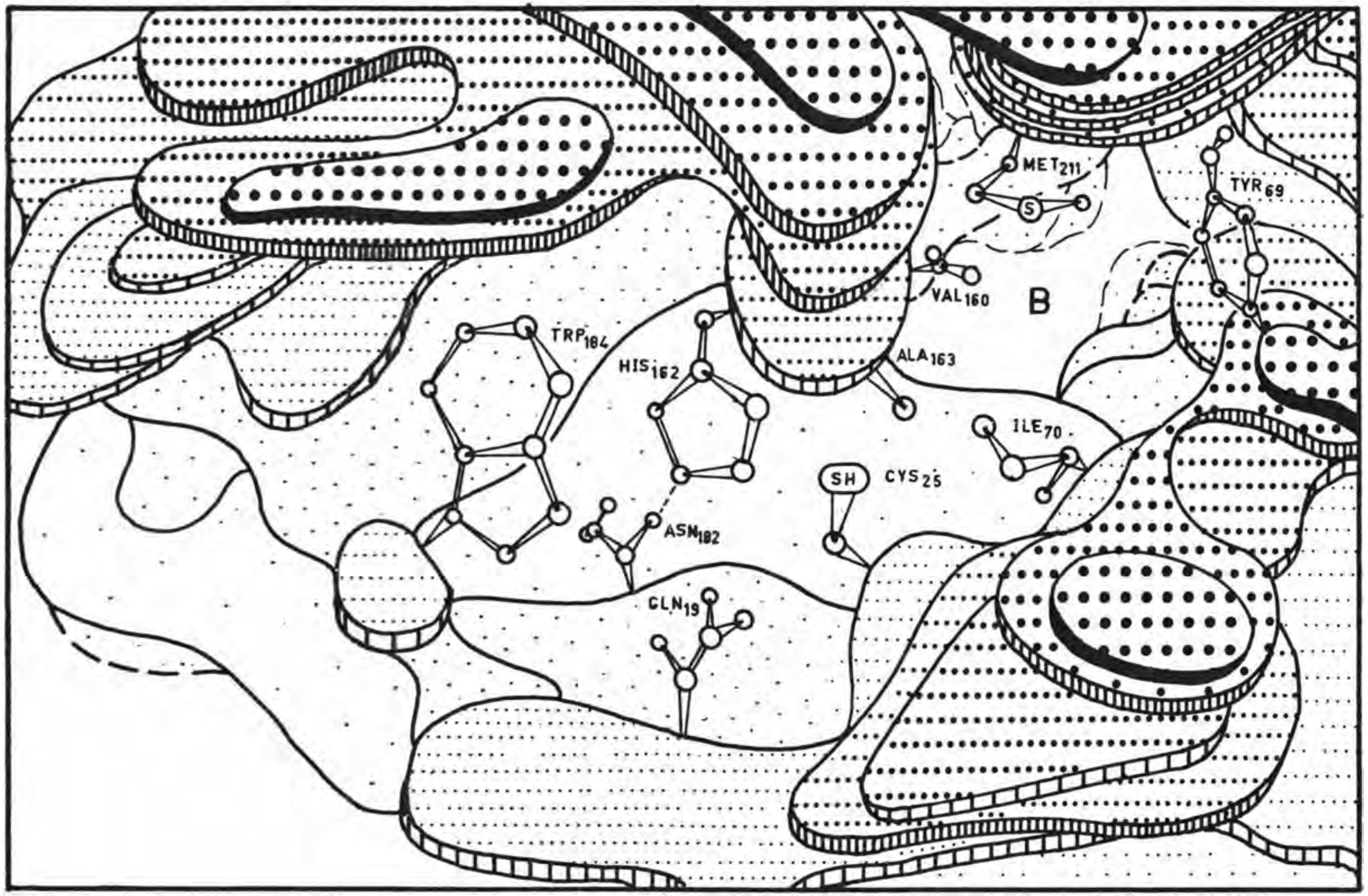


FIG. 1.3. GENERAL ACTIVE SITE STRUCTURE OF THE PLANT THIOL PROTEASE ENZYMES.

The amino acid residue numbers refer to the sequence of actinidin.

'B' is the hydrophobic binding site.

hydrophobic binding site, has been demonstrated with the aid of peptide substrates and inhibitors (Berger and Schechter, 1970).

The X-ray crystallographic studies of papain (Drenth et al, 1968b) also showed that several amino acid residues formed a hydrophobic binding site or pocket at one end of the active site of papain (labelled B in Fig. 1.3.), and that these residues came from widely different regions of the primary sequence. X-ray crystallographic studies of actinidin (Baker, 1973, 1976a, 1976b) show that the three dimensional structure of actinidin is similar to that of papain and that the amino acid residues in the active site of actinidin also come from widely different regions of the primary sequence (Fig. 1.3.).

Although fragments of sequence are available for other plant thiol proteases, papain remains the only one for which a complete sequence is available. Since amino acid residues from many different parts of the amino acid sequence are likely to be vital, both for the activity of a protein and for maintenance of its proper three dimensional structure, the determination of the complete amino acid sequence of a protein is essential for a fuller understanding of its biological function. Where a group of proteins have similar structures and functions, amino acid sequence information can be especially rewarding, because the changes and similarities of individual residues can indicate their relative importance to structure and function [as has been shown by species variations in cytochrome c (Dayhoff, 1972)]. Furthermore, where two proteins are closely related, as actinidin and papain appear to be, a detailed analysis of any differences in conformation accompanying changes in specific amino acid residues, provides invaluable data for attempts to predict the three dimensional structure of proteins from amino acid sequence information alone.

Determination of the amino acid sequence and the three dimensional structure of actinidin were therefore undertaken, in parallel, to determine the degree of homology between actinidin and papain, and to give a better understanding of their physical and chemical properties.

1.2. Determination of a protein primary sequence:

Protein sequence determination has been almost entirely an achievement of the years since 1945. The first, and because of its pioneering character, the most important achievement in this field, was the determination of the complete sequence of amino acids in the A and B chains of insulin by Sanger (Sanger, 1945; Sanger and Tuppy, 1951a, 1951b; Sanger and Thompson, 1953a, 1953b). Until this time, only a few simple peptides had been clearly identified from proteins (Synge, 1943).

The great problem in peptide chemistry had always been to find methods of fractionating complex peptide mixtures produced by the partial degradation of a protein. In 1941 Martin and Synge developed partition chromatography, which, when used in the form of paper chromatography (Consden et al, 1944), enabled the separation of complex peptide mixtures.

To determine the primary sequence of insulin, Sanger used both partial acid hydrolysis and enzymic hydrolysis to give a number of shorter peptide chains which were separated by chromatography or by migration in an electric field. The amino acid composition of each purified peptide was determined using acid hydrolysis. The amino terminal residues were identified by reaction with 1-fluoro-2,4-dinitrobenzene (FDNB) (Sanger, 1945; Porter and Sanger, 1948) and the dinitrophenyl amino acids produced were analysed by chromatography. Enzymic hydrolysis was developed further by investigators working on the structure of ribonuclease (Hirs et al, 1956a, 1956b; Redfield and Anfinsen, 1956), which had the advantage of giving a more specific splitting of the protein chain than did partial acid hydrolysis.

These earlier methods were suitable for small proteins such as insulin, but for larger proteins, there was a requirement for techniques to enable the specific breakdown of these proteins into large peptides and for fractionation of such peptides. It was not until 1949 that Moore and Stein described the determination of the amino acid composition of a protein hydrolysate by column chromatography. The original starch column used was superceded by sulphonated polystyrene resins with aqueous citrate buffer systems in 1951 (Moore and Stein). The automatic amino acid analyser was introduced in 1956-58 (Spackman et al, 1958), and has been a key instrument in the determination of primary structure of proteins and peptides. Improvements in resolving power and analysis speed have come with modifications to resins, buffers, temperature and columns (Hamilton, 1963, 1966).

Methods for determining the amino acid with a free α-amino group were initially developed to characterise small peptide fragments from partial acid hydrolyses, or from naturally occurring peptides. The proliferation of procedures for amino terminal determination is demonstrated by a recent review listing 42 methods (Rosmus and Deyl, 1971).

Modern aspects of this work began with the development of 1-fluoro-2,4-dinitrobenzene (FDNB) (Sanger, 1945), which unlike the 1-chloro derivative developed by Abderhalden and Blumberg (1910), reacts under mild conditions. FDNB reacts with α-amino and other reactive groups of amino acids as was shown with insulin (Sanger and Tuppy, 1951a, 1951b). In the years following, procedures were developed for the separation of the dinitrophenyl amino acid derivatives by two dimensional chromatography (Narita et al, 1975).

Hartley and Massey's (1956) research on the reaction of the dye 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride or DNS-Cl) (Weber, 1952) with α-chymotrypsin, resulted in the development of its use as a means of studying α-amino and other reactive groups of proteins and peptides. The main advantages of the dansyl chloride method over Sanger's FDNB procedure were the increased stability of the dansyl derivatives to that of the dinitrophenyl derivatives, and the 100-fold increase in sensitivity, making the dansyl chloride method readily applicable to less than one nanomole of sample (Gray, 1967a, 1967b). Subsequently, a suitable two dimensional chromatographic system on polyamide layers was developed for separation of the dansyl-amino acids (Woods and Wang, 1967).

In 1930, Abderhalden and Brockman suggested the use of phenylisocyanate as a possible method for the sequential removal of amino acids from proteins. It was not until 1950, however, that Edman described a method for determining amino acid sequences by stepwise degradation of a polypeptide chain from the amino terminal using phenylisothiocyanate (PITC). This method, in its modified forms, still provides the basis for the current systems used for amino terminal sequence analysis of peptides and proteins. Application of the Edman degradation (Edman, 1956) to small peptides required the extraction of the phenylthiocarbamyl peptide from the reaction by-products. In some cases, this also resulted in the extraction and consequent loss of hydrophobic peptides. In addition, the chromatographic separation of amino acid phenylthiohydantoins was always less satisfactory to those of other derivatives such as dansyl or dinitrophenyl amino acids. The difficulties of detecting phenylthiohydantoin derivatives from the Edman degradation led Gray (1967b) to develop a combined method known as the dansyl-Edman procedure for sequencing peptides. This was the method chosen for the sequence work of actinidin. Most new methods of amino terminal analysis have aimed either at quantitating the dansyl reaction, or replacing dansyl chloride with a new fluorescent reagent. A more sensitive amino terminal reagent, fluorescamine, which reacts with primary amines, was reported recently for the determination of peptides on paper (Udenfriend et al, 1972; Mendez and Lai, 1975).

In contrast to amino terminal determination, there are no satisfactory chemical methods available to obtain the carboxyl terminal sequence of a protein or peptide. As a result, enzymic procedures are widely used since they are generally the simplest and most convenient. Carboxypeptidase A was found to hydrolyse most carboxyl terminal amino acids at varying rates from proteins, except for carboxyl terminal proline and arginine (Ambler, 1972a, 1972b). This problem was partially overcome by using carboxypeptidase B (Folk and Gladner, 1958), which rapidly releases carboxyl terminal lysine and arginine from proteins, but not proline. Recently, a carboxypeptidase C was isolated (Zuber, 1964, 1968) which releases carboxyl terminal proline, and a carboxypeptidase Y (Hayashi et al, 1973) which releases all carboxyl amino acids from proteins.

Amino terminal exopeptidases are also available. Smith and Hill (1960) reported a leucine aminopeptidase that sequentially removed amino acids, at varying rates, from unblocked amino termini of peptides and proteins, except for peptide bonds on the amino terminal side of proline. Another enzyme, leucine aminopeptidase M is more stable and has a similar specificity (Wachsmuth et al, 1966).

The present procedures of primary sequence determination, whether they be manual or automated, require to some extent fragmentation of the polypeptide chain. A need for methods to cleave a polypeptide chain at specific sites has become a necessity with the advent of structural studies on larger protein molecules. A variety of nonenzymic methods involving peptide bond cleavage have been reviewed (Witkop, 1961), and of these, the most specific and generally applicable method available is the cyanogen bromide reaction (Gross and Witkop, 1961). This method has been popular because of the specificity for methionine residues (which are usually rare in proteins) enabling the production of large fragments. Oxidation of methionine to methionine sulphoxide prevents the cyanogen bromide reaction, but a report showed that oxidised methionine residues could be reduced to methionine with β-mercaptoethanol (Naider and Bohak, 1972). The cyanogen bromide reaction was used on actinidin to provide information on the carboxyl terminal region, information on overlaps of the tryptic peptides, and confirmation of sequence.

The studies of Bergmann et al (1939) and Bergmann and Fruton (1941), which revealed the high specificity of the proteolytic enzyme trypsin, opened the way for the application of proteolytic enzymes in the specific cleavage of polypeptide chains for the determination of protein structure. Trypsin is the most specific of the proteolytic enzymes generally employed, as the enzyme hydrolyses proteins and peptides only on the carboxyl side of lysine and arginine. The sites of cleavage can be restricted, with blocking of the $\underline{\epsilon}$-amino groups of lysine by carbamylation or acetylation. Reversible blocking methods such as trifluoroacetylation or maleylation are also used (Kasper, 1975a). Sites of cleavage by trypsin have been extended by aminoethylation of the cysteine residues with ethyleneimine (Raftery and Cole, 1966). Preparations of trypsin are usually contaminated with chymotrypsin which can be removed by reaction with L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) (Schoellman and Shaw, 1963), or diphenyl carbamyl chloride (DCC) (Erlanger and Cohen, 1963).

Most other enzymes commonly used have broader specificities, and so are useful for further degradation of large peptides, or for the determination of overlapping sequence. The large tryptic peptides obtained from the digestion of actinidin were further degraded with chymotrypsin, thermolysin and pepsin. Staphylococcus aureus V8 protease, a microbial enzyme recently described as specifically cleaving at the carboxyl side of glutamic acid (Houmard and Drapeau, 1972), was also found to be useful for further digestion of tryptic peptides from actinidin.

In general, native proteins are not good substrates for extensive proteolytic degradation because of the globular or fibrous nature of the protein. Methods for unfolding the polypeptide chain have included heating, trichloroacetic acid precipitation, reduction and S-carboxymethylation, and performic acid oxidation. Disulphide reduction in 8M to 10M-urea or 5M to 6M-guanidine hydrochloride in the presence of $\underline{\beta}$-mercaptoethanol, followed by S-carboxymethylation of the liberated reduced thiol groups with iodoacetic acid or iodoacetamide has been employed (Sela et al, 1959). With actinidin, reduction in 6M-guanidine hydrochloride in the presence of dithiothreitol (Cleland, 1964) was used instead of $\underline{\beta}$-mercaptoethanol because of the large equilibium constant enabling the use of low concentrations of dithiothreitol for reduction. The reduced actinidin was then S-carboxymethylated with iodo[^{14}C]acetic acid (Anfinsen and Haber, 1961). The determination of the active site thiol group in actinidin was carried out by enzymic digestion of S-carboxymethylated actinidin that was radioactively labelled only at the active site cysteine and isolation of the radioactive peptide.

Several techniques have been necessary for the separation of peptide mixtures resulting from either chemical or enzymic degradation. Tryptic peptide mixtures from the digestion of actinidin were initially separated on columns of Sephadex gel, and on a diethylaminoethyl (DEAE)-cellulose column. The Sephadex gels were also used to fractionate other peptide mixtures. Further purification of peptides from actinidin was obtained using high voltage electrophoresis on paper, and paper chromatography. The ninhydrin-cadmium reagent and the chlorine tolidine method were used to detect peptides on paper. Specific stains were used to detect arginine, tryptophan and tyrosine/ histidine in peptides.

To determine the primary sequence of actinidin, S-carboxymethylated actinidin was digested with trypsin and the tryptic peptides were purified, characterised and sequenced using a combination of the procedures detailed in Chapter II.

Further digestion of the large tryptic peptides with either chymotrypsin, thermolysin, pepsin, or S. aureus V8 protease provided smaller fragments, that after purification, could be sequenced and then aligned to reconstruct each tryptic peptide. S. aureus V8 protease was particularly useful for the determination of amide residues in the large tryptic peptides because of the enzyme specificity for the carboxyl side of glutamic acid.

The results obtained for the sequence of each tryptic peptide of actinidin, tabulated in Chapter III, are discussed in Chapter IV. The tryptic peptides are aligned to reconstruct the primary sequence of actinidin using information from cyanogen bromide fragments, information from tryptic peptides obtained from maleylated S-carboxymethyl actinidin,and information from the

X-ray crystallographic structure of actinidin determined by Dr E.N. Baker.

Finally, the primary sequence of actinidin is compared with the sequence of papain and fragments of the ficin and stem bromelain sequences that have so far been determined, and the degree of sequence homology between these plant thiol proteolytic enzymes is discussed.

CHAPTER II

MATERIALS AND METHODS

MATERIALS:

General laboratory chemicals were supplied by British Drug Houses Ltd., England and by May and Baker, England. Supplies of other chemicals and materials were obtained as follows:

Bovine serum albumin - Sigma Chemical Company, U.S.A..
N-*α*-carbobenzoxy-L-lysine-*p*-nitrophenyl ester - Vega-Fox Biochemicals, U.S.A..
Chinese gooseberries - Fruit and vegetable market, Palmerston North.
Chromatography No.1 and 3MM paper - Whatman, England.
Cyanogen bromide - Ajax Chemicals, Australia.
Dialysis membrane - Union Carbide Corporation, U.S.A..
DEAE-cellulose - Whatman, England.
p-dimethylamino benzaldehyde - Fluka, Switzerland.
DNS-amino acids and DNS-Cl - Sigma Chemical Company, U.S.A..
2,5-diphenyloxazole (PPO) - Sigma Chemical Company, U.S.A..
1,4-di[2-(5-phenyloxazolyl)]benzene (POPOP) - Koch-Light Laboratories Ltd., England.
Disodium tetrathionate - prepared from sodium thiosulphate using the method of Liu and Inglis (1972).
Dithiothreitol (DTT) - Sigma Chemical Company, U.S.A..
Filter papers - Whatman, England.
Iodo[$^{14}C_2$]acetic acid - The Radiochemical Centre, Amersham, England.
Maleic anhydride - (B.D.H.), was recrystallized from chloroform and resublimed under vacuum.
β-mercaptoethanol - Fluka, Switzerland.
Nitrogen (oxygen free) and oxygen - Industrial Gases Ltd., New Zealand.
2-nitrophenylsulphenyl chloride - Sigma Chemical Company, U.S.A..
Phenanthren-9,10-quinone - Fluka, Switzerland.

Polyamide sheets - Cheng Chin Trading Co. Ltd., Taiwan.
Sephadex gels - Pharmacia Fine Chemicals, Sweden.
Trifluoroacetic acid (TFA) - Hallocarbon, U.S.A..
X-ray film, X-ray plate liquid fixer and developer - Kodak, Australia.

Proteolytic enzymes:

α-chymotrypsin, 4 x crystallized - British Drug Houses, England.
Pepsin - Sigma Chemical Company, U.S.A..
S. aureus V8 protease - Miles Laboratories Ltd., England.
Thermolysin, B grade - Calbiochem, U.S.A..
TPCK-trypsin, Type XI, 1 x crystallized, and soybean trypsin inhibitor - Sigma Chemical Company, U.S.A..

Purification of solvents:

Glacial acetic acid - redistilled after refluxing over either potassium dichromate or potassium permanganate.
Hydrochloric acid - redistilled in an all glass distillation apparatus. For amino acid analyses, the redistilled HCl was standardised to 6M.
Phenylisothiocyanate (PITC) and *n*-butyl acetate - Koch-Light Laboratories Ltd., England. - purification by the methods of Edman and Begg (1967).
Pyridine - redistilled after refluxing over ninhydrin and then redistilled after refluxing over KOH.

All other solvents used were redistilled before use.

METHODS:

Section 2.1. Protein purification:

Actinidin was extracted from firm ripe chinese gooseberry fruit using a modification of the method of Boland and Hardman (1972). All operations were carried out at 4°C and centrifuging was done in an RC2-B Sorvall centrifuge. Two kg of peeled sliced fruit was blended for 30 s with 2.5 l of extraction medium containing 10 mM disodium tetrathionate and 1 mM EDTA. The enzyme was extracted by stirring for 30 min. The S-sulphenyl thiosulphate derivative of the enzyme was prepared to prevent autodigestion during purification.

The extraction solution containing the reversibly inactivated actinidin was centrifuged at 12,500 x g for 10 min to remove the hard fruit cores and pips from the solution. The enzyme was precipitated from the supernatant by the addition of finely ground ammonium sulphate to 70 % saturation and stirred for 30 min. The precipitate was collected by centrifugation at 8,500 x g for 10 min and resuspended in 300 ml of a solution containing 2 parts disodium tetrathionate extraction medium to 1 part KH_2PO_4 buffer (0.1M, pH 6.0), followed by dialysis against distilled water (20 l) for 16 h to remove the remaining ammonium sulphate. The residual precipitate was removed by centrifugation at 12,500 x g for 15 min.

The solution was clarified by forming a calcium phosphate gel. $CaCl_2$ was added to the supernatant to a concentration of 40 mM, followed by the immediate addition of an aqueous solution of K_2HPO_4 to a concentration of 100 mM. The calcium phosphate precipitated solution was allowed to stand for 30 min and then centrifuged at 12,500 x g for 10 min to produce a clear supernatant.

The clarified supernatant was loaded onto a DE-23 cellulose column contained in a 4.5 x 20 cm glass tube and was washed into the column with KH_2PO_4 buffer (0.1M, pH 6.0), containing 0.025M KCl. Elution of the enzyme from the column was accomplished with a linear gradient consisting of 0.025M-KCl (1 l) and 1.0M KCl (1 l) containing KH_2PO_4 buffer (0.1M, pH 6.0), at a flow rate of 50 ml.h^{-1}.

The fractions containing enzyme of specific activity greater than 30 were pooled, and the enzyme solution concentrated to 2 % by precipitation in 55 % saturated ammonium sulphate. The precipitate was resuspended in KH_2PO_4 buffer (0.1M, pH 6.0). The concentrated enzyme solution was dialysed for 16 h against 20 % saturated ammonium sulphate in KH_2PO_4 buffer (0.1M, pH 6.0), to produce crystals of actinidin. The crystals were centrifuged down from the supernatant at 12,000 x g for 10 min and stored at 0°C. Before use, the crystals were dissolved in KH_2PO_4 buffer (0.1M, pH 6.0), to give a 2 % protein solution and exhaustively dialysed against distilled water.

2.1.1. Enzyme assay:

Actinidin was assayed by measuring the rate of hydrolysis of N-α-carbobenzoxy-L-lysine-p-nitrophenyl ester to give p-nitrophenol which was determined at 348 nm.

The enzyme solution was activated by mixing 50 µl of the enzyme solution with 50 µl 0.1M-dithiothreitol. 50 µl of this solution was added to the reaction mixture which contained 2.55 ml KH_2PO_4 buffer (0.1M, pH 6.0), and 0.1 ml 0.1mM-substrate in a 3 ml quartz cuvette. KH_2PO_4 buffer (0.1M, pH 6.0) was used as the blank.

The rate of hydrolysis at 20°C was followed at 348 nm using a dual beam Beckman Acta III recording spectrophotometer. The specific activity of the purified actinidin (µmol substrate transformed.min^{-1}.mg $protein^{-1}$) was determined using a value for $\epsilon_{p\text{-nitrophenol}} = 5400\ l.mol^{-1}.cm^{-1}$ (Boland and Hardman, 1972) and a value of $E^{1\%}_{1cm} = 21.2\ g^{-1}.cm^{-1}$ for actinidin at 280 nm (McDowall, 1970).

In other cases, protein was assayed by the Lowry method (Lowry et al, 1951), with a Hitachi 101 manual spectrophotometer. Bovine serum albumin was used for the protein standard.

2.1.2. Gel electrophoresis of purified actinidin:

Disc gel electrophoresis of purified actinidin was carried out with an Ortec disc gel electrophoresis apparatus and power pack, using the 15 % gel system of Davis (1964) and Ornstein (1964). The mobility of actinidin was measured relative to the dye marker, bromophenol blue. The gels were stained with 0.25 % coomassie blue in 50 % (v/v) ethanol/ 7 % (v/v) acetic acid, and destaining was carried out in 50 % (v/v) ethanol/ 7 % (v/v) acetic acid.

Section 2.2. Chemical modification reactions:

2.2.1. Reduction and S-carboxymethylation of actinidin:

Reduction:

Actinidin (10 μmol) was unfolded in a solution containing guanidine hydrochloride (6M), EDTA (1mM) and Tris-HCl (0.5M-Tris) at pH 8.0. A five-fold molar excess of dithiothreitol (DTT) (Cleland, 1964), over the total thiol groups was added to the solution, and reduction allowed to proceed at 4°C under a nitrogen atmosphere for 16 h. The reduced protein solution was then dialysed at 4°C against 2 l 6M-guanidine hydrochloride in Tris-HCl buffer (0.5M-Tris, pH 8.0) overnight.

S-carboxymethylation:

A three-fold molar excess of neutralised iodoacetic acid containing 100 μCi iodo[$^{14}C_2$]acetic acid was added to the reduced actinidin solution and allowed to react for 30 min under a nitrogen atmosphere in the dark at 20°C. The excess iodoacetic acid was reacted with 2 ml β-mercaptoethanol and the S-carboxymethylated actinidin solution was exhaustively dialysed against 4 x 5 l distilled water. As the guanidine hydrochloride concentration decreased with dialysis, the S-carboxymethylated protein precipitated, but the material redissolved on exhaustive dialysis over 2 days. The solution was diluted to 0.5 % for tryptic digestion.

2.2.2. Reaction of cyanogen bromide with actinidin:

S-carboxymethylated actinidin (5 µmol) was dissolved in 14 ml 100 % formic acid and diluted to 20 ml with distilled water. The acid conditions were used to denature the protein and ensure only the methionine residues reacted.

A 100-fold molar excess of crystalline cyanogen bromide was added to the reaction mixture and the reaction was carried out in a stoppered vessel for 24 h at 20°C. After diluting the reaction mixture ten-fold, the excess reagent and formic acid were removed by lyophilization. The protein material was redissolved in 8M-urea and the cyanogen bromide fragments were separated on a Sephadex G-25 (10 to 40 µm particle size) column (95 cm long) supported in a Pharmacia K-26 x 100 cm chromatographic tube using 0.2M-ammonium bicarbonate as the eluant.

2.2.3. Reaction of maleic anhydride with actinidin:

(Butler and Hartley, 1972)

A solution of S-carboxymethyl actinidin (10 µmol)(radioactively labelled at the active site with iodo[$^{14}C_2$]acetic acid) at a concentration of 7 mg.ml^{-1} in 6M-guanidine hydrochloride was reacted with 140 µmol of recrystallized and resublimed maleic anhydride (2000-fold molar excess). The maleic anhydride was added in 20 aliquots over a period of 1 h and the pH was maintained at pH 9.0 with additions of 2M-NaOH. When the addition of the maleic anhydride was complete, and there was no further pH change, the maleylated protein solution was dialysed against distilled water (4 x 5 l).

The dialysed maleylated S-carboxymethyl protein was digested with trypsin and the maleyl tryptic peptides were initially separated on a Sephadex G-75 (10 to 40 µm particle size) column (95 cm long) supported in a Pharmacia K-26 x 100 cm chromatographic tube using 0.2M-ammonium bicarbonate as the eluant. Removal of the maleyl groups was accomplished in one of the following two ways:

1. By exposing the maleyl peptides on paper to the vapour of pyridinium acetate buffer (1 % pyridine, 5 % glacial acetic acid, pH 3.5) at 60°C in a dessicator overnight.
2. By incubation of the peptide material in pyridinium acetate buffer overnight at 60°C.

Section 2.3. Enzyme digestion of actinidin:

2.3.1. Trypsin:

S-carboxy[$^{14}C_2$]methyl actinidin was digested with TPCK-trypsin using the titrimetric method (Kasper, 1975b). A radiometer pH-26 pH meter was used to measure the pH of the digestion solution which was maintained at pH 8.0 with 0.05M-NaOH.

A 0.5 % solution of the protein was digested at 37°C under nitrogen. TPCK-trypsin, at an enzyme/substrate ratio of 1:100 (w/w) was added at the start of the digestion, and after 1 h, a further 1:100 (w/w) trypsin was added. The digestion was continued for a further 1 h. The enzyme digestion was stopped by the addition of soybean trypsin inhibitor in an equimolar quantity with respect to trypsin. The digestion solution was lyophilized and redissolved in 5 ml to 10 ml 0.2M-ammonium bicarbonate for column chromatography.

2.3.2. Chymotrypsin, thermolysin and *S. aureus* V8 protease:

Chymotrypsin, thermolysin and *S. aureus* V8 protease were used to further degrade the larger tryptic peptides to produce smaller peptides for sequence determination and for sequence overlaps within the tryptic peptides. All digestions were performed in 1 % ammonium bicarbonate at 37°C with an enzyme/substrate ratio of 1:100 (w/w) for 2 to 3 h. The digestion was stopped by lyophilization and the complex peptide mixture was initially separated by either column chromatography or high voltage paper electrophoresis.

2.3.3. Pepsin:

Pepsin was used to degrade the larger *S. aureus* V8 protease peptides derived from the digestion of the larger tryptic peptides. The peptide was dissolved in 100 % formic acid and diluted to 5 % formic acid with distilled water to give a solution of about pH 2. Pepsin, at an enzyme/substrate ratio of 1:100 (w/w) was added and the digestion carried out at 37°C for 3 h. The formic acid was removed by lyophilization and the material redissolved in 0.02M-ammonium hydroxide for paper electrophoresis.

2.3.4. Carboxypeptidase A:

Carboxypeptidase A was used to confirm the carboxyl terminus of actinidin.

Three samples of actinidin (300 nmol per sample)were treated in the following manner prior to carboxypeptidase A digestion.

Sample 1. Control.

300 nmol actinidin was added to 1 ml pyridinium acetate buffer

(0.1M-pyridine, pH 6.0).

Sample 2. Dithiothreitol activated.

300 nmol actinidin was added to 1 ml pyridinium acetate buffer (0.1M-pyridine, pH 6.0) and 1 µmol dithiothreitol was added

Sample 3. Acid denatured.

300 nmol actinidin in solution was adjusted to pH 1.0 with 0.5M-HCl.

After the three samples had stood for 40 min, a 0.2 ml aliquot was taken from each sample and mixed with 0.2 ml 0.4M N-ethyl morpholine. Carboxypeptidase A solution (50 µl) prepared by the method of Ambler (1972a, 1972b) (adapted from the method of Harris et al, 1955) was added, and the digestion carried out at 37°C for 1 h. The digestions were dried down under high vacuum and redissolved in 50 µl distilled water. 10 µl of this solution was taken for polyacrylamide disc gel electrophoresis using 15 % gels (Davis, 1964; Ornstein, 1964) and the remaining 40 µl were used for amino acid analysis.

Section 2.4. Column chromatography:

The columns used for the purification of peptides were contained in either a K-26 x 100 cm or K-16 x 60 cm Pharmacia jacketed chromatographic tube, pumped in either the ascending or descending direction using an LKB RecyChrom type 4912-A 2-channel peristaltic pump. Eluate fractions were collected from the columns using an LKB 7000 Ultrorac fraction collector and the eluate was monitored at 280 nm using an LKB 8300 Uvicord II monitor.

2.4.1. Sephadex gel filtration:

Sephadex G-25 (10 to 40 µm particle size) was used for peptide fractionation, and Sephadex G-25 (40 to 120 µm particle size) for desalting peptide samples in 0.2M-ammonium bicarbonate using either a K-26 (95 cm long) or K-16 (55 cm long) chromatographic tube. A flow rate of 50 $ml.h^{-1}$ was used for desalting samples, while peptides were fractionated at a flow rate of 15 to 20 $ml.h^{-1}$.

The tryptic peptides from carboxymethyl actinidin were initially fractionated on two Sephadex G-50 (10 to 40 µm particle size) columns (each column 95 cm long) packed in K-26 x 100 cm chromatographic tubes which were linked together with 1 mm (i.d.) Portex tubing. The system was pumped at an ascending flow rate of 20 $ml.h^{-1}$.

The tryptic peptides from maleylated carboxymethyl actinidin were fractionated on a Sephadex G-75 (10 to 40 µm particle size) column (95 cm long) and the system was pumped at an ascending flow rate of 20 $ml.h^{-1}$.

2.4.2. DEAE-cellulose chromatography:

DE-32 cellulose was used to further fractionate the large tryptic peptides derived from carboxymethyl actinidin. The DE-32 column (95 cm long) was contained in a K-26 x 100 cm chromatographic tube and was equilibrated with Tris-HCl buffer (0.05M-Tris, pH 8.0, 0.025M-NaCl) and was pumped at an ascending flow rate of 25 $ml.h^{-1}$ using a continuous flow gradient system to elute the peptides from the column.

The gradient system consisted of Tris-HCl buffer (0.05M-Tris, pH 8.0) containing the following concentrations of NaCl:

Gradient 1.	550 ml 0.025M-NaCl	x	550 ml 0.2M-NaCl.
Gradient 2.	350 ml 0.2M-NaCl	x	350 ml 0.5M-NaCl.

The NaCl gradient was checked by using a Radiometer type CDM2e conductivity meter.

Section 2.5. Peptide purification by paper techniques:

2.5.1. High voltage paper electrophoresis:

Small peptides derived from actinidin were further purified by high voltage electrophoresis on paper using a tank electrophoresis apparatus (Michl, 1951) with Shell Sol-T as the coolant. Three electrophoresis tanks containing pH 6.5, pH 3.5 and pH 2.1 buffer were used: (Tang and Hartley, 1967)

pH 6.5: Pyridine/glacial acetic acid/distilled water, (25:1:225 by vol.) for separating peptides that, at pH 6.5, have a net basic or acidic charge.

pH 3.5: Pyridine/glacial acetic acid/distilled water, (1:10:90 by vol.) useful for further purification of peptides containing carboxymethyl cysteine.

pH 2.1: Formic acid/glacial acetic acid/distilled water, (1:4:45 by vol.) for separating peptides that are neutral at pH 6.5.

The pH 6.5, pH 3.5 and pH 2.1 electrophoretograms were subjected to 50 V.cm^{-1}. Whatman No.1 chromatography paper (46 cm x 57 cm) was used for quantitative runs and peptide mapping, while Whatman 3MM chromatography paper was used for the preparative purification of peptides. To aid the comparison and identification of peptides, two 5 mM marker amino acid solutions in 10 % isopropanol were used:

R marker: Tyr Phe Met Leu Val Arg Lys.
T marker: Asp Glu Thr Ser Ile Ala His Gly.

5 µl of each marker amino acid solution was applied to the chromatogram. DNS-Arg and DNS-OH were used as fluorescent markers for electrophoresis and chromatography. For electrophoresis at pH 6.5, mobilities of peptides were measured relative to aspartic acid, and at pH 3.5, pH 2.1 and chromatography,

relative to DNS-Arg. Guide strips were cut from all electrophoretograms for staining to locate the positions of the peptides.

2.5.2. Paper chromatography:

Descending chromatography on sheets of Whatman No. 1 and 3MM chromatography paper was carried out in Shandon chromatography tanks for two dimensional peptide mapping and for the purification of some peptides that would not separate by electrophoresis. Two solvent systems were used:

BAW: butanol/glacial acetic acid/distilled water, (4:1:5 by vol.) using upper phase. (Katz *et al*, 1959)
BAWP: butanol/pyridine/glacial acetic acid/distilled water, (75:50:15:60 by vol.) (Light and Smith, 1962).

To map the peptides produced by further digestion of large tryptic peptides, 100 nmol of the peptide mixture was separated by pH 6.5 electrophoresis, the neutral band was rerun at pH 2.1, and strips from both electrophoretograms were chromatographed in the BAWP solvent to produce a two dimensional map.

2.5.3. Peptide staining on paper:

All electrophoretograms and chromatograms were checked for fluorescence under a 366 nm wavelength UV light before staining. Guide strips and two dimensional peptide maps were stained with the ninhydrin-cadmium acetate reagent and occasionally , the chlorine-tolidine reagent was used.

2.5.3.1. Ninhydrin-cadmium reagent:

(Atfield and Morris, 1961; Heathcote and Haworth, 1969)

Solution A:

15 g cadmium acetate + 300 ml glacial acetic acid + 600 ml distilled water.

Solution B:

1 % ninhydrin in acetone.

Solutions A and B were mixed in the ratio 3:17 prior to dipping the paper and air drying at 20°C.

The papers were stored in a chromatography tank containing a small vessel of concentrated sulphuric acid to produce an ammonia free atmosphere to reduce background colour development. To prevent ninhydrin spots fading, the papers were dipped in cupric nitrate reagent (Kawerau and Wieland, 1951) which was prepared by mixing 100 ml 95 % ethanol with 0.2 ml 10 % nitric acid and 1 ml saturated cupric nitrate.

2.5.3.2. Chlorine-tolidine reagent:

(Reindel and Hoppe, 1954)

The paper to be stained was washed with acetone/ethanol (1:1 v/v) and blotted dry. The damp paper was exposed to a chlorine atmosphere for 15 min which was prepared by mixing equal volumes of 1M-HCl and 0.05M-potassium permanganate in a sealed perspex container prior to chlorination of the paper. After chlorination, the excess chlorine was removed from the paper in a fume hood. The chlorinated paper was dipped in a solution containing freshly mixed equal volumes of saturated tolidine in 2M-acetic acid and 0.05M-potassium iodide solution. The paper was immediately blotted, revealing the location of peptides as blue spots.

2.5.3.3. Specific amino acid staining on paper:

To obtain extra information from peptide maps and guide strips, specific staining reagents were used.

(a) Ehrlich reagent for tryptophan:

(Smith, 1953)

10 % p-dimethylamino benzaldehyde in concentrated HCl was mixed with acetone (1:9 v/v) just before dipping the paper. The purple colour development at 20°C due to tryptophan was observed from 0 to 30 min after staining. 5 µl of a standard 5 mM tryptophan solution was applied as a control spot before staining.

(b) Arginine reagent:

(Yamada and Itano, 1966)

Equal volumes of 0.02 % phenanthrene-9,10-quinone in absolute ethanol, and a fresh solution of 10 % NaOH in 60 % (v/v) ethanol were mixed prior to dipping the paper. Arginine spots showed up with a green/ blue fluorescence under 366 nm wavelength UV light after air drying at 20°C for 20 min. 5 µl of a standard 5 mM-arginine solution was applied to the paper as a control spot before staining.

(c) Pauly reaction for histidine and tyrosine:

(Frank and Petersen, 1955)

Solution A: 1 % sulphanilic acid in 1M-HCl.
Solution B: 5 % sodium nitrite.
Solution C: 15 % sodium carbonate.

Equal volumes of solutions A and B were mixed and allowed to stand for 10 min at 0°C. Two volumes of solution C were added to the cooled mixture. The paper was immediately dipped and blotted dry. Histidine gives a cherry-red colour and tyrosine a weak rust colour. The spots were marked immediately as they faded rapidly.

2.5.4. Peptide elution from paper:

Following electrophoresis or chromatography, peptides were located using the stained guide strips and were eluted from the paper with 0.02M-ammonium hydroxide. In one case, a peptide required elution from the paper with the BAWP solvent.

Paper strips containing peptides to be eluted were supported vertically between glass slides in a perspex peptide elution apparatus. The peptides were eluted in the descending direction into glass test tubes (6 cm x 1 cm). Normally 2 ml of eluate was collected and lyophilized. The peptide material was redissolved in 250 µl 0.02M-ammonium hydroxide and stored at -10°C until required for analysis.

Section 2.6. Amino acid analysis:

2.6.1. Determination of the amino acid composition of peptide and protein samples:

Peptide samples to be hydrolysed for amino acid analysis (20 to 50 nmol) were dried down in thick walled acid washed pyrex test tubes (10 mm x 75 mm), followed by the addition of 0.2 ml 6M-HCl containing 0.1 % phenol to reduce losses of tyrosine. The contents of the tubes were cooled and the tubes were sealed under vacuum (0.5 to 0.1 mm Hg).

For protein samples, 900 nmol norleucine was added as an internal standard to a stock solution of 300 nmol actinidin. Protein samples for amino acid analysis (15 nmol) were oxidised with performic acid and hydrolysed at 110±1°C in triplicate for 24, 48 and 72 h with 1 ml 6M-HCl containing 0.1 % phenol, and the results were extrapolated back to zero time. Performic acid was made by mixing 1 volume hydrogen peroxide (35 % w/v) with 19 volumes 100 % formic acid and adding 500 µl of this solution to the protein sample (300 nmol). The reaction was carried out for 3 h and the performic acid removed under high vacuum.

2.6.2. Determination of tryptophan:

The number of tryptophan residues in actinidin were determined colorimetrically by reaction with 2-nitrophenylsulphenyl chloride according to the method of Scoffone et al (1968).

S-carboxymethyl actinidin (1.0 µmol) was dissolved in 2.5 ml 100 % formic acid and then diluted to 50 % formic acid with 2.5 ml distilled water to give an approximately 0.5 % solution of actinidin. A ten-fold molar excess of 2-nitrophenylsulphenyl chloride (NpS-Cl) dissolved in 1.0 ml glacial acetic acid was added to the protein solution and allowed to react for 20 min in an ice bath. The NpS-actinidin was precipitated from the reaction mixture by the addition of 150 ml acetone and the precipitate was centrifuged down from the solution and washed twice with acetone. The washed precipitate was dissolved in 0.1M-ammonium hydroxide and dialysed against distilled water (2 x 5 l).

The absorbance of the dialysed NpS-actinidin solution was determined at 365 nm and the amount of the NpS moiety bound to the protein was determined using a value of $\varepsilon_{NpS} = 4000\ l.mol^{-1}.cm^{-1}$. The protein content in samples of the dialysed NpS-actinidin was determined by amino acid analysis.

Section 2.7. Determination of radioactivity:

^{14}C radioactivity was determined either by liquid scintillation spectrometry or by radioautography.

2.7.1. Liquid scintillation spectrometry:

Radioactive peptides in eluate fractions from peptide separation by column chromatography were determined by transferring 10 µl of solution from each fraction tube to glass screw capped scintillation vials containing 5 ml Triton X-100/ toluene scintillation solvent. The vials were counted in a model 3375 Packard Tri-Carb liquid scintillation spectrometer and the counts corrected for quenching.

Scintillation solvent:

4 g 2,5-diphenyloxazole (PPO) and 100 mg (1,4-di[2-(5-phenyloxazolyl)] benzene (POPOP) were dissolved in 667 ml toluene, followed by the addition of 333 ml Triton X-100.

2.7.2. Radioautography:

Preparative paper electrophoretograms, chromatograms and peptide maps containing radioactive peptides were exposed to Kodak X-ray plates (35 cm x 43 cm). The X-ray plates were developed manually using standard Kodak X-ray plate developer and fixer.

Section 2.8. Peptide sequencing:

2.8.1. Dansyl N-terminal procedure for peptides:

(Gray, 1967a)

5 to 10 nmol (10 µl) of peptide was dried down in a 5 mm x 30 mm tapered glass tube. 10 µl 0.2M-sodium bicarbonate and 10 µl 1-dimethylaminonaphthalene-5-sulphonyl chloride (DNS-Cl) (2.5 $mg.ml^{-1}$ in acetone) were added to the tube to give 5 mM DNS-Cl, 1 mM peptide and 50 % acetone at about pH 9.8. The solution in the tube was mixed, the tube sealed with parafilm, and incubated for 30 min at 45°C. The reaction mixture was evaporated and the peptide was hydrolysed under vacuum in 6M-HCl for 16 h at 110°C. The tube was opened and the contents evaporated over NaOH pellets. The DNS-amino acid was dissolved in 95 %

ethanol (5 µl) and equal amounts were spotted on each side of a polyamide sheet (7.5 cm x 7.5 cm) (Woods and Wang, 1967) for two dimensional chromatography. 1 µl of a DNS-amino acid marker solution (containing 0.1 mg.ml^{-1} DNS-Gly, DNS-Glu, DNS-Ile, DNS-Phe, DNS-Pro, DNS-Ser and DNS-Arg) was applied to one side of the plate.

Chromatography solvents:

1. 1.5 % (v/v) formic acid in distilled water.
2. Toluene/glacial acetic acid (9:1 v/v).
3. Ethyl acetate/methanol/glacial acetic acid (20:1:1 by vol.).
4. (glacial acetic acid 40: pyridine 22.5: water 2500, pH 4.4)/ ethanol, mixed in a ratio of 75:25 (by vol.).
5. 0.05M-Na_3PO_4/ethanol (3:1 v/v) (pH about 11), no separation of arginine and lysine at pH above 12.

The plates were run in solvent 1 in one dimension for 12 min, dried 15 min, then run in solvent 2 in the second dimension for 15 min, dried and examined under 366 nm wavelength UV light. Solvent 3 was then run in the same dimension as solvent 2 for 10 min, dried and examined under UV light (giving separation of DNS-Thr/Ser, DNS-Glu/Asp and DNS-CMCys from DNS-OH). Solvent 4 was run in the same dimension as solvent 2 for seven min to separate DNS-His from DNS-Lys and DNS-Arg, and solvent 5 was run for 45 min to separate DNS-Lys and DNS-Arg.

2.8.2. Dansyl-Edman procedure for peptide sequencing:

(Gray, 1967b; Hartley, 1970)

The peptide sample (100 to 500 nmol) in 100 to 200 µl distilled water was transferred to a screw capped pyrex test tube (1 cm x 4.5 cm) and dried down under vacuum ready for Edman degradation. 150 µl distilled water and 150 µl 5 % (v/v) phenylisothiocyanate (PITC) in pyridine were added and the tube was gassed with oxygen-free nitrogen before being sealed with a screw cap lined with aluminium foil. The tube was incubated at

45°C for 1.5 h. The tube contents were then dried over P_2O_5 and NaOH pellets under high vacuum at 60°C for 30 min. 200 µl trifluoroacetic acid (TFA) was added and the tube was gassed with oxygen-free nitrogen before being sealed. The tube was incubated at 45°C for 30 min to cleave the N-terminal phenylthiocarbamyl amino acid from the peptide. The TFA was removed under vacuum over NaOH pellets. To remove the by-products, the material in the tube was suspended in 200 µl distilled water and was extracted with 3 x 1 ml n-butyl acetate. The top n-butyl acetate layer was discarded and the peptide in the aqueous phase dried down. The peptide sample, now one residue shorter, was dissolved in 150 µl distilled water, from which a 5 or 10 µl sample was taken for N-terminal analysis by dansylation. The remaining material was subjected to another cycle of the Edman degradation.

2.8.3. Rapid Edman degradation: (Gray, 1972a)

This procedure was used to obtain the sequence of the first three to five residues from the N-terminal end of the larger tryptic peptides. 10 nmol (10 µl) of the peptide to be analysed was placed in each of four tapered glass test tubes (6 mm x 40 mm) and the contents dried down. 25 µl distilled water and 25 µl 5 % (v/v) PITC in pyridine were added to each tube and the tubes were gassed with oxygen-free nitrogen before being sealed with a rubber cap lined with aluminium foil. The tubes were incubated for 1 h at 45°C and the tube contents were then dried over NaOH pellets and P_2O_5 under high vacuum at 60°C for 30 min. 50 µl TFA was added to each tube and the tubes were gassed with oxygen-free nitrogen before being sealed. The tubes were incubated at 45°C for 30 min to cleave the N-terminal phenylthiocarbamyl amino acid from the peptide. The TFA was removed under vacuum over NaOH pellets.

One of the tubes was set aside and the Edman degradation was repeated on the other three tubes which was followed by removal of another tube and the Edman reaction repeated on the remaining two tubes until the peptide samples in each of the tubes were each degraded a different number of residues. The peptide samples in the tubes were dissolved in distilled water (25 µl) and extracted with n-butyl acetate (3 x 25 µl). The contents of each tube were dansylated and the N-terminals analysed by two dimensional chromatography to give the N-terminal sequence of amino acids in the peptide.

2.8.4. Determination of amide groups:

The Offord mobility procedure for peptides was used to determine amide groups and for comparison of the relative mobility (from electrophoresis) and molecular weight of a peptide with the net charge on the peptide (Offord, 1966; Gray, 1972b). The mobility of a peptide can be described by the equation

$$\log_{(\mathrm{mobility})} = 1.44 + \log(-e) - 0.6\log_{(\mathrm{MW})}$$

where e is the net ionic charge at a given pH (e.g. pH 6.5 or pH 2.1). From a graph of $\log_{(\mathrm{mobility})}$ Vs. $\log_{(\mathrm{MW})}$, the charge on a peptide can be determined.

By removing a sample of the peptide after each Edman degradation cycle for determination of the mobility of the peptide by high voltage electrophoresis, it is possible to determine whether or not a charged amino acid has been lost from the peptide.

Section 2.9. Determination of the active site cysteine residue in actinidin:

Actinidin (10 µmol) in 20 ml Tris-HCl buffer (0.1M-Tris, pH 8.0) was reduced with a five-fold molar excess of DTT for 4 h at 4°C. The reduced protein (reduced only at the active site cysteine residue) solution was dialysed for 4 h against 2 l Tris-HCl buffer (0.1M-Tris, pH 8.0) to remove excess DTT. Sufficient Tris-HCl buffer (3M-Tris, pH 8.0) was added to make the buffer concentration of the protein solution to 0.5M.

A three-fold molar excess of neutralised iodoacetic acid containing 20 µCi iodo[$^{14}C_2$]acetic acid was added to the reaction mixture. The iodoacetic acid was reacted with the reduced actinidin for 30 min at 20°C in the dark under a nitrogen atmosphere. The reaction was stopped by the addition of 1 ml β-mercaptoethanol, and the S-carboxymethylated protein was exhaustively dialysed against distilled water. This active site cysteine S-carboxy[$^{14}C_2$]methylated actinidin was unfolded in a solution containing guanidine hydrochloride (6M), EDTA (1 mM) and Tris-HCl buffer (0.5M-Tris) at pH 8.0. A five-fold molar excess of DTT was added to the solution and reduction allowed to proceed at 20°C under a nitrogen atmosphere for 4 h. The reduced protein solution was then dialysed at 20°C against 2 l 6M-guanidine hydrochloride in Tris-HCl buffer (0.5M-Tris, pH 8.0) for 4 h. A three-fold molar excess of neutralised iodoacetic acid was added to the reduced actinidin solution and was allowed to react for 30 min under nitrogen in the dark at 20°C. The excess iodoacetic acid was reacted with 2 ml β-mercaptoethanol and the protein solution was exhaustively dialysed against 4 x 5 l distilled water.

The protein was digested with chymotrypsin at an enzyme/substrate ratio of 1:100 (w/w) in 0.2M-ammonium bicarbonate for 3 h. The complex peptide mixture from the enzymic digestion was separated on a column (95 cm long) of Sephadex G-25 (10 to 40 µm particle size), supported in a K-26 x 100 cm Pharmacia chromatographic tube. The radioactive peptide fraction was pooled and further purified by high voltage electrophoresis.

CHAPTER III

RESULTS

Section 3.1. Purification of actinidin:

The absorbance profile at 280 nm for the elution of actinidin from the DE-23 cellulose column using KH_2PO_4 buffer (0.1M, pH 6.0) with a 0.025M to 1.0M-KCl gradient is given in Fig. 3.1..
The course of the purification of actinidin, starting with 2 kg peeled chinese gooseberries,is shown in Table 3.1..

Table 3.1. Purification of actinidin.

Procedure	Total protein (mg)	Total activity (E.U.)	Specific activity
Centrifuged extract.	29,000	66,700	2.3
Ammonium sulphate resuspended ppt. dialysed & centrifuged.	3,400	54,740	16.1
Calcium phosphate gel supernatant.	3,200	52,800	16.5
Pooled DE-23 eluate.	600	45,000	75.0
Ammonium sulphate crystallized enzyme.	460	36,800	80.0

The specific activity of alternate fractions collected from the DE-23 cellulose column was determined and is plotted in Fig. 3.1..

Polyacrylamide gel electrophoresis of crystallized actinidin gave a single band with a mobility of 0.79 relative to bromophenol blue. Dansylation of crystallized actinidin gave a single N-terminal leucine.

Fig. 3.1. DEAE-cellulose chromatography of actinidin.

The resuspended dialysed solution containing actinidin that had been precipitated from the extraction medium with 70 % saturated ammonium sulphate was loaded onto a DE-23 cellulose column (4.5 cm x 20 cm) that was equilibrated with KH_2PO_4 buffer (0.1M, pH 6.0) containing 0.025M-KCl. The protein was washed into the column with KH_2PO_4 buffer (0.1M, pH 6.0, 0.025M-KCl) and then eluted from the column with a linear gradient consisting of 0.025M-KCl (1 l) and 1.0M-KCl (1 l). The protein was monitored at 280 nm and the enzyme activity was determined from the rate of hydrolysis of N-α-CBZ-lys-p-NP at pH 6.0 and 20°C. The highest specific activity (μmol substrate transformed. min^{-1}.mg $protein^{-1}$) was found in the later portion of the protein peak. The fractions containing protein with a specific activity greater than 30 were pooled.

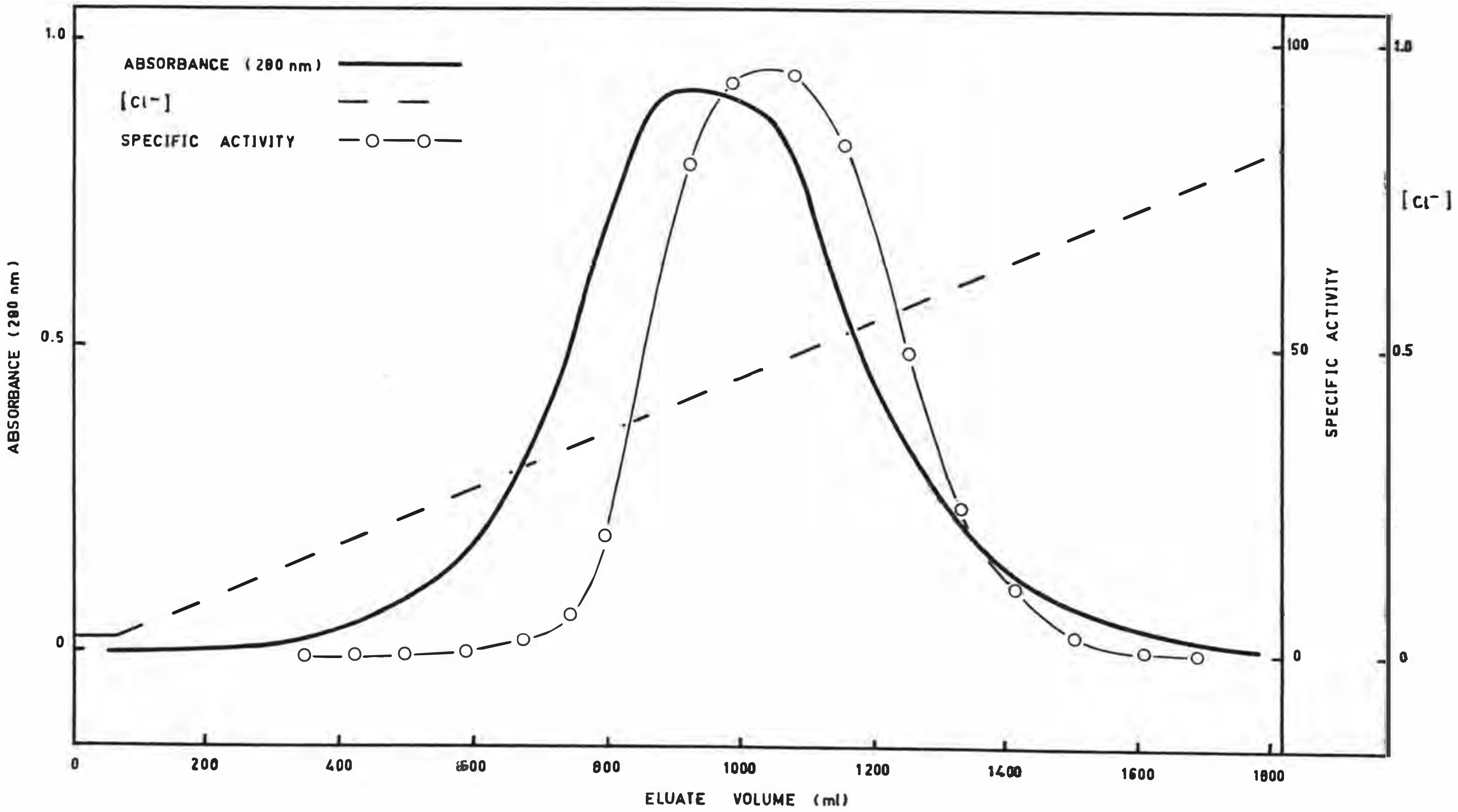


FIG. 3.1. DEAE-CELLULOSE CHROMATOGRAPHY OF ACTINIDIN.

Section 3.2. Amino acid composition of actinidin:

Samples of actinidin (0.5 mg) were hydrolysed for 24, 48 and 72 h. Each hydrolysate was analysed in triplicate on an automatic amino acid analyser.

The quantities of amino acids from each hydrolysate were standardised using norleucine as an internal standard. The residue number of each amino acid was calculated based on a molecular weight for actinidin of 23,500 (an approximate molecular weight obtained from the sequence data).

To determine this composition, the values for the amino acids threonine and serine were obtained by extrapolating the results to zero time, while the values for alanine, valine, leucine and isoleucine were obtained from the maximum readings after 72 h hydrolysis. The content of the other amino acids was determined from the average of the hydrolysis values.

Cysteine and cystine were determined together as cysteic acid because carboxymethyl cysteine did not always resolve completely from aspartic acid on the amino acid analyser under the conditions used. Tryptophan was determined colorimetrically by the method of Scoffone et al (1968), from an average of three estimations.

The amino acid analysis data for actinidin are given in Table 3.2..

Table 3.2. Amino acid composition of actinidin[g]

Amino acid	Hydrolysis time[a]			Average residue No.
residue	24 h	48 h	72h	per MW 23,500[d]
His	1.4	1.5	1.3	1.4
Lys	6.4	6.2	6.5	6.3
Arg	5.0	5.1	5.0	5.1
Cys[e]	6.7	6.4	6.5	6.6
Asp	29.1	29.4	28.5	28.7
Thr	17.4	16.9	16.5	17.8[b]
Ser	11.6	11.0	10.2	12.4[b]
Glu	20.3	19.8	20.6	20.4
Pro	6.8	7.0	6.5	6.9
Gly	27.5	27.4	28.1	27.6
Ala	17.5	17.4	17.7	17.7[c]
Val	16.1	16.5	16.7	16.7[c]
Met	2.1	2.1	1.9	2.1
Ile	16.6	16.5	16.9	16.9[c]
Leu	7.4	7.3	7.7	7.7[c]
Tyr	14.4	14.5	14.0	14.3
Phe	5.6	4.9	5.2	5.3
Trp	-	-	-	5.8[f]

[a] The values for the 24, 48 and 72 h hydrolysates were each an average of three amino acid analyses.

[b] Results extrapolated to zero time.

[c] Result taken as value at 72 h.

[d] Approximate molecular weight for actinidin based on sequence data.

[e] Cysteine and cystine determined together as CySO$_3$H.

[f] Tryptophan was determined colorimetrically by the method of Scoffone et al (1968), from an average of three estimations.

[g] A statistical analysis of the data was not carried out as the data were confirmed by sequence information.

Section 3.3. Purification of the tryptic peptides of actinidin:

A tryptic digest of S-carboxy[$^{14}C_2$]methyl actinidin (10 μmol)[a] was subjected to gel chromatography in two separate 5 μmol fractions on two Pharmacia K-26 columns of Sephadex G-50 linked together in series (Fig. 3.2.).

An electrophoresis scan at pH 6.5 of aliquots from each fraction tube from the Sephadex G-50 column showed that peaks V, VI and VII contained electrophoretically mobile peptides. These three peaks were pooled separately, lyophilized, and the peptides were purified by paper electrophoresis. Fraction V was later shown to contain peptide T_2, fraction VI to contain peptides T_5 and T_9, and fraction VII to contain peptides T_1, T_{10} and T_{12} (Fig. 3.3.) for which the sequences were determined. T represents tryptic peptide and the number refers to the order of alignment in the primary sequence of actinidin.

Fractions I, II, III and IV were pooled separately and further purified on DEAE-cellulose. Fraction I was later shown to contain peptide T_6, fraction II to contain peptide T_7, fraction III to contain peptide T_8, and fraction IV to contain peptides T_3 and T_{11}. The composite elution profile for these peptides from DEAE-cellulose is given in Fig. 3.4.. Peptide T_4 could not be located in either the Sephadex G-50 or the DEAE-cellulose column fractions and the way in which this peptide was obtained is detailed in Section 3.4.4..

The purified peptides from fractions I, II, III and IV were digested with other enzymes to produce smaller peptides which were purified by paper electrophoresis and chromatography. These small peptides were sequenced and aligned to enable the reconstruction of each tryptic peptide.

[a] 10 μmol actinidin of specific activity 8.4×10^6 dpm/μmol. (1.2×10^6 dpm/μmol of cysteine)

Fig. 3.2. Sephadex G-50 chromatography of the tryptic peptides of carboxymethyl actinidin.

A tryptic digest of S-carboxy[$^{14}C_2$]methyl actinidin (10 µmol) was subjected to gel filtration on two Sephadex G-50 (10 to 40 µm particle size) columns connected in series (each 95 cm long) contained in Pharmacia K-26 x 100 cm chromatographic tubes. The digest was chromatographed as two equal samples and the material separated into seven fractions (I to VII). The peptide material was eluted from the column with 0.2M-ammonium bicarbonate at an ascending flow rate of 20 ml.h^{-1}. The ^{14}C radioactivity was determined in each fraction and expressed as dpm. Fractions I, II, III and IV were pooled separately and rechromatographed on DEAE-cellulose (Fig. 3.4.). Fractions V, VI and VII contained peptides that could be purified by paper techniques (Fig. 3.3.).

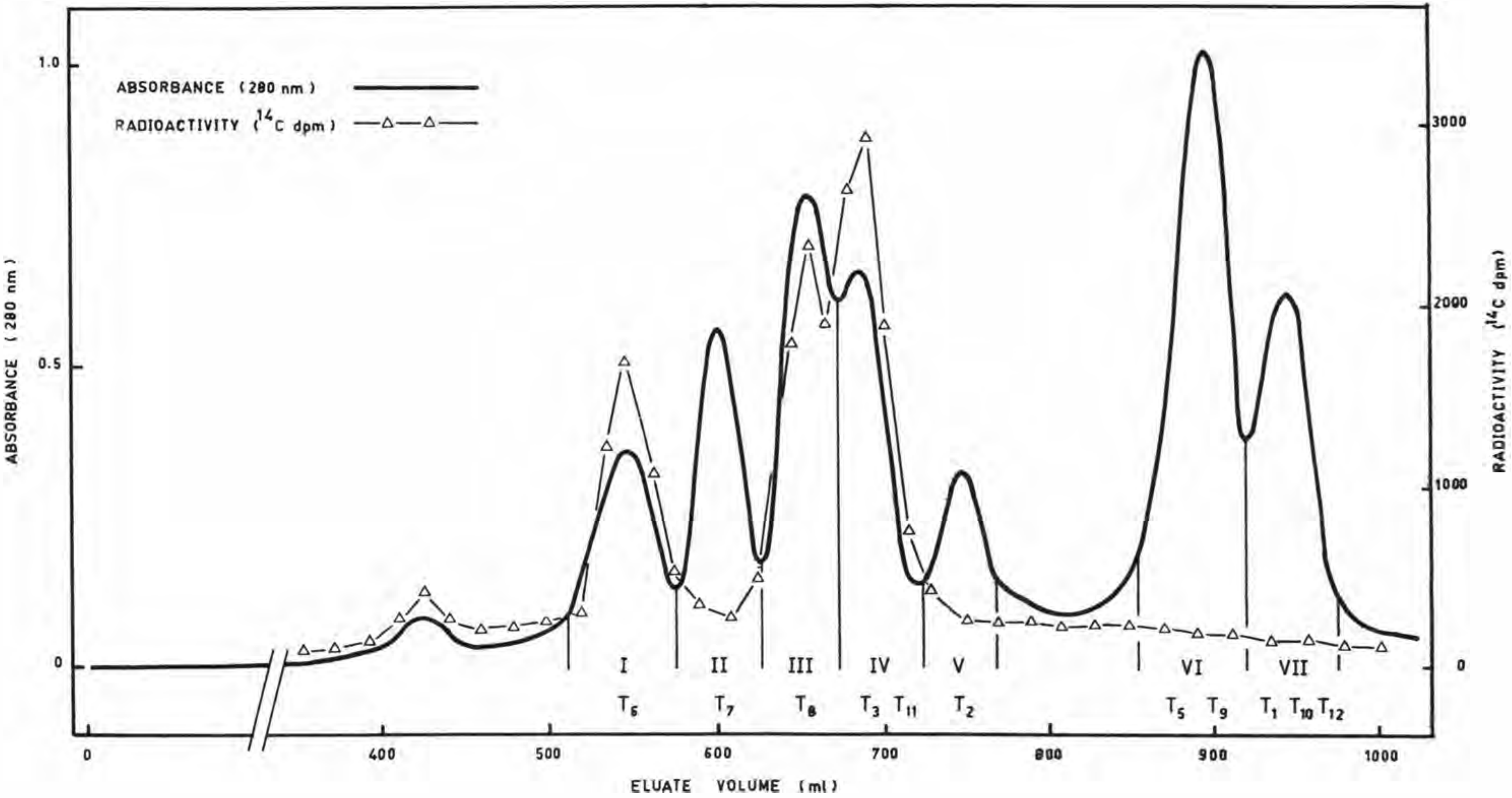
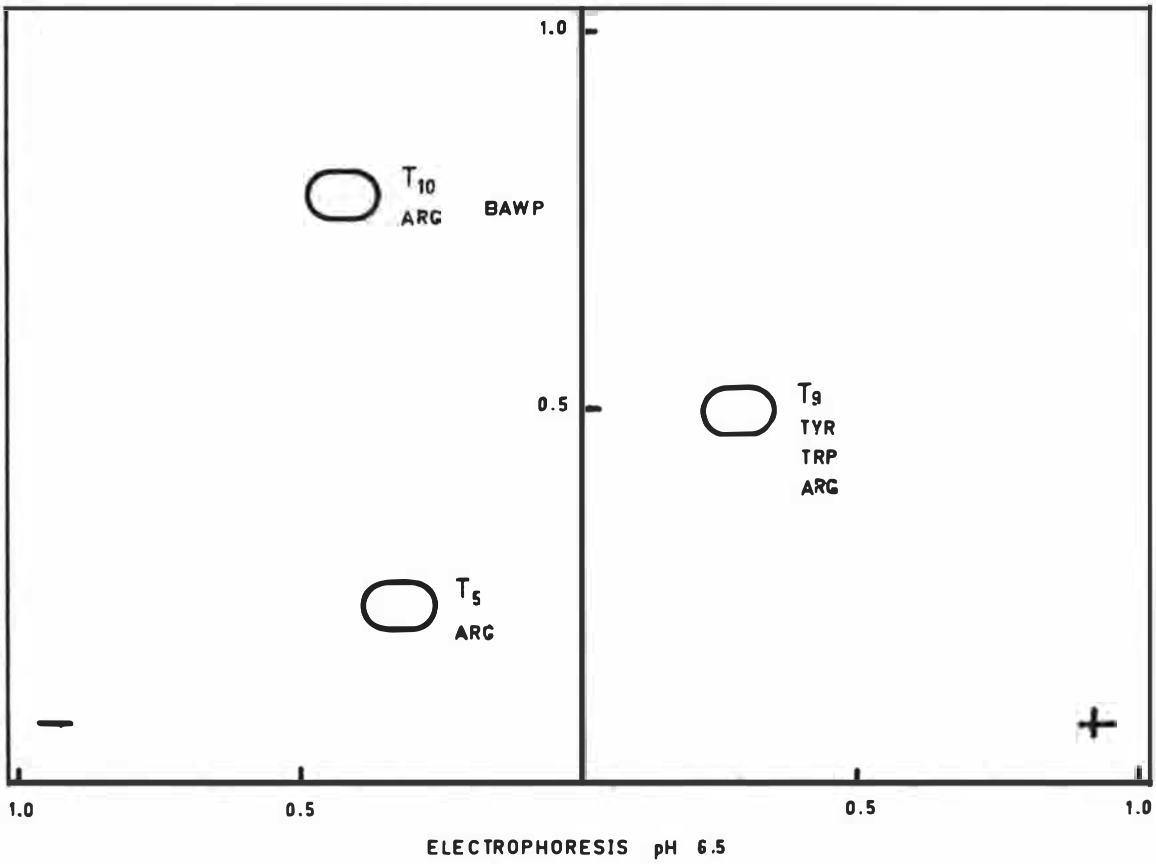


FIG. 3.2. SEPHADEX G-50 CHROMATOGRAPHY OF THE TRYPTIC PEPTIDES OF CARBOXYMETHYL ACTINIDIN.

Fig. 3.3. Composite peptide maps of the electrophoretically mobile tryptic peptides of carboxymethyl actinidin.

The tryptic peptides in fractions V, VI and VII from the Sephadex G-50 elution profile (Fig. 3.2.) were sufficiently small to enable purification by paper techniques. Fraction V was shown to contain peptide T_2, fraction VI to contain peptides T_5 and T_9, and fraction VII to contain peptides T_1, T_{10} and T_{12}, which are shown in the peptide maps. Peptide mobility for electrophoresis at pH 6.5 was measured relative to aspartic acid. Peptide mobility for electrophoresis at pH 2.1, and for chromatography in the BAWP solvent was measured relative to DNS-Arg. Separate peptide maps were stained for tyrosine, tryptophan and arginine. Radioautographs of the peptide maps were prepared to check for radioactive peptides.
For electrophoresis, (+) indicates anode and (-) indicates cathode.



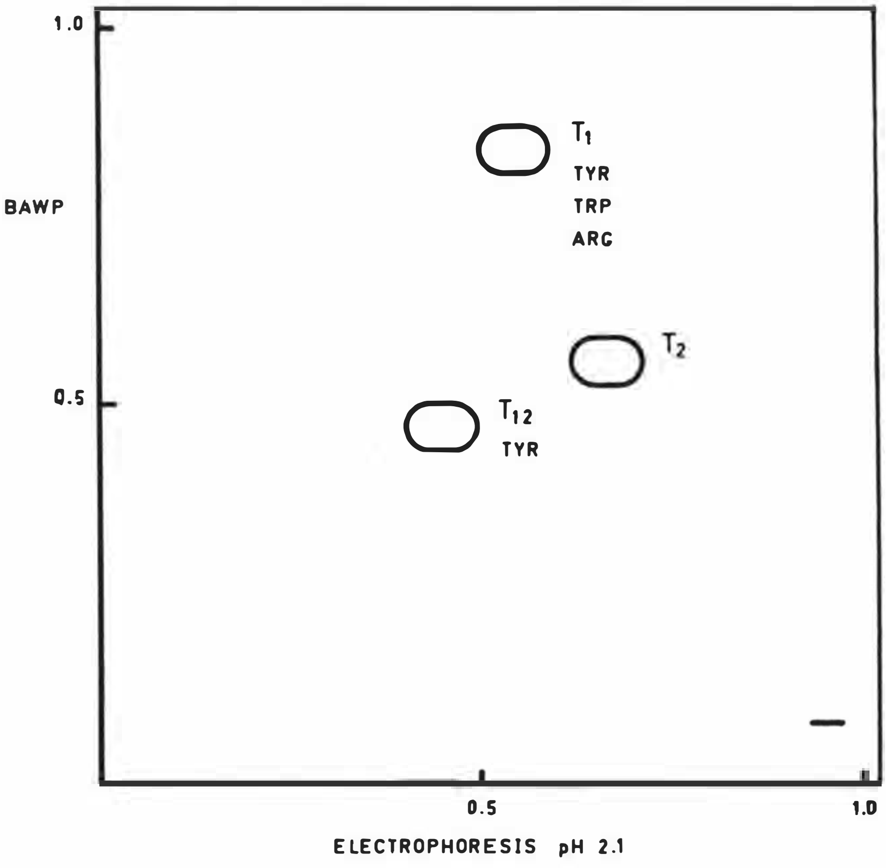


FIG. 3.3. COMPOSITE PEPTIDE MAPS OF THE ELECTROPHORETICALLY MOBILE TRYPTIC PEPTIDES OF CARBOXYMETHYL ACTINIDIN.

Fig. 3.4. Composite DEAE-cellulose chromatography of the tryptic peptides of carboxymethyl actinidin.

Fractions I, II, III and IV from the Sephadex G-50 column elution profile (Fig. 3.2.) were pooled separately and further purified on a DE-32 cellulose column (95 cm long) contained in a Pharmacia K-26 x 100 cm chromatographic tube. The column was equilibrated with Tris-HCl buffer (0.05M-Tris, pH 8.0, containing 0.025M-NaCl) and pumped at ascending flow rate of 25 $ml.h^{-1}$. Each peptide fraction sample (I,II,III and IV) was washed into the column with Tris-HCl buffer (0.05M-Tris, pH 8.0, 0.025M-NaCl) and the peptide material was eluted from the column with a continuous NaCl gradient system containing Tris-HCl buffer (0.05M-Tris, pH 8.0) and the following concentrations of NaCl:

Gradient 1: 550 ml 0.025M-NaCl x 550 ml 0.2M-NaCl.
Gradient 2: 350 ml 0.2M-NaCl x 350 ml 0.5M-NaCl.

Fraction I was shown to contain peptide T_6, fraction II to contain peptide T_7, fraction III to contain peptide T_8 and fraction IV to contain peptides T_3 and T_{11}. The ^{14}C radioactivity was determined in each fraction and expressed as dpm. The numbering of the fractions (I to IV) is the order in which they eluted from the Sephadex G-50 column (Fig. 3.2.).

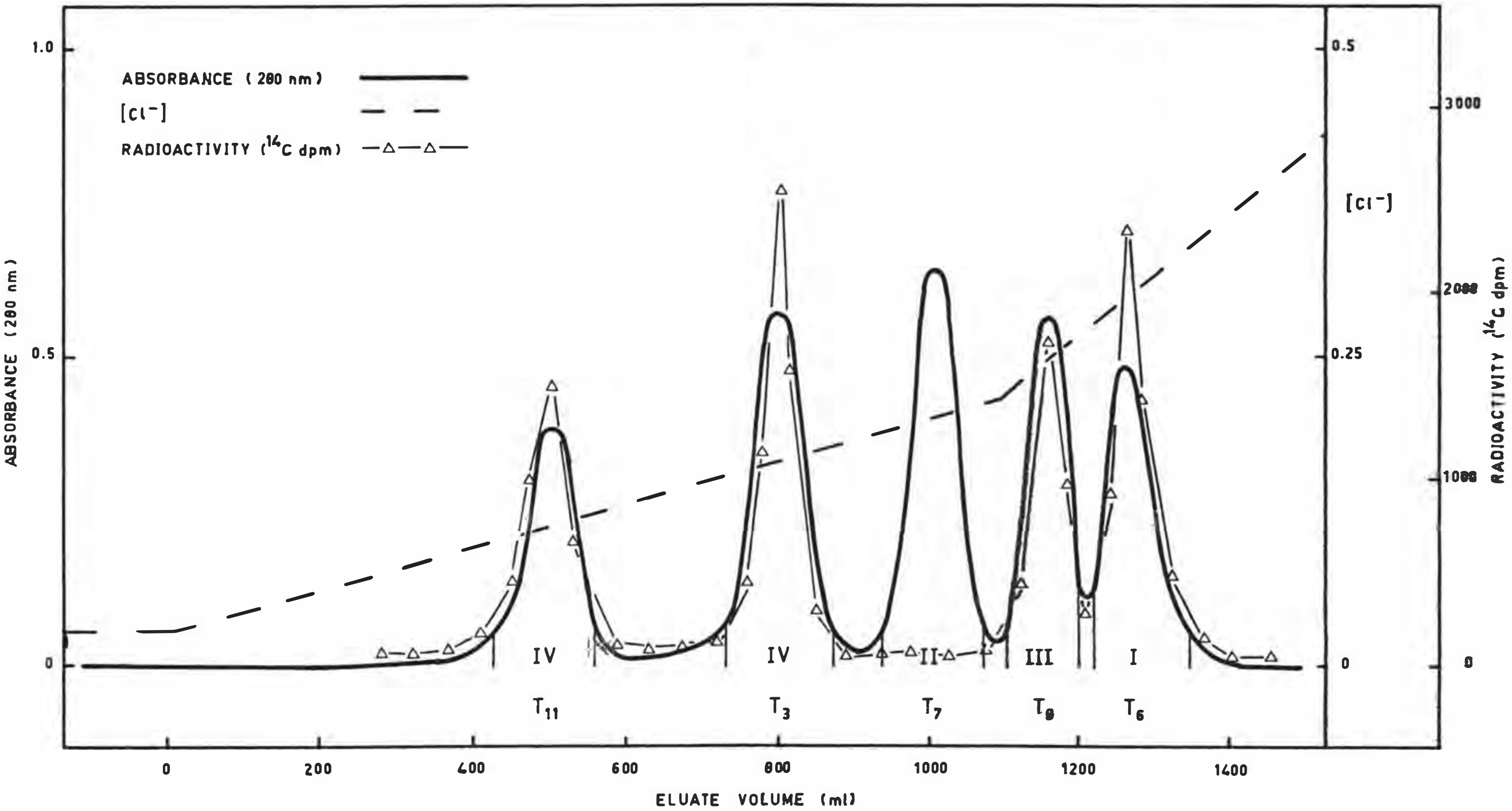


FIG. 3.4. COMPOSITE DEAE-CELLULOSE CHROMATOGRAPHY OF THE TRYPTIC PEPTIDES OF CARBOXYMETHYL ACTINIDIN.

Section 3.4. The sequences of the tryptic peptides:

The results of the determination of the amino acid sequence of each tryptic peptide are tabulated in the following sections (3.4.1. to 3.4.12.), in the order that they were aligned to reconstruct the primary amino acid sequence of actinidin (peptides T_1 to T_{12}).

The course of purification of each peptide is detailed and the data tabulated where possible. The mobility of each peptide for electrophoresis at pH 6.5 was measured relative to aspartic acid. For electrophoresis at pH 2.1, and for chromatography in the BAWP solvent, the mobility of each peptide was measured relative to DNS-Arg.

The colour of each peptide when stained with ninhydrin, as determined from stained peptide maps or marker strips, is tabulated as:

R = red, O = orange, Y = yellow.

Observation of the colour of each peptide when stained with ninhydrin provided useful information, as a yellow N-terminal gave an indication of either a glycine, serine, threonine, asparagine or CM-cysteine N-terminal amino acid in the peptide. Serine, threonine and CM-cysteine often change colour to orange with time. The N-terminal amino acid of each peptide was confirmed by taking a sample of the peptide for dansylation. The amino acid composition and the yield of each peptide was determined and is tabulated showing the number of residues of each amino acid that are present in the peptide.

All paper electrophoretograms and chromatograms were checked for fluorescence under 366 nm wavelength UV light prior to staining marker strips with ninhydrin. Each peptide is recorded as either containing (+) or not containing fluorescence. Fluorescence in a peptide indicated that aromatic residues (in particular tryptophan) were likely to be present. The specific radioactivity (dpm ^{14}C/ µmol) of each peptide was determined to give an indication of the number of S-carboxy[$^{14}C_2$]methyl cysteine residues present in the peptide. Peptide maps were stained for tyrosine, tryptophan and arginine, and the results are recorded as the peptide either containing (+) or not containing (-) one of these residues.

Determination of the sequence of a peptide directly by the dansyl-Edman method is indicated by the symbol (¬) under the amino acid sequence of each peptide.
For large peptides, a rapid Edman degradation of each peptide was carried out, and the extent of this degradation is also indicated by the symbol (¬).

The Offord (1966) mobility procedure was used to determine the amide residues in each peptide. Where it was not possible to determine the amide residues in a peptide from the peptide net charge, the peptide was sequentially degraded by the Edman method, and the peptide electrophoretic mobility was determined after the removal of each residue. The results are expressed diagramatically, showing at which residue a charge change occurred in the peptide during degradation.

Peptides obtained from the enzyme digests are named in the following manner:
T = trypsin, ChT = chymotrypsin, Th = thermolysin, P = pepsin and GE = *S. aureus* V8 protease digest peptide.
The letters a, b and N indicate whether the peptide, at pH 6.5, is acidic, basic or neutral respectively.

The methods used to obtain the results are discussed in Chapter 4.

3.4.1. Peptide T_1:

Peptide T_1 was isolated from fraction VII of the Sephadex G-50 column elution profile (Fig. 3.2.). Peptide T_1 was purified by paper electrophoresis (Fig. 3.3.). The peptide purification data and amino acid composition of peptide T_1 are given in Table 3.3..

Table 3.3. Peptide isolation data and amino acid composition of peptide T_1.

Peptide	T_1
Mobility:	
pH 6.5	-
pH 2.1	0.60
BAWP	0.86
Ninhydrin colour	R
N-terminal	Leu
Fluorescence	+
^{14}C (dpm/μmol)	-
Yield (μmol)	6.0
Stained for:	
Tyr	+
Trp	+
Arg	+

Amino acid composition

Amino acid	No. of residues
His	-
Lys	-
Arg	1.0
CMCys	1.0
Asp	
Thr	-
Ser	0.9
Glu	-
Pro	1.0
Gly	-
Ala	-
Val	0.9
Met	-
Ile	-
Leu	1.0
Tyr	1.0
Phe	-

Peptide T_1 was sequenced by the dansyl-Edman method to give the sequence:

$$\underset{\rightharpoondown}{\text{Leu}}-\underset{\rightharpoondown}{\text{Pro}}-\underset{\rightharpoondown}{\text{Ser}}-\underset{\rightharpoondown}{\text{Tyr}}-\underset{\rightharpoondown}{\text{Val}}-\underset{\rightharpoondown}{\text{Asp}}-\underset{\rightharpoondown}{\text{Trp}}-\underset{\rightharpoondown}{\text{Arg}}$$

The absence of amide residues from peptide T_1 was confirmed by the Offord (1966) mobility procedure.

3.4.2. Peptide T_2:

Peptide T_2 was isolated from fraction V of the Sephadex G-50 column elution profile (Fig. 3.2.). Peptide T_2 was purified by paper electrophoresis (Fig. 3.3.). The peptide purification data and amino acid composition for peptide T_2 are given in Table 3.4..

Table 3.4. Peptide isolation data and amino acid composition of peptide T_2.

Peptide	T_2
Mobility:	
pH 6.5	-
pH 2.1	0.73
BAWP	0.58
Ninhydrin colour	Y/O
N-terminal	Ser
Fluorescence	-
^{14}C (dpm/μmol)	-
Yield (μmol)	5.0
Stained for:	
Tyr	-
Trp	-
Arg	-

Amino acid composition	
Amino acid	No. of residues
His	-
Lys	1.0
Arg	-
CMCys	1.0
Asp	
Thr	-
Ser	0.8
Glu	-
Pro	-
Gly	1.0
Ala	2.0
Val	1.8
Met	-
Ile	1.0
Leu	-
Tyr	-
Phe	-

Peptide T_2 was sequenced by the dansyl-Edman method to give the sequence:

$$\underset{\rightharpoondown}{\text{Ser}}-\underset{\rightharpoondown}{\text{Ala}}-\underset{\rightharpoondown}{\text{Gly}}-\underset{\rightharpoondown}{\text{Ala}}-\underset{\rightharpoondown}{\text{Val}}-\underset{\rightharpoondown}{\text{Val}}-\underset{\rightharpoondown}{\text{Asp}}-\underset{\rightharpoondown}{\text{Ile}}-\underset{\rightharpoondown}{\text{Lys}}$$

The absence of amide residues from peptide T_2 was confirmed by the Offord (1966) mobility procedure.

3.4.3. Peptide T_3:

Peptide T_3 from fraction IV of the Sephadex G-50 column elution profile (Fig. 3.2.) was rechromatographed on DEAE cellulose to separate peptide T_3 from peptides T_{11} and T_8 that were present in this fraction. The second peak of the fraction IV material eluting from the DEAE-cellulose column contained peptide T_3 and was pooled (Fig. 3.4.). The amino acid composition of peptide T_3 is given in Table 3.5., and the N-terminal was determined as serine. The yield of the peptide was 7 µmol. The specific radioactivity of peptide T_3 (2.5×10^6 dpm/µmol) indicated two cysteine residues were present in the peptide.

Table 3.5. Amino acid composition of peptide T_3.

Amino acid	No. of residues
His	-
Lys	1.0
Arg	-
CMCys Asp	2.9
Thr	1.0
Ser	1.8
Glu	3.0
Pro	-
Gly	3.9
Ala	3.4
Val	1.0
Met	-
Ile	2.0
Leu	-
Tyr	-
Phe	0.9

A rapid Edman degradation of peptide T_3 provided the following N-terminal sequence:

N-terminal	Residue 2	Residue 3
Ser	Glx	Gly

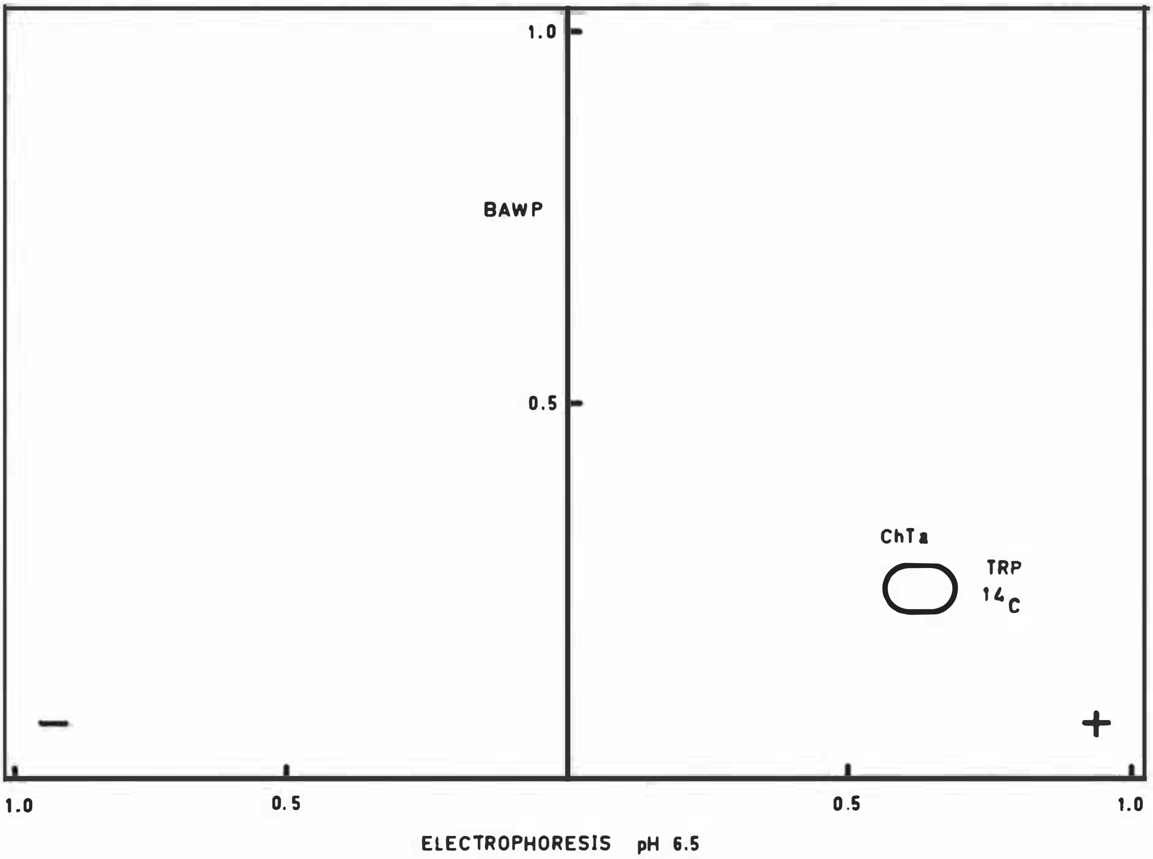
Chymotryptic digestion of peptide T_3:

Peptide T_3 (2 µmol) was digested with chymotrypsin and

100 nmol was used to map the chymotryptic peptides (Fig. 3.5.). The chymotryptic peptides were purified from the remainder of the digest (1.9 µmol) by paper electrophoresis. The peptide purification data and the amino acid compositions of the peptides are given in Table 3.6..

Table 3.6. Peptide isolation data and amino acid compositions of the chymotrypsin digest peptides of peptide T_3.

Peptide	ChTa	ChTN$_1$	ChTN$_2$
Mobility:			
pH 6.5	-0.72	-	-
pH 2.1	-	0.58	0.70
BAWP	0.29	0.60	0.46
Ninhydrin colour	Y/O	Y/O	R
N-terminal	Ser	Ser	Ala
Fluorescence	+	-	-
^{14}C (dpm/µmol)	2.6×10^6	-	-
Yield (nmol)	700	300	400
Stained for:			
Tyr	-	-	-
Trp	+	-	-
Arg	-	-	-
Amino acid composition:			
Amino acid.	Residues.		
His	-	-	-
Lys	-	1.0	-
Arg	-	-	-
CMCys Asp	1.8	1.0	-
Thr	-	0.9	-
Ser	0.9	0.9	-
Glu	2.3	1.0	-
Pro	-	-	-
Gly	3.3	0.9	-
Ala	0.3	2.3	1.3
Val	-	0.9	-
Met	-	-	-
Ile	-	2.0	-
Leu	-	-	-
Tyr	-	-	-
Phe	-	-	1.0



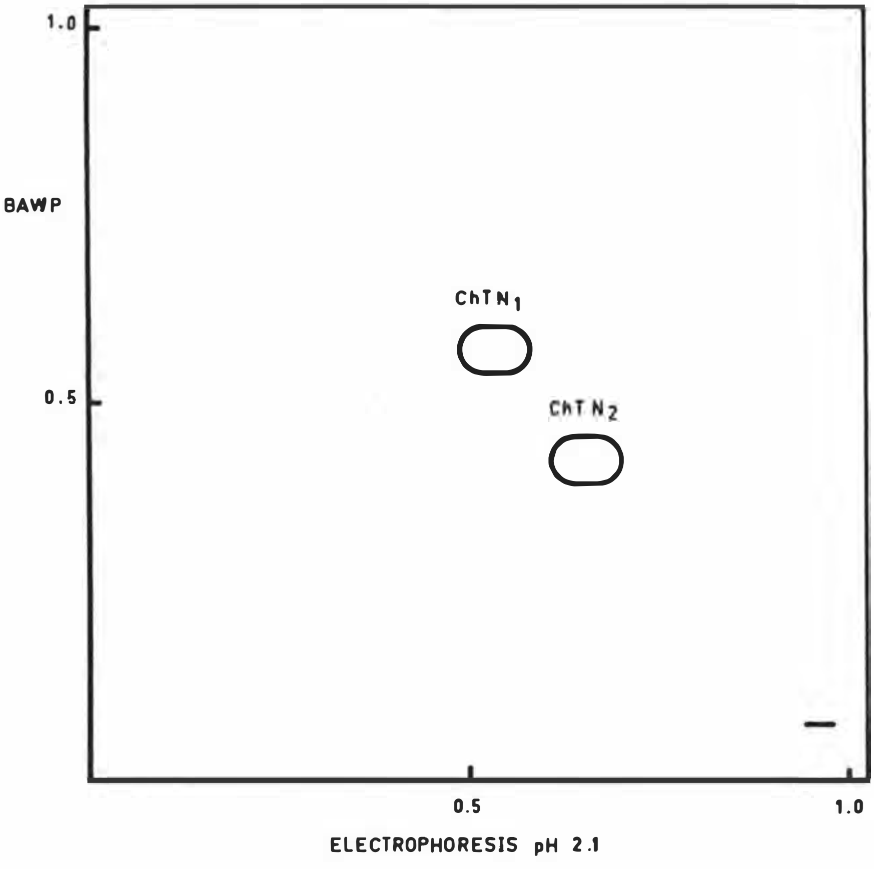


FIG. 3.5. CHYMOTRYPSIN DIGEST PEPTIDE MAPS OF PEPTIDE T_3.

Thermolysin digestion of peptide T_3:

Peptide T_3 (2 μmol) was digested with thermolysin and the peptides were mapped (Fig. 3.6.) and purified by paper electrophoresis (Table 3.7.).

Table 3.7. Peptide isolation data and amino acid compositions of the thermolysin digest peptides from peptide T_3.

Peptide	Tha_1	Tha_2	ThN_1	ThN_2	Thb
Mobility:					
pH 6.5	-0.60	-0.70	-	-	+0.55
pH 3.5	-	+0.14	-	-	-
pH 2.1	-	-	0.60	0.67	-
BAWP	0.47	0.57	0.71	0.76	0.36
Ninhydrin colour	R	Y/O	R	R	R
N-terminal	Val	Ser	Ile	Phe	Ile
Fluorescence	-	+	-	-	-
^{14}C (dpm/μmol)	-	2.4×10^6	-	-	-
Yield (nmol)	730	300	550	250	800
Stained for:					
Tyr	-	-	-	-	-
Trp	-	+	-	-	-
Arg	-	-	-	-	-

Amino acid composition:

Amino acid.	Residues.				
His	-	-	-	-	-
Lys	-	-	-	-	1.1
Arg	-	-	-	-	-
CMCys Asp	-	1.9	-	-	1.0
Thr	-	-	0.9	-	-
Ser	-	0.8	-	0.9	-
Glu	1.0	2.0	-	-	-
Pro	-	-	-	-	-
Gly	1.0	3.1	-	-	-
Ala	-	1.3	1.0	1.0	-
Val	1.0	-	-	-	-
Met	-	-	-	-	-
Ile	-	-	1.1	-	1.0
Leu	-	-	-	-	-
Tyr	-	-	-	-	-
Phe	-	-	-	1.0	-

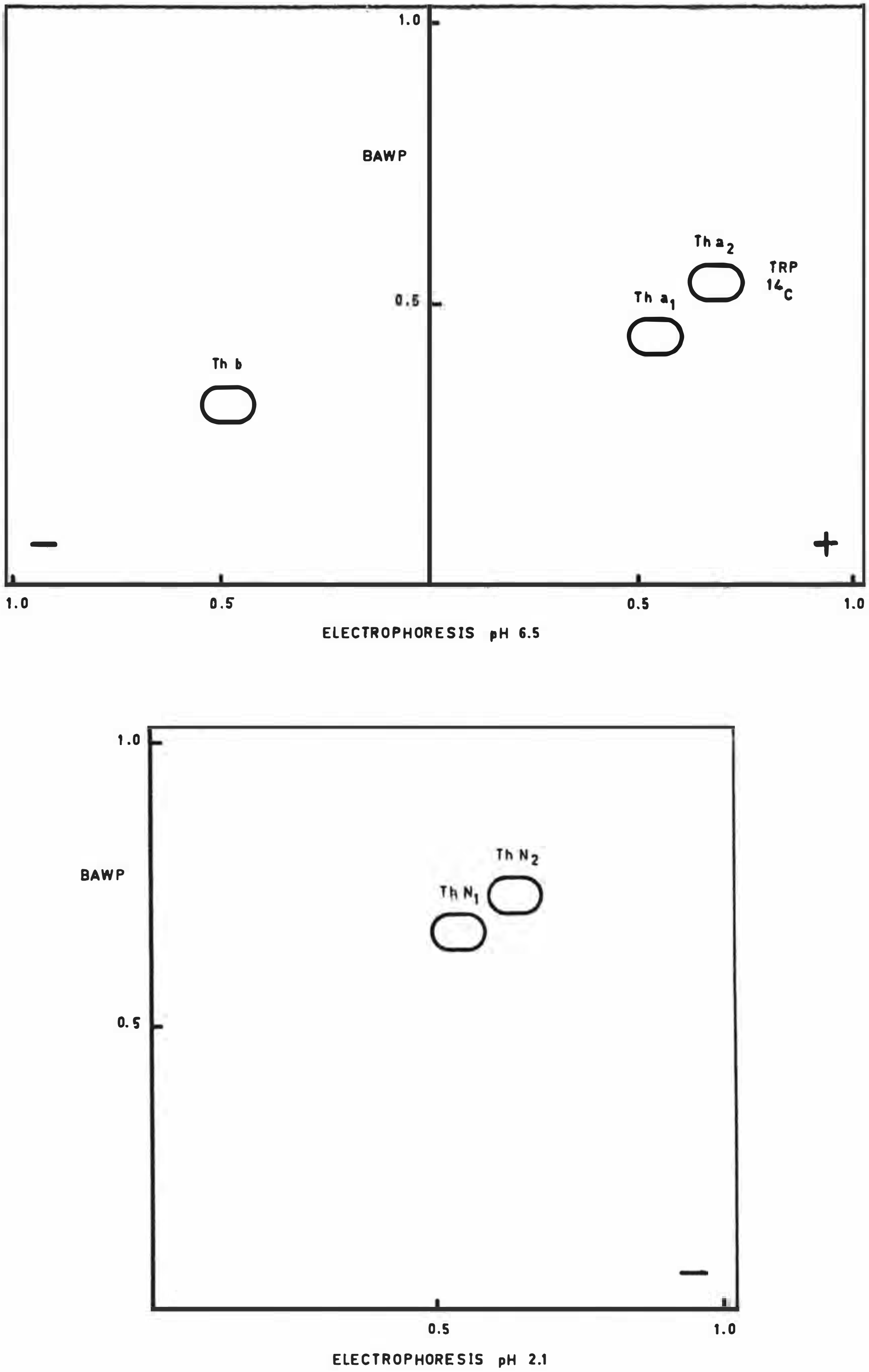


FIG. 3.6. THERMOLYSIN DIGEST PEPTIDE MAPS OF PEPTIDE T_3.

The amino acid compositions of the thermolysin peptides are given in Table 3.7.. The peptides from the chymotrypsin and thermolysin digests of peptide T_3 were sequenced and aligned to reconstruct the sequence of peptide T_3 (Fig. 3.7.).

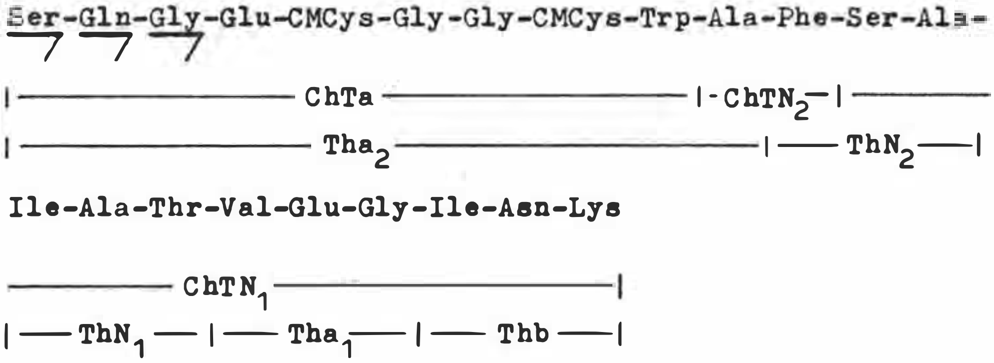


Fig. 3.7. Reconstruction of peptide T_3 from chymotrypsin and thermolysin digest peptides.

Amide and acid residues in peptide T_3 were confirmed by the Offord (1966) mobility procedure. Peptide ChTa was subjected to a sequential Edman degradation and the electrophoretic mobility was determined after each Edman cycle. The results (plotted in Fig. 3.8.) show that the second residue in peptide ChTa is glutamine and the fourth residue is glutamic acid.

Fig. 3.8. Determination of the amide and acid residues in peptide T_3ChTa by the Offord mobility procedure.

The mobility and molecular weight of peptide ChTa indicated that the net charge on the peptide at pH 6.5 was -3, as determined by the Offord mobility procedure, which showed one amide residue was present in the peptide. To determine the amide residue, peptide ChTa was subjected to a sequential Edman degradation and samples of the peptide after each Edman cycle were removed for the determination of the electrophoretic mobility by paper electrophoresis at pH 6.5. The molecular weight and mobility of the peptide after each Edman cycle was plotted on an Offord mobility diagram. The point during the Edman degradation at which a charged residue is removed is indicated by a charge change in the diagram. The results show the second residue in peptide ChTa is glutamine as there is no charge change after removal of this residue from the peptide. The fourth residue is glutamic acid as there is a charge change from -3 to -2 on removal of this residue from the peptide.

e is the net charge on the peptide at pH 6.5.

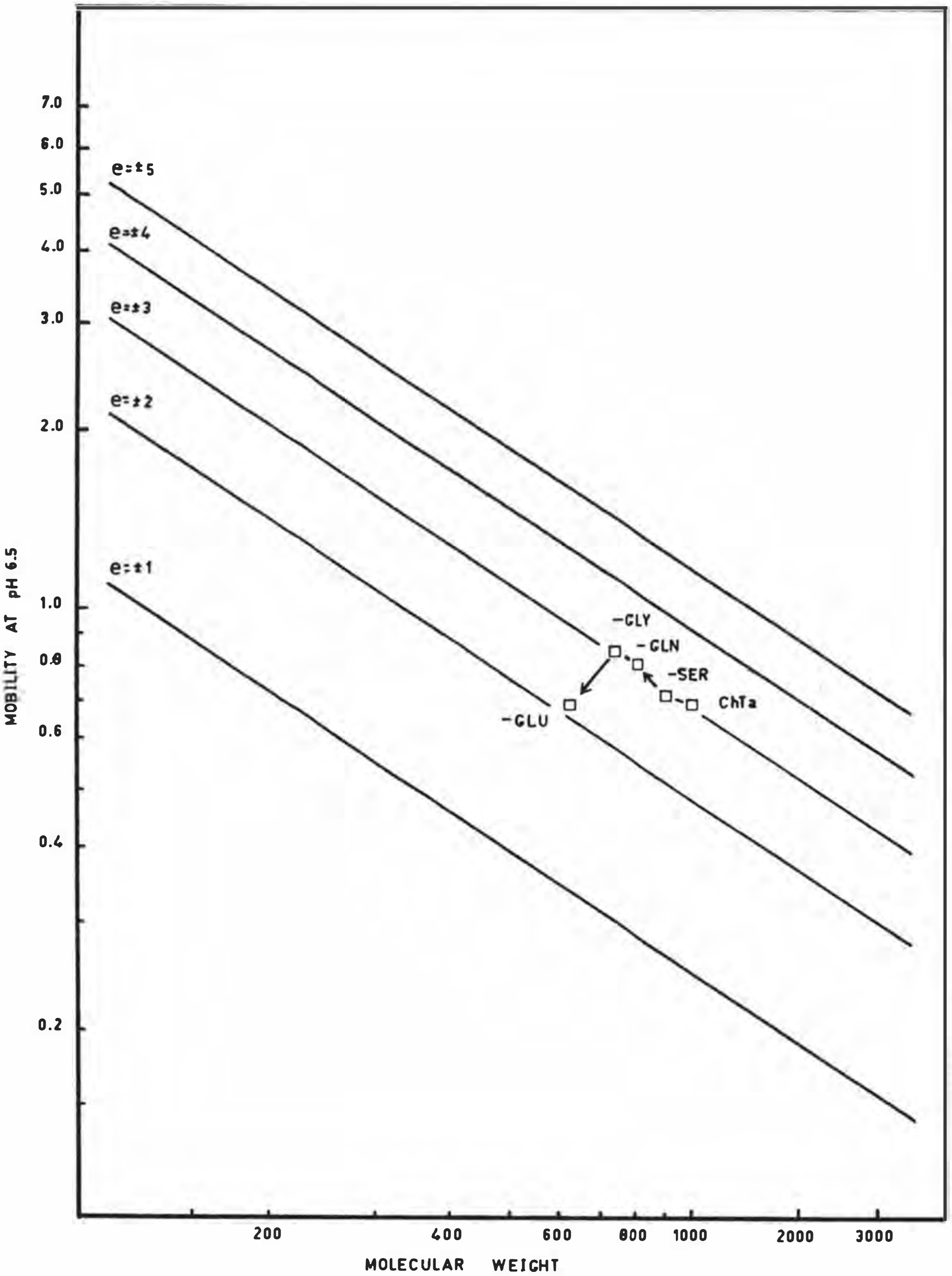


FIG. 3.8. DETERMINATION OF THE AMIDE AND ACID RESIDUES IN PEPTIDE T_3ChTa BY THE OFFORD MOBILITY PROCEDURE.

3.4.4. Peptide T_4:

Peptide T_4 was the only tryptic peptide that could not be isolated from the tryptic digest of S-carboxy[$^{14}C_2$]methyl actinidin. Comparison of the amino acid composition of actinidin with the sequences of the eleven tryptic peptides isolated, indicated that peptide T_4 should contain in the peptide sequence 1 cysteine and 1 arginine, but no aromatic residues (no tyrosine, phenylalanine or tryptophan). Tentative alignment of the sequences of the eleven tryptic peptides to reconstruct the sequence of actinidin, using information from the X-ray crystallographic three dimensional structure of actinidin, indicated peptide T_4 was located between peptides T_3 and T_5 [peptide T_3 was shown to contain the active site cysteine residue (Section 3.6., page 103)].

To obtain the amino acid sequence of peptide T_4, S-carboxy[$^{14}C_2$]methyl actinidin (labelled with ^{14}C only at the active site cysteine) was maleylated and then digested with trypsin. The tryptic peptides from the maleylated carboxymethyl actinidin were separated on a Sephadex G-75 Pharmacia K-26 column (Fig. 3.9.).

Two of the fractions (A and B) from the Sephadex G-75 column elution profile were radioactive. The radioactive peptide in fraction B was found to have the same sequence as peptide T_3, indicating that the maleylation of carboxymethyl actinidin had been incomplete. One half of fraction A material was digested with chymotrypsin. One tenth of the chymotryptic digest was subjected to electrophoresis at pH 6.5 as a 4 cm wide band. The electrophoretogram was exposed to the vapour of pH 3.5 buffer to demaleylate the lysine containing peptides on the paper. A 2 cm strip was then subjected to electrophoresis at pH 6.5 in the second dimension to produce a maleyl diagonal peptide map. A second map was prepared and stained for arginine.

The diagonal map (Fig. 3.10.) showed that the only spot staining for arginine (ChTA) was located on the diagonal.

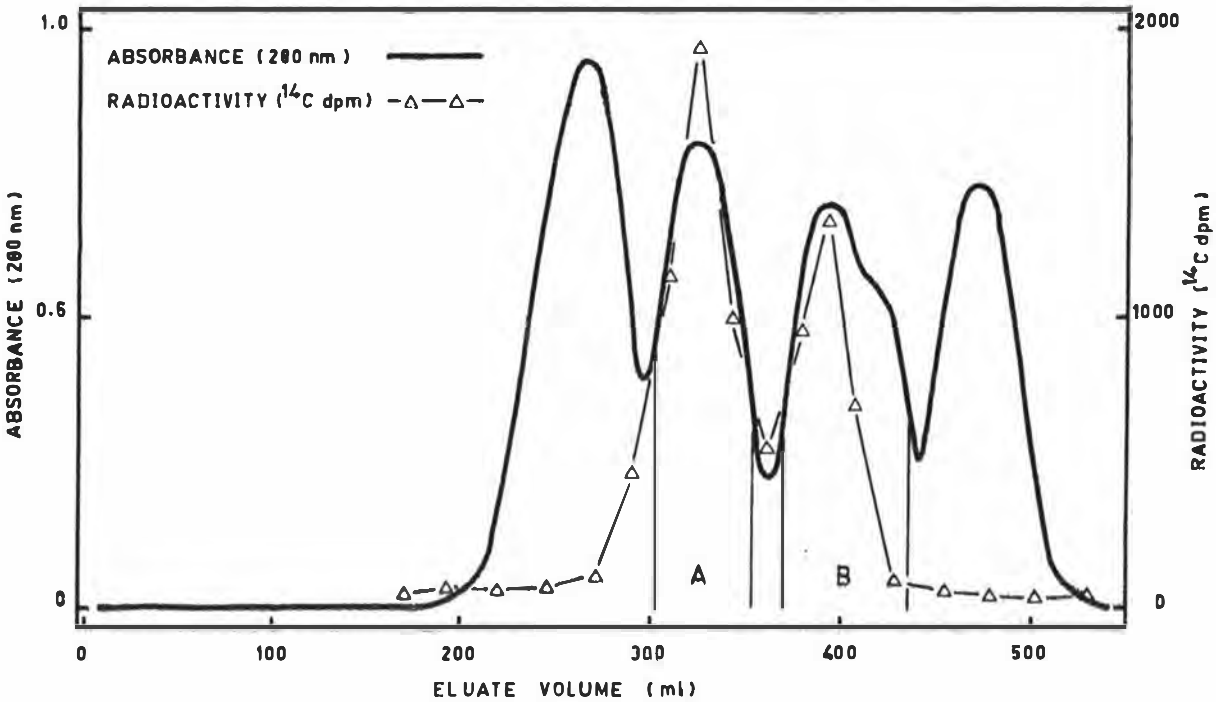


Fig. 3.9. Sephadex G-75 chromatography of the tryptic peptides of maleylated carboxymethyl actinidin.

Two ninhydrin spots ($ChTM_1$ and $ChTM_2$) appeared off of the diagonal. Both peptide $ChTM_1$ and $ChTM_2$ stained yellow-orange with ninhydrin on paper, and did not migrate as confined bands. Using information from the maleyl diagonal, the three peptides (ChTA, $ChTM_1$ and $ChTM_2$) were purified by preparative electrophoresis at pH 6.5. The peptide isolation data and amino acid compositions of peptides ChTA, $ChTM_1$ and $ChTM_2$ are given in Table 3.8..

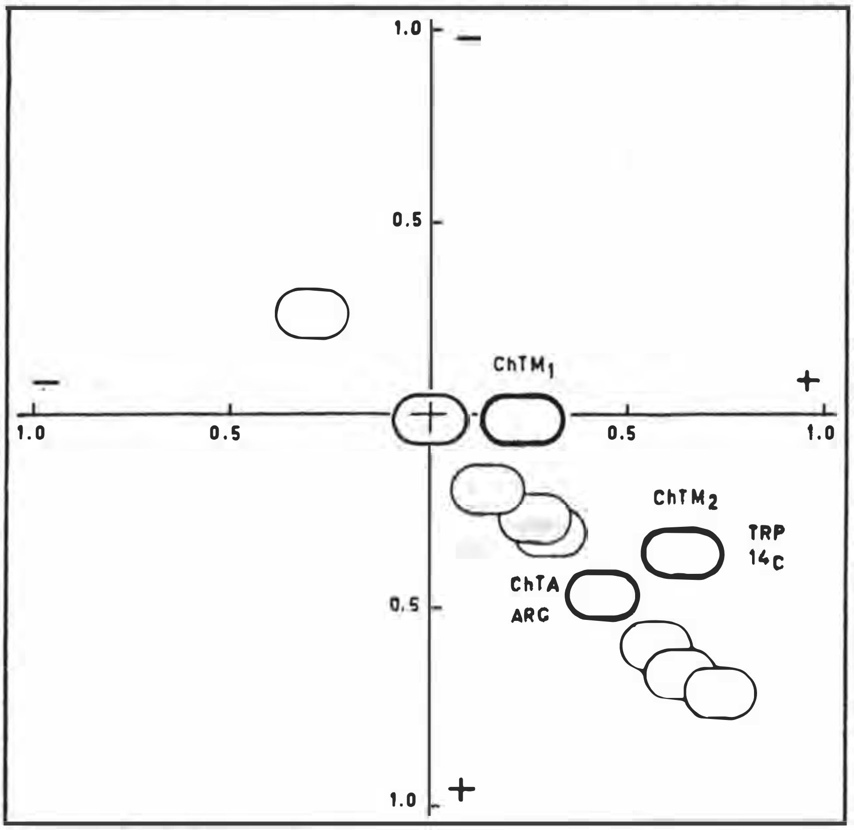


Fig. 3.10. Maleyl diagonal peptide map at pH 6.5 of a chymotrypsin digest of the maleyl tryptic peptide containing peptide T_4.

One tenth of the chymotrypsin digest material of fraction A from the Sephadex G-75 elution profile (Fig. 3.9.) was subjected to electrophoresis at pH 6.5 as a 4 cm band and was exposed to the vapour of pyridinium acetate buffer (1 % pyridine, 5 % glacial acetic acid, pH 3.5) at 60°C overnight to remove the maleyl groups from the peptides on paper. A 2 cm strip was then subjected to electrophoresis at pH 6.5 in the second dimension to produce the maleyl diagonal map. Removal of a maleyl group from a peptide results in a charge change of +2. Therefore after demaleylation, peptides lying off the diagonal towards the cathode contain lysine.
Peptide mobilities at pH 6.5 were measured relative to aspartic acid.

Table 3.8. Peptide isolation data and amino acid compositions of the chymotrypsin digest peptides from the maleyl tryptic peptide containing peptide T_4.

Peptide	ChTA	$ChTM_1$	$ChTM_2$
Mobility:			
pH 6.5			
before demaleylation	-0.60	-0.35	-0.75
after demaleylation	-0.60	neutral	-0.44
pH 2.1	-	0.48	-
Ninhydrin colour	R	Y/O	Y/O
N-terminal	Ile	Ser	Ser
Fluorescence	-	-	-
^{14}C (dpm/µmol)	-	-	0.9×10^{6}
Yield (nmol)	800	400	350
Stained for:			
Tyr	-	-	-
Trp	-	-	+
Arg	+	-	-

Amino acid composition:

Amino acid. Residues.	ChTA	$ChTM_1$	$ChTM_2$
His	-	-	-
Lys	-	1.0	1.0
Arg	1.0	-	-
CMCys Asp	1.8	1.0	2.7
Thr	-	1.9	-
Ser	1.7	2.6	1.9
Glu	3.0	1.0	2.1
Pro	-	-	-
Gly	1.1	2.1	4.2
Ala	-	2.0	2.3
Val	-	1.1	2.0
Met	-	-	-
Ile	1.9	3.0	1.0
Leu	1.9	1.1	-
Tyr	-	-	-
Phe	-	-	-

One third of peptide ChTA material was sequenced directly by the dansyl-Edman method. The remainder of peptide ChTA was digested with S. aureus V8 protease and the peptides were purified by paper electrophoresis. The peptide isolation data and amino acid compositions of these peptides are given in Table 3.9.. The peptides obtained from the digestion were sequenced, and from this information, the sequence of peptide ChTA was reconstructed:

Ile-Ser-Leu-Ser-Glu-Gln-Glu-Leu-Ile-Asp-CMCys-Gly-Arg
⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁

Peptide ChTM$_1$ was sequenced directly by the dansyl-Edman method which provided the following sequence:

Ser-Ala-Ile-Ala-Thr-Val-Glu-Gly-Ile-Asn-Lys-Ile-Thr-Ser-Gly-Ser-Leu
⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁

The first eleven residues in this peptide corresponded to the C-terminal sequence of the tryptic peptide T$_3$.

The amino acid composition, specific radioactivity and N-terminal of peptide ChTM$_2$ indicated that this peptide contained the sequence of tryptic peptide T$_2$ and the N-terminal sequence of peptide T$_3$. Peptide ChTM$_2$ was not sequenced.

The remaining half of the fraction A material from the Sephadex G-75 elution profile was digested with S. aureus V8 protease. Samples of the digest subjected to electrophoresis at pH 6.5 and to BAWP chromatography showed that a nonradioactive peptide which stained yellow with ninhydrin remained at the origin (peptide GE/Y). Demaleylation of the remainder of the digest in solution resulted in precipitation of some of the material. The precipitate contained all of the peptide GE/Y material, and from the amino acid composition (Table 3.10.) and the N-terminal (glycine), the peptide was sufficiently pure to sequence.

Table 3.9. The peptide isolation data and amino acid compositions of the S. aureus V8 protease digest peptides of peptide ChTA that was obtained by chymotrypsin digestion of the maleyl tryptic peptide containing peptide T_4.

Peptide	GEa_1	GEa_2	GEa_3	GEa_4
Mobility:				
pH 6.5	-0.33	-0.46	-0.55	-0.65
Ninhydrin colour	R	R	R	R
N-terminal	Leu	Ile	Glx	Ile
Fluorescence	-	-	-	-
^{14}C (dpm/μmol)	1.0×10^6	-	0.9×10^6	-
Yield (nmol)	200	120	100	170
Stained for:				
Tyr	-	-	-	-
Trp	-	-	-	-
Arg	+	-	+	-

Amino acid composition:

Amino acid.	Residues.			
His	-	-	-	-
Lys	-	-	-	-
Arg	1.0	-	1.0	-
CMCys Asp	1.8	-	1.9	-
Thr	-	-	-	-
Ser	-	1.9	-	1.9
Glu	-	1.0	2.0	3.2
Pro	-	-	-	-
Gly	1.1	-	1.0	-
Ala	-	-	-	-
Val	-	-	-	-
Ile	0.9	1.1	1.0	1.0
Leu	0.9	1.0	0.9	1.0
Tyr	-	-	-	-
Phe	-	-	-	-

Table 3.10. Amino acid composition of peptide GE/Y obtained by *S. aureus* V8 protease digestion of the maleyl tryptic peptide containing peptide T_4.

Amino acid	No. of residues
His	-
Lys	1.0
Arg	-
CMCys	1.0
Asp	
Thr	0.9
Ser	3.7
Glu	3.1
Pro	-
Gly	2.1
Ala	-
Val	-
Met	-
Ile	2.9
Leu	2.0
Tyr	-
Phe	-

Peptide GE/Y was sequenced directly by the dansyl-Edman method to provide the following sequence:

$$\underset{\rightharpoondown}{\text{Gly}}\text{-}\underset{\rightharpoondown}{\text{Ile}}\text{-}\underset{\rightharpoondown}{\text{Asn}}\text{-}\underset{\rightharpoondown}{\text{Lys}}\text{-}\underset{\rightharpoondown}{\text{Ile}}\text{-}\underset{\rightharpoondown}{\text{Thr}}\text{-}\underset{\rightharpoondown}{\text{Ser}}\text{-}\underset{\rightharpoondown}{\text{Gly}}\text{-}\underset{\rightharpoondown}{\text{Ser}}\text{-}\underset{\rightharpoondown}{\text{Leu}}\text{-}\underset{\rightharpoondown}{\text{Ile}}\text{-}\underset{\rightharpoondown}{\text{Ser}}\text{-}\underset{\rightharpoondown}{\text{Leu}}\text{-}\underset{\rightharpoondown}{\text{Ser}}\text{-}\underset{\rightharpoondown}{\text{Glu}}\text{-}\underset{\rightharpoondown}{\text{Gln}}\text{-}\underset{\rightharpoondown}{\text{Glu}}$$

Peptides ChTA, ChTM_1, ChTM_2 and GE/Y were aligned with tryptic peptides T_2 and T_3 to reconstruct the sequence of the maleyl tryptic peptide obtained from fraction A of the Sephadex G-75 elution profile to provide the sequence of tryptic peptide T_4 (Fig. 3.11.).

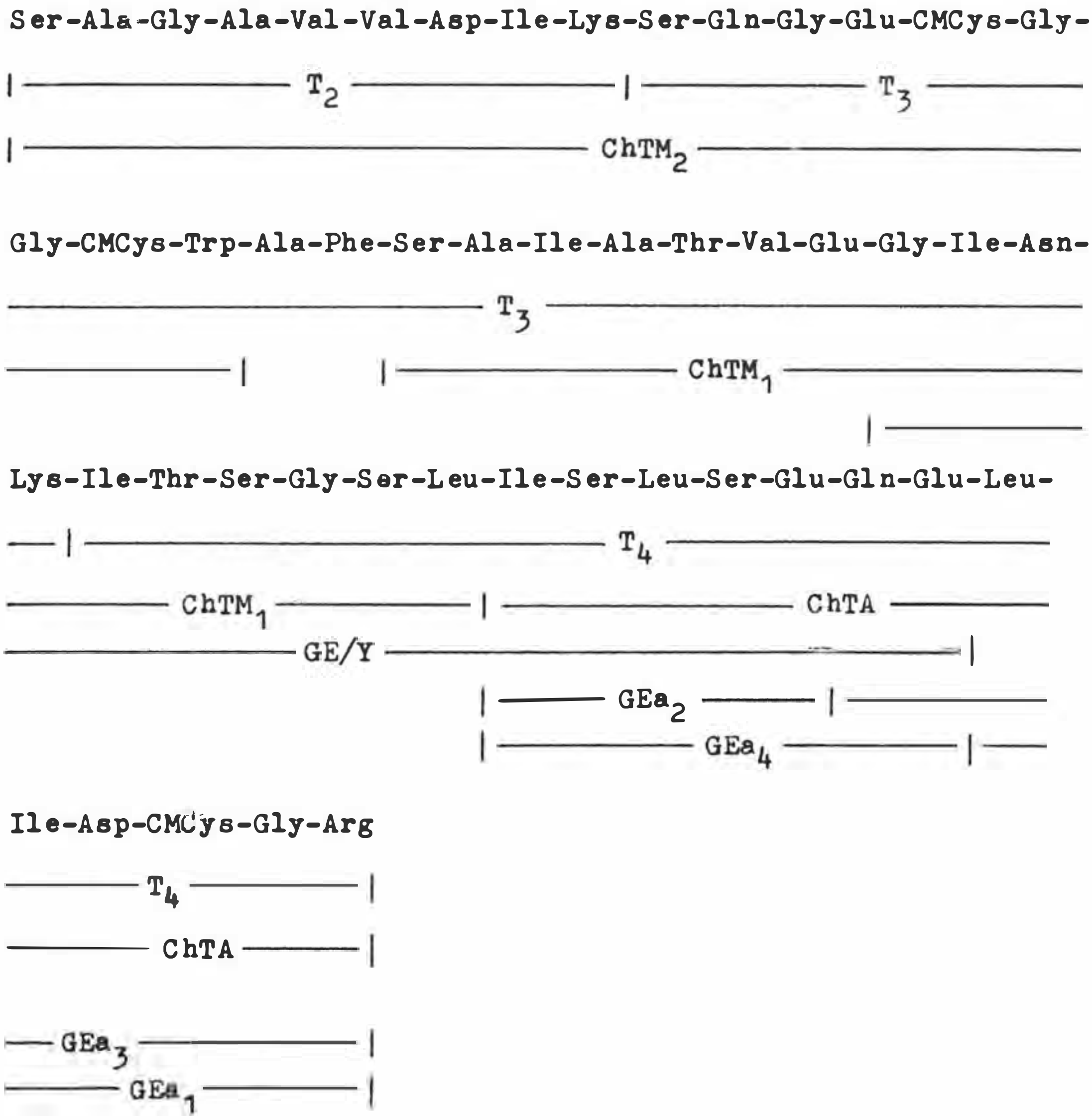


Fig. 3.11. Reconstruction of the maleyl tryptic peptide obtained from fraction A of the Sephadex G-75 elution profile from chymotrypsin and *S. aureus* V8 protease digest peptides to provide the sequence of peptide T_4.

3.4.5. Peptide T_5:

Peptide T_5 was isolated from fraction VI of the Sephadex G-50 column elution profile (Fig. 3.2.). Peptide T_5 was purified by paper electrophoresis (Fig. 3.3.). The peptide purification data and amino acid composition of peptide T_5 are given in Table 3.11..

Table 3.11. Peptide isolation data and amino acid composition of peptide T_5.

Peptide	T_5
Mobility:	
pH 6.5	+0.37
pH 2.1	-
BAWP	0.26
Ninhydrin colour	Y/O
N-terminal	Thr
Fluorescence	-
^{14}C (dpm/μmol)	-
Yield (μmol)	5.5
Stained for:	
Tyr	-
Trp	-
Arg	+

Amino acid composition

Amino acid	No. of residues
His	-
Lys	-
Arg	0.9
CMCys Asp	1.0
Thr	1.8
Ser	-
Glu	1.1
Pro	-
Gly	-
Ala	-
Val	-
Met	-
Ile	-
Leu	-
Tyr	-
Phe	-

Peptide T_5 was sequenced by the dansyl-Edman method to give the sequence:

$$\underset{\rightharpoondown}{\text{Thr}}\text{-}\underset{\rightharpoondown}{\text{Gln}}\text{-}\underset{\rightharpoondown}{\text{Asn}}\text{-}\underset{\rightharpoondown}{\text{Thr}}\text{-}\underset{\rightharpoondown}{\text{Arg}}$$

The absence of acid residues from peptide T_5 was confirmed by the Offord (1966) mobility procedure.

3.4.6. Peptide T_6:

Peptide T_6 from fraction I of the Sephadex G-50 column elution profile (Fig. 3.2.) was rechromatographed on DEAE-cellulose. The purified peak eluting from the DE-32 column was pooled (Fig. 3.4.). The amino acid composition was determined (Table 3.12.) and the yield of the peptide was 7.5 µmol. The specific radioactivity (2.2 x 10^6 dpm/µmol) of peptide T_6 indicated two cysteine residues were present in the peptide.

Table 3.12. Amino acid composition of peptide T_6.

Amino acid	No. of residues
His	-
Lys	1.0
Arg	-
CMCys Asp	12.4
Thr	2.8
Ser	-
Glu	5.2
Pro	0.9
Gly	6.7
Ala	1.9
Val	1.0
Met	-
Ile	3.9
Leu	1.2
Tyr	3.0
Phe	2.0

A rapid Edman degradation of peptide T_6 provided the following N-terminal sequence:

N-terminal	Residue 2	Residue 3
Gly	CMCys	Asx

Chymotrypsin digestion of peptide T_6:

Peptide T_6 (2 µmol) was digested with chymotrypsin and 100 nmol was used to map the peptides (Fig. 3.12.). From the remainder of the digest (1.9 µmol), the peptides were purified using electrophoresis. The peptide purification data and the amino acid compositions of the peptides are given in Table 3.13..

Table 3.13. Peptide isolation data and the amino acid compositions of the chymotrypsin digest peptides from peptide T_6.

Peptide	$ChTa_1$	$ChTa_2$	$ChTa_3$	$ChTa_4$	$ChTa_5$	$ChTN_1$	$ChTN_2$
Mobility:							
pH 6.5	-0.32	-0.42	-0.85	-0.71	-0.71	-	-
pH 3.5	-	-	+0.38	-0.19	-0.37	-	-
pH 2.1	-	-	-	-	-	0.58	0.89
BAWP	0.83	0.77	0.20	0.35	0.50	0.65	0.40
Ninhydrin colour	R	R	O	Y	Y	R	R
N-terminal	Ile	Ile	Thr	Gly	Gly	Glx	Glx
Fluorescence	-	-	-	-	-	-	-
^{14}C (dpm/μmol)	-	-	0.9×10^6	1.2×10^6	1.1×10^6	-	-
Yield (nmol)	500	600	300	300	400	450	200
Stained for:							
Tyr	-	+	-	+	+	-	-
Trp	-	-	-	-	-	-	-
Arg	-	-	-	-	-	-	-

Amino acid composition:

Amino acid.	Residues.						
His	-	-	-	-	-	-	-
Lys	-	-	-	-	-	-	1.0
Arg	-	-	-	-	-	-	-
CMCys Asp	}1.3	4.9	3.8	2.7	2.0	-	1.0
Thr	1.0	1.0	1.0	1.0	-	-	-
Ser	-	-	-	-	-	-	-
Glu	-	1.1	1.0	1.0	-	1.0	1.9
Pro	-	1.0	-	-	-	-	-
Gly	1.2	2.3	1.1	4.3	3.2	-	-
Ala	-	-	1.8	-	-	-	-
Val	-	-	0.7	-	-	-	-
Met	-	-	-	-	-	-	-
Ile	1.0	2.9	-	1.0	-	-	-
Leu	-	-	1.0	-	-	-	-
Tyr	-	2.0	-	1.1	1.0	-	-
Phe	1.0	-	-	1.9	-	1.0	-

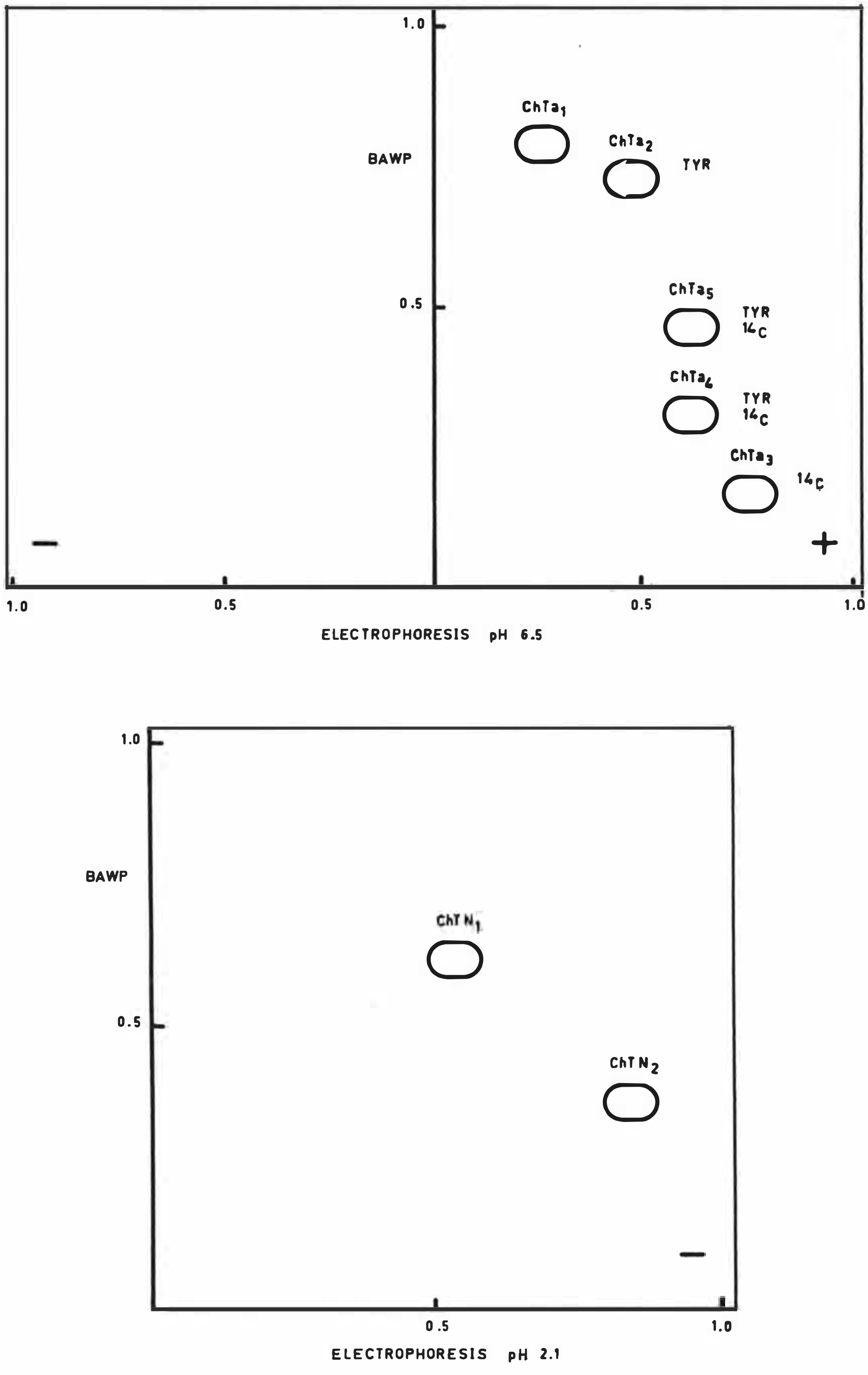


FIG.3.12. CHYMOTRYPSIN DIGEST PEPTIDE MAPS OF PEPTIDE T_6.

S. aureus V8 protease digestion of peptide T_6:

Peptide T_6 (3 μmol) was digested with *S. aureus* V8 protease and the peptides were subjected to gel filtration on a Sephadex G-25 Pharmacia K-26 column (Fig. 3.13.). All of the material eluted from the G-25 column as a single broad peak.

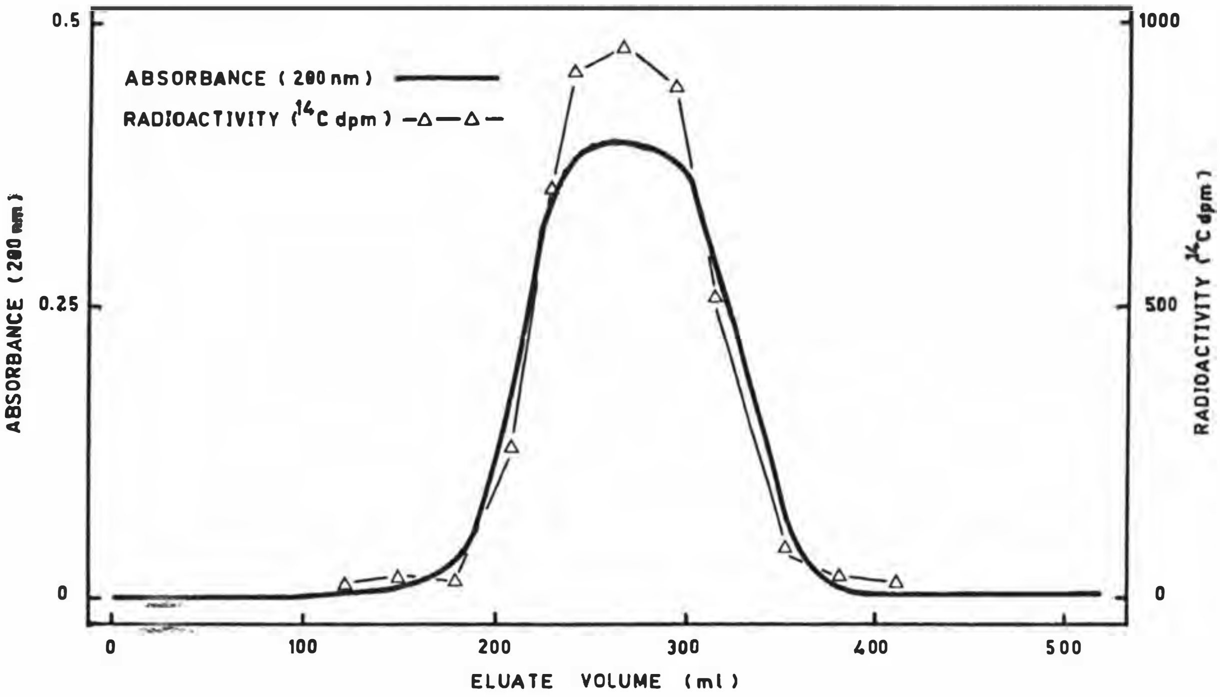


Fig. 3.13. Sephadex G-25 chromatography of peptides obtained by digestion of peptide T_6 with *S. aureus* V8 protease.

The G-25 peak was pooled and a sample (20 nmol) was subjected to electrophoresis at pH 6.5 which produced a single broad radioactive yellow-orange band of mobility -0.54. After electrophoresis of another 20 nmol sample at pH 3.5, a radioactive yellow-orange band appeared with a mobility of +0.1 and a radioactive yellow band remained at the origin. The pH 3.5 nonmobile peptide ran as a very broad band of mobility 0.30 to 0.57 in BAWP and the pH 3.5 soluble band had a mobility of 0.37 in BAWP.

The pooled G-25 peak was lyophilized and dissolved in distilled water and the pH was dropped to 3.5 with dilute acetic acid. The acid precipitated material was centrifuged down, the supernatant removed, and the precipitate resuspended in distilled water. The two fractions were lyophilized to remove the acetic acid. The acid soluble fraction gave a single N-terminal asparagine and the acid insoluble fraction gave a single N-terminal glycine. The amino acid compositions of the acid soluble (GEa) and acid insoluble (GEb) fractions were determined and are given in Table 3.14. .

Table 3.14. Amino acid compositions of fractions GEa and GEb obtained by *S. aureus* V8 protease digestion of peptide T_6.

Amino acid	No. of residues	
	GEa	GEb
His	-	-
Lys	1.0	-
Arg	-	-
CMCys Asp	6.1	6.9
Thr	0.9	1.9
Ser	-	-
Glu	2.9	2.0
Pro	0.8	-
Gly	1.1	6.4
Ala	1.9	-
Val	0.9	-
Met	-	-
Ile	-	3.8
Leu	1.0	-
Tyr	2.0	1.0
Phe	-	2.1

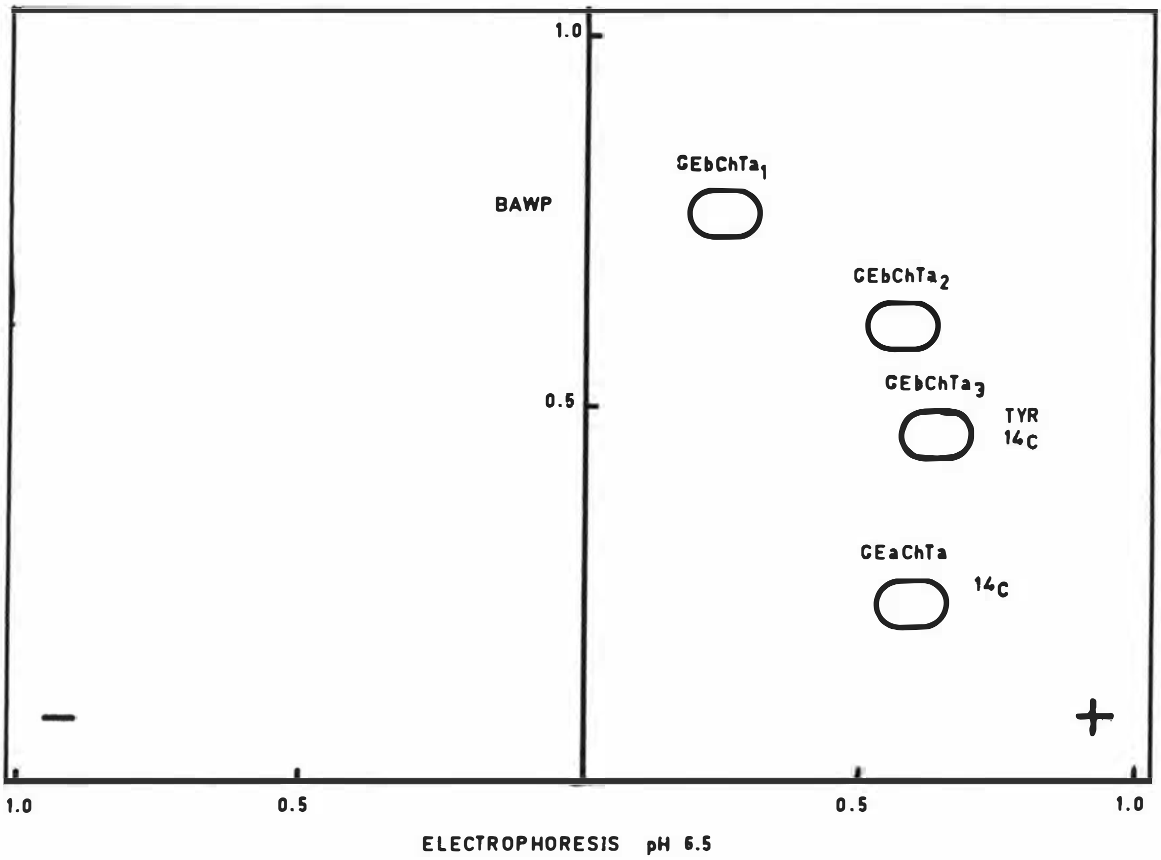
Chymotrypsin digestion of peptides GEa and GEb:

Peptide GEa was digested with chymotrypsin, which produced only two peptides; GEaChTa and GEaChTN. These peptides were purified by electrophoresis and sequenced. Peptide GEb was likewise digested with chymotrypsin, which produced four peptides; $GEbChTa_1$, $GEbChTa_2$, $GEbChTa_3$ and $GEbChTN_1$. These peptides were purified by electrophoresis and sequenced. The peptide data and amino acid compositions of these peptides are given in Table 3.15. and Fig.3.14..

Table 3.15. Peptide isolation data and amino acid compositions of the chymotryptic peptides from peptides GEa and GEb that were obtained by S. aureus V8 protease digestion of peptide T_6.

	GEa		GEb			
Peptide	ChTa	ChTN	$ChTa_1$	$ChTa_2$	$ChTa_3$	$ChTN_1$
Mobility:						
pH 6.5	-0.66	-	-0.33	-0.62	-0.70	-
pH 2.1	-	0.40	-	-	-	0.56
BAWP	0.27	0.76	0.80	0.65	0.49	0.64
Ninhydrin colour	O	Y/O	R	R	Y	R
N-terminal	Thr	Asn	Ile	Ile	Gly	Glx
Fluorescence	-	-	-	-	-	-
^{14}C (dpm/µmol)	1.0×10^6	-	-	-	1.2×10^6	-
Yield (nmol)	600	650	400	500	450	500
Stained for:						
Tyr	-	+	-	-	+	-
Trp	-	-	-	-	-	-
Arg	-	-	-	-	-	-
Amino acid composition:						
Amino acid.	Residues.					
His	-	-	-	-	-	-
Lys	1.0	-	-	-	-	-
Arg	-	-	-	-	-	-
CMCys Asp	} 5.0	1.0	1.1	3.9	2.0	-
Thr	1.0	-	1.0	0.9	-	-
Ser	-	-	-	-	-	-
Glu	3.1	-	-	1.0	-	1.0
Pro	-	0.9	-	-	-	-
Gly	1.0	-	1.2	2.2	3.3	-
Ala	1.8	-	-	-	-	-
Val	0.9	-	-	-	-	-
Met	-	-	-	-	-	-
Ile	-	-	1.0	3.1	-	-
Leu	1.0	-	-	-	-	-
Tyr	-	2.0	-	-	1.0	-
Phe	-	-	1.1	-	-	1.0

The peptides from the chymotrypsin and S. aureus V8 protease digestions of peptide T_6 were sequenced and the T_6 peptide reconstructed (Fig. 3.15.).



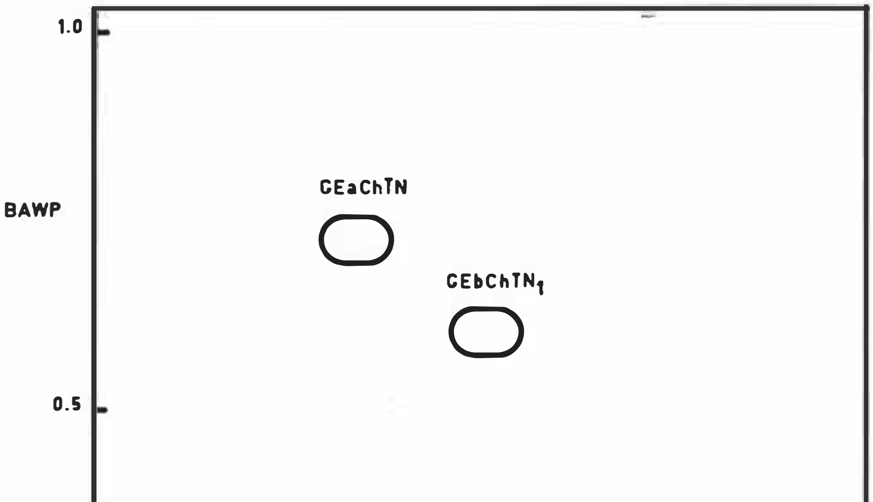


FIG. 3.14. COMPOSITE PEPTIDE MAPS OF THE CHYMOTRYPSIN DIGEST PEPTIDES FROM PEPTIDES GEa AND GEb FROM PEPTIDE T_6.

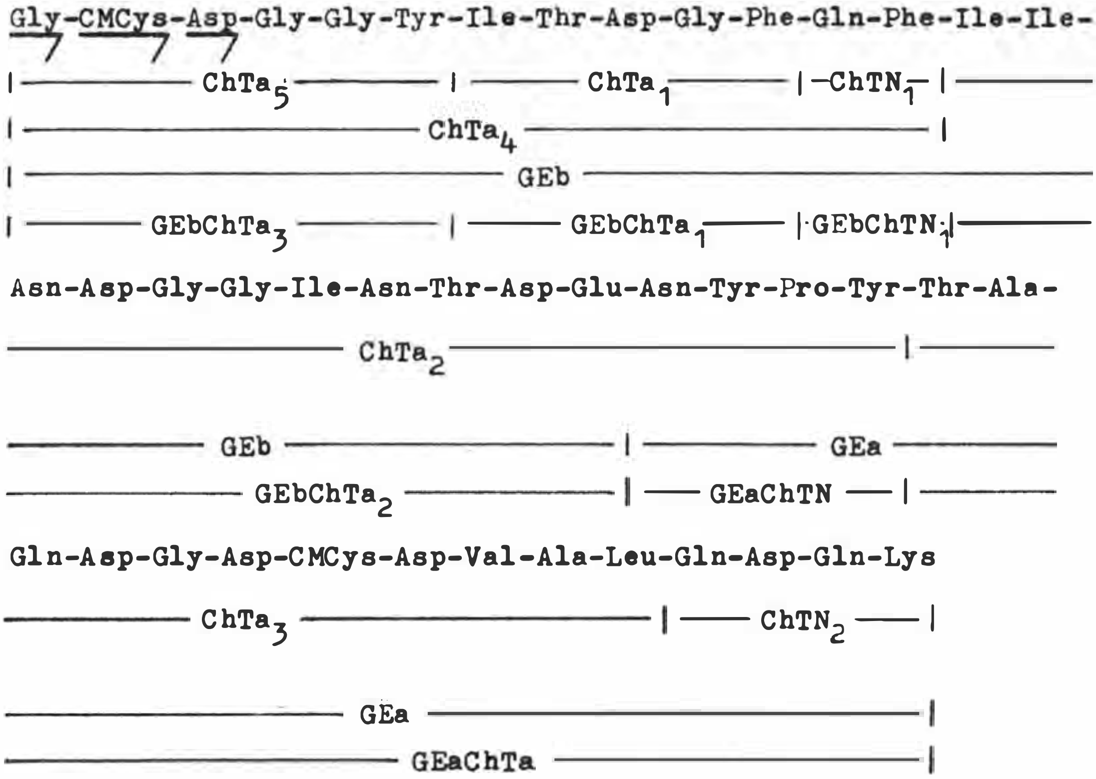


Fig. 3.15. Reconstruction of peptide T_6 from chymotrypsin and S. aureus V8 protease digest peptides.

Amide and acid residues in peptide T_6 were confirmed by the Offord (1966) mobility procedure. Peptide GEbChTa$_2$ (300 nmol) was digested with thermolysin which produced two peptides (a_1 and a_2) that contained the following sequences:

peptide a_1: Ile-Ile-Asx-Asx-Gly-Gly
peptide a_2: Ile-Asx-Thr-Asx-Glu

Peptides a_1 and a_2 were subjected to a sequential Edman degradation and the electrophoretic mobility was determined after each Edman cycle. The results (plotted in Fig. 3.16.) show that the location of the amide residues are as follows:

peptide a_1: Ile-Ile-Asn-Asp-Gly-Gly
peptide a_2: Ile-Asn-Thr-Asp-Glu

Fig. 3.16. Determination of the amide and acid residues in peptide $T_6GEbChTa_2$ by the Offord mobility procedure.

The mobility and molecular weight of peptide $GEbChTa_2$ indicated that the net charge on the peptide at pH 6.5 was -3, as determined by the Offord mobility procedure, which showed two amide residues were present in the peptide. To determine the two amide residues, peptide $GEbChTa_2$ was digested with thermolysin which produced two peptides (a_1 and a_2). Peptide a_1 had the sequence: Ile-Ile-Asx-Asx-Gly-Gly , and an electrophoretic mobility at pH 6.5 of -0.40 which indicated that the peptide had a net charge of -1.
Peptide a_2 had the sequence: Ile-Asx-Thr-Asx-Glu , and an electrophoretic mobility at pH 6.5 of -0.69 which indicated that the peptide had a net charge of -2.
Peptides a_1 and a_2 were subjected to a sequential Edman degradation and samples of each peptide after each Edman cycle were removed for the determination of the electrophoretic mobility by paper electrophoresis at pH 6.5. The molecular weight and mobility of each peptide after each Edman cycle was plotted on an Offord mobility diagram. The point during the Edman degradation at which a charged residue is removed is indicated in the diagram by a charge change. The results show that the location of the amide residues in peptides a_1 and a_2 are as follows:

peptide a_1: Ile-Ile-Asn-Asp-Gly-Gly
peptide a_2: Ile-Asn-Thr-Asp-Glu

There is no charge change in either of the peptides during degradation until the removal of the fourth residue in both peptides.

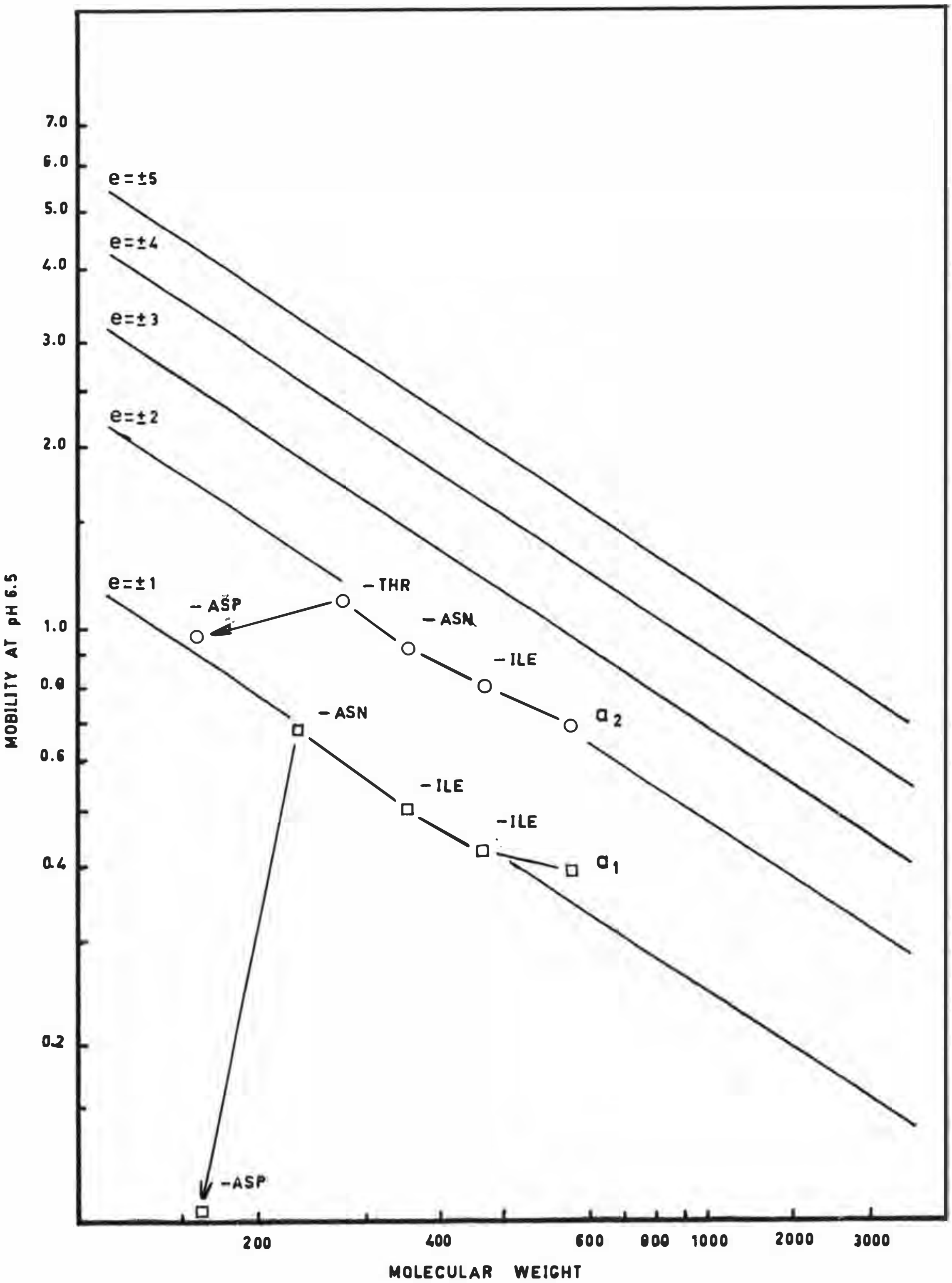


FIG. 3.16. DETERMINATION OF THE AMIDE AND ACID RESIDUES IN PEPTIDE $T_6GEbChTa_2$ BY THE OFFORD MOBILITY PROCEDURE.

3.4.7. Peptide T_7:

Peptide T_7 from fraction II of the Sephadex G-50 column elution profile (Fig. 3.2.) was rechromatographed on DEAE cellulose. The purified peak eluting from the DE-32 column was pooled (Fig. 3.4.). The amino acid composition was determined (Table 3.16.) and the yield of the peptide was 6 μmol.

Table 3.16. Amino acid composition of peptide T_7.

Amino acid	No. of residues
His	-
Lys	1.0
Arg	-
CMCys Asp	6.1
Thr	3.8
Ser	1.0
Glu	4.2
Pro	1.9
Gly	1.1
Ala	5.9
Val	4.8
Met	-
Ile	1.0
Leu	2.0
Tyr	4.0
Phe	1.0

A rapid Edman degradation of peptide T_7 provided the following N-terminal sequence:

N-terminal	Residue 2	Residue 3	Residue 4	Residue 5
Tyr	Val	Thr	Ile	Asx

Chymotrypsin digestion of peptide T_7:

Peptide T_7 (2 μmol) was digested with chymotrypsin and 100 nmol was used to map the chymotryptic peptides (Fig. 3.17.). The chymotryptic peptides were purified from the remainder of the digest (1.9 μmol) by electrophoresis. The peptide purification data and the amino acid compositions of the peptides are given in Table 3.17..

Table 3.17. Peptide isolation data and amino acid compositions of the chymotrypsin digest peptides from peptide T_7.

Peptide	ChTa$_1$	ChTa$_2$	ChTa$_3$	ChTa$_4$	ChTN$_1$	ChTN$_2$
Mobility:						
pH 6.5	-0.35	-0.35	-0.42	-0.42	-	-
pH 2.1	-	-	-	-	0.32	0.46
BAWP	0.39	0.57	0.43	0.57	0.67	0.76
Ninhydrin colour	R	R	Y	R	R	R
N-terminal	Asp	Tyr	Asn	Glx	Ala	Glx
Fluorescence	-	-	+	-	-	-
^{14}C (dpm/µmol)	-	-	-	-	-	-
Yield (nmol)	750	1000	1100	1050	600	700
Stained for:						
Tyr	-	+	-	+	+	-
Trp	-	-	+	-	-	-
Arg	-	-	-	-	-	-

Amino acid composition:

Amino acid.	Residues.					
His	-	-	-	-	-	-
Lys	1.0	-	-	-	-	-
Arg	-	-	-	-	-	-
CMCys Asp	} 2.1	1.1	2.0	1.2	-	-
Thr	-	1.8	-	-	1.7	-
Ser	-	-	-	-	-	1.0
Glu	-	-	1.0	1.0	1.1	1.0
Pro	-	-	-	1.0	-	0.9
Gly	1.3	-	-	-	-	-
Ala	3.2	-	-	-	2.0	1.0
Val	-	1.0	-	1.1	1.2	1.9
Met	-	-	-	-	-	-
Ile	-	1.0	-	-	-	-
Leu	-	-	-	-	1.0	1.0
Tyr	-	2.2	-	1.0	1.0	-
Phe	1.0	-	-	-	-	-

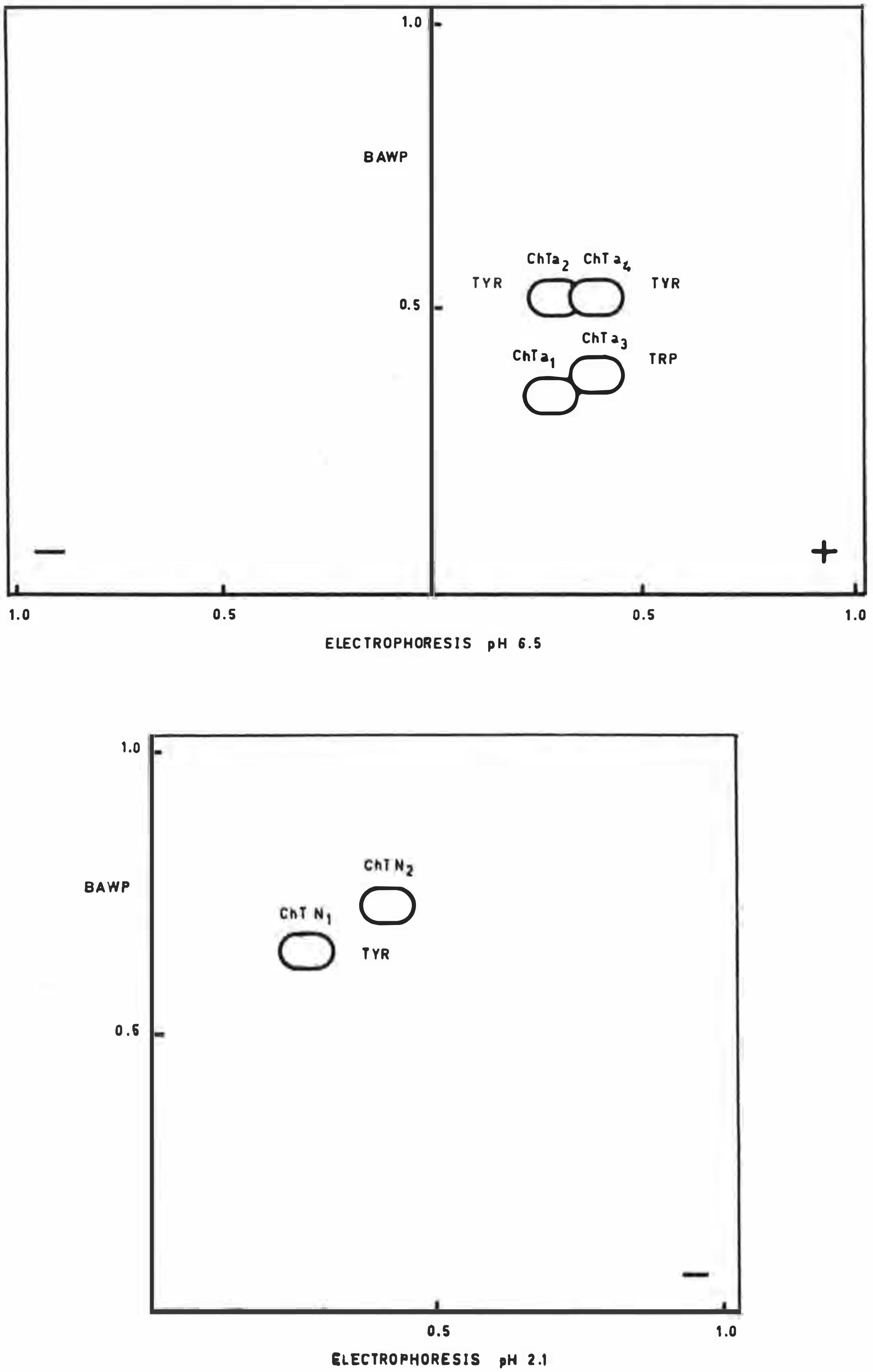


FIG. 3.17. CHYMOTRYPSIN DIGEST PEPTIDE MAPS OF PEPTIDE T_7.

S. aureus V8 protease digestion of peptide T_7:

Peptide T_7 (3 µmol) was digested with S. aureus V8 protease * and the peptides were separated on a Sephadex G-25 Pharmacia K-16 column (Fig. 3.18.). The peptide eluting in fraction GE_1 was subjected to a rapid Edman degradation to provide the N-terminal sequence:

N-terminal	Residue 2	Residue 3
-	Ala	Leu

Fraction GE_1 was further digested with pepsin and the peptic peptides were purified by paper electrophoresis to provide overlaps for the chymotryptic peptides. The peptide purification data and amino acid compositions of the peptic peptides from peptide GE_1 are given in Table 3.18. and Fig. 3.19. The two peptides eluting in fraction GE_{2+3} were further purified by paper electrophoresis for which the peptide data and amino acid compositions are given in Table 3.19. and Fig. 3.19..

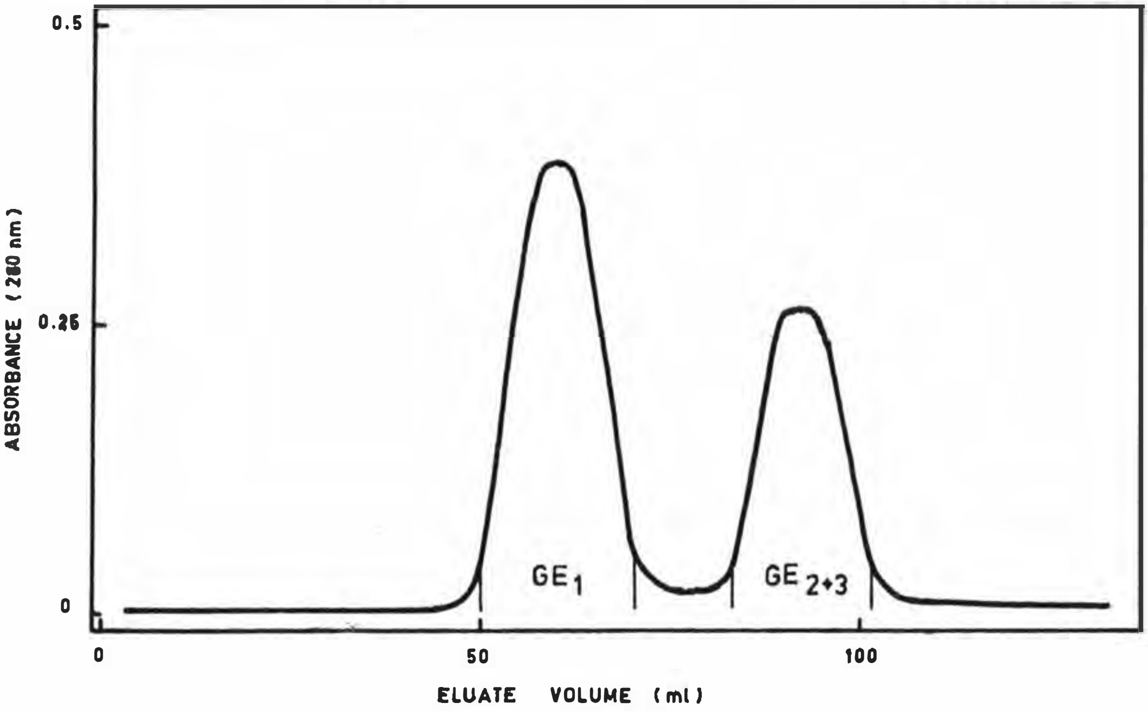


Fig. 3.18. Sephadex G-25 chromatography of peptides obtained by digestion of peptide T_7 with S. aureus V8 protease.

Table 3.18. Peptide isolation data and amino acid compositions of the pepsin digest peptides of peptide GE_1 that was obtained by S. aureus V8 protease digestion of peptide T_7.

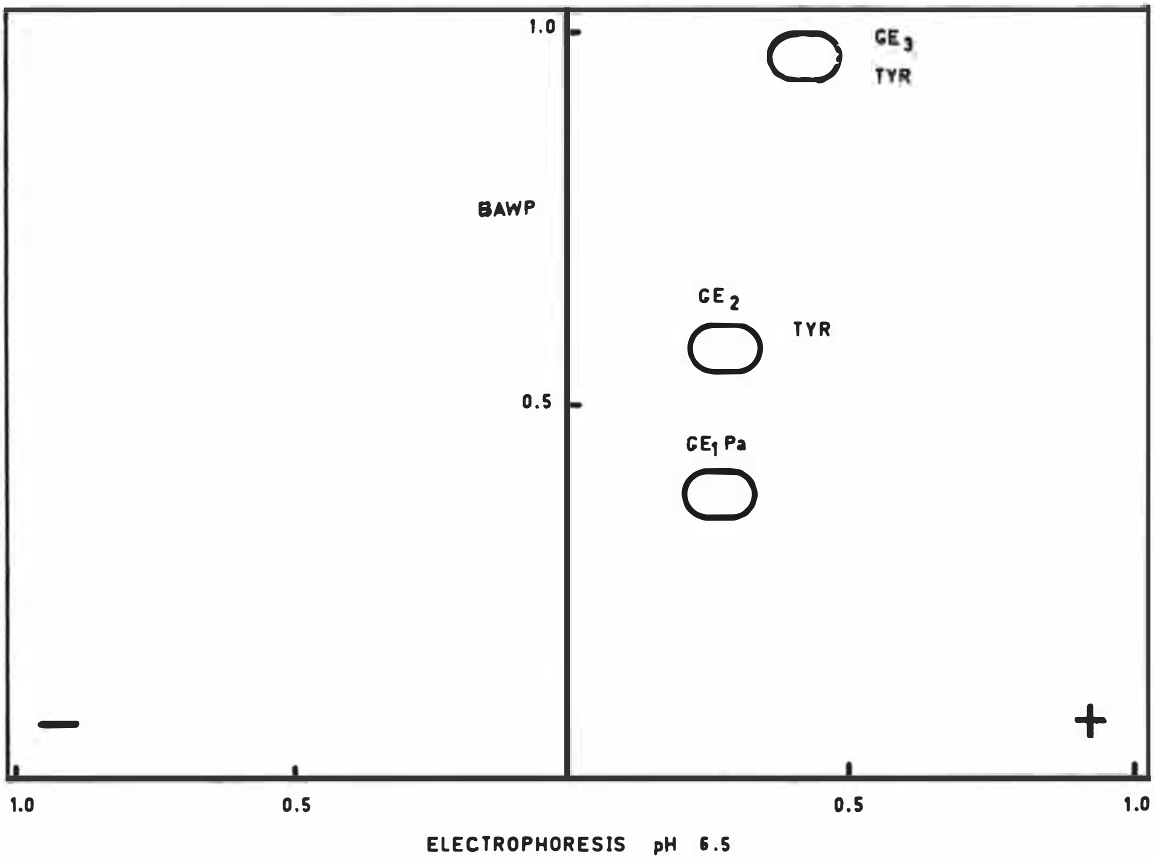
Peptide	GE_1Pa	GE_1PN_1	GE_1PN_2	G-25 fraction GE_1
Mobility:				
pH 6.5	-0.36	-	-	
pH 3.5	+0.57	-	-	
pH 2.1	-	0.32	0.60	
BAWP	0.42	0.77	1.00	
Ninhydrin colour	R	R	R	
N-terminal	Asp	Ala	Val	
Fluorescence	-	-	-	
^{14}C (dpm/µmol)	-	-	-	
Yield (nmol)	250	200	220	
Stained for:				
Tyr	-	+	-	
Trp	-	-	-	
Arg	-	-	-	
Amino acid composition:				
Amino acid.	Residues.			
His	-	-	-	-
Lys	1.0	-	-	1.0
Arg	-	-	-	-
CMCys Asp	} 2.1	-	-	2.0
Thr	-	1.0	-	1.8
Ser	-	0.9	-	0.9
Glu	-	1.0	-	1.9
Pro	-	0.8	-	0.8
Gly	1.2	-	-	1.1
Ala	2.9	1.0	1.0	5.5
Val	-	1.9	1.0	3.0
Met	-	-	-	-
Ile	-	-	-	-
Leu	-	-	1.0	1.9
Tyr	-	1.0	-	1.1
Phe	1.2	-	-	1.0

Table 3.19. Peptide isolation data and amino acid compositions of peptides GE_2 and GE_3 that were obtained by digestion of peptide T_7 with S. aureus V8 protease.

Peptide	GE_2	GE_3
Mobility:		
pH 6.5	-0.37	-0.48
pH 2.1	-	-
BAWP	0.61	1.00
Ninhydrin colour	Y	R
N-terminal	Asn	Tyr
Fluorescence	-	-
^{14}C (dpm/μmol)	-	-
Yield (nmol)	500	800
Stained for:		
Tyr	+	+
Trp	-	-
Arg	-	-

Amino acid composition:

Amino acid.	Residue.	
His	-	-
Lys	-	-
Arg	-	-
CMCys Asp	} 3.0	1.3
Thr	-	1.8
Ser	-	-
Glu	1.1	1.2
Pro	0.9	-
Gly	-	-
Ala	-	-
Val	1.0	1.0
Met	-	-
Ile	-	1.0
Leu	-	-
Tyr	1.0	2.0
Phe	-	-



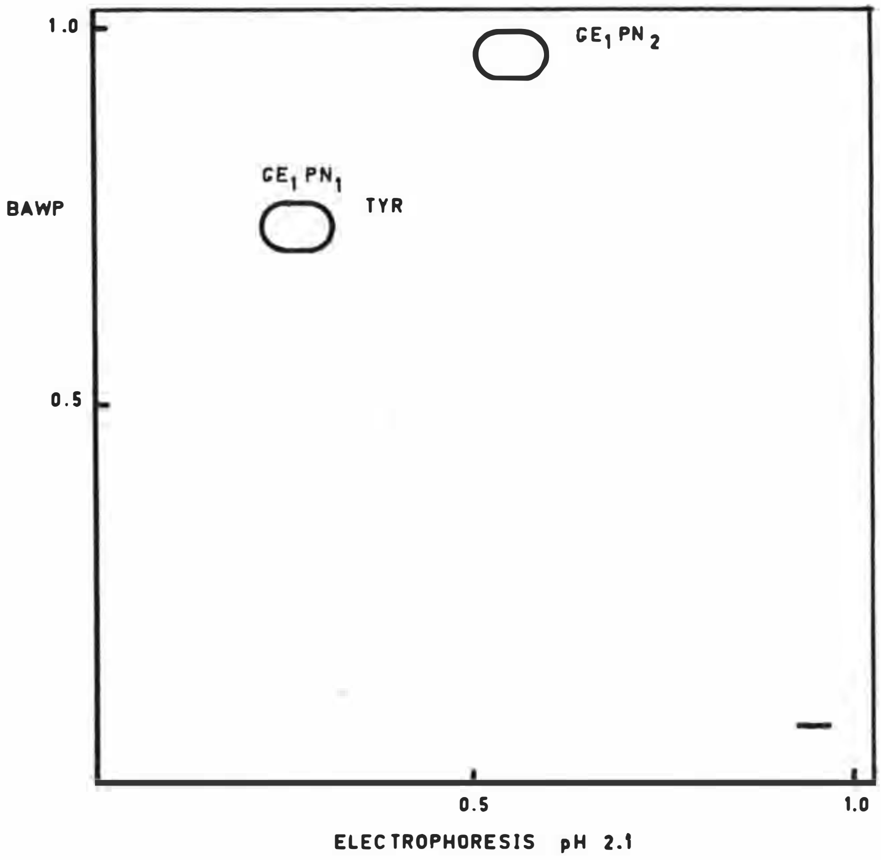


FIG. 3.19. COMPOSITE PEPTIDE MAPS OF S.AUREUS V8 PROTEASE AND PEPSIN DIGEST PEPTIDES FROM PEPTIDE T_7.

The peptides from the chymotrypsin, S. aureus V8 protease and pepsin digestions of peptide T_7 were sequenced and the T_7 peptide reconstructed (Fig. 3.20.).

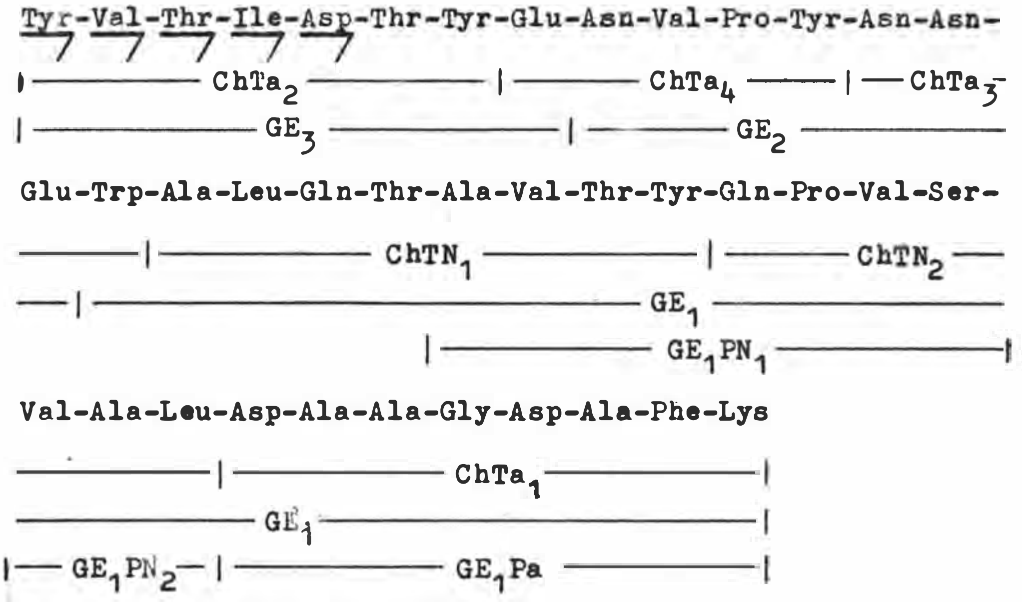


Fig. 3.20. Reconstruction of peptide T_7 from chymotrypsin, pepsin and S. aureus V8 protease digest peptides.

Amide and acid residues in peptide T_7 were confirmed by the Offord (1966) mobility procedure.

3.4.8. Peptide T_8:

Peptide T_8 from fraction III of the Sephadex G-50 column elution profile (Fig. 3.2.) was rechromatographed on DEAE cellulose. The purified peak eluting from the DE-32 column was pooled (Fig. 3.4.). The amino acid composition was determined (Table 3.20.) and the yield of the peptide was 6.5 µmol. The specific radioactivity (1.1×10^6 dpm/µmol) of peptide T_8 indicated that one cysteine residue was present in the peptide.

Table 3.20. Amino acid composition of peptide T_8.

Amino acid	No. of residues
His	1.0
Lys	1.0
Arg	-
CMCys Asp	3.0
Thr	2.8
Ser	1.0
Glu	2.0
Pro	0.9
Gly	7.2
Ala	2.9
Val	4.8
Met	-
Ile	4.0
Leu	-
Tyr	2.9
Phe	1.0

A rapid Edman degradation of peptide T_8 provided the following N-terminal sequence:

N-terminal	Residue 2	Residue 3
Glx	Tyr	Ala

Chymotrypsin digestion of peptide T_8:

Peptide T_8 (2 µmol) was digested with chymotrypsin and 100 nmol was used to map the peptides (Fig. 3.21.). The chymotryptic peptides were purified from the remainder of the digest (1.9 µmol) by paper electrophoresis. The peptide purification data and amino acid compositions of the peptides are given in Table 3.21..

Table 3.21. Peptide isolation data and amino acid compositions of the chymotrypsin digest peptides from peptide T_8.

Peptide	ChTa$_1$	ChTa$_2$	ChTN$_1$	ChTb
Mobility:				
pH 6.5	-0.33	-0.55	-	+0.50
pH 2.1	-	-	0.36	-
BAWP	0.52	0.77	0.65	0.59
Ninhydrin colour	O	Y	R	R
N-terminal	Thr	Gly	Glx	Ile
Fluorescence	-	+	-	-
^{14}C (dpm/μmol)	1.2×10^6	-	-	-
Yield (nmol)	600	800	150	500
Stained for:				
Tyr	+	+	+	-
Trp	-	+	-	-
Arg	-	-	-	-

Amino acid composition:

Amino acid.	Residues.			
His	1.0	-	-	-
Lys	-	-	-	1.3
Arg	-	-	-	-
CMCys Asp	} 1.9	1.2	-	-
Thr	2.4	1.0	-	-
Ser	-	-	0.9	-
Glu	-	1.1	1.0	-
Pro	1.0	-	-	-
Gly	3.0	3.0	1.1	-
Ala	2.1	-	1.0	-
Val	2.9	1.0	-	0.9
Met	-	-	-	-
Ile	1.8	-	0.9	1.0
Leu	-	-	-	-
Tyr	1.0	0.9	1.1	-
Phe	-	-	0.8	-

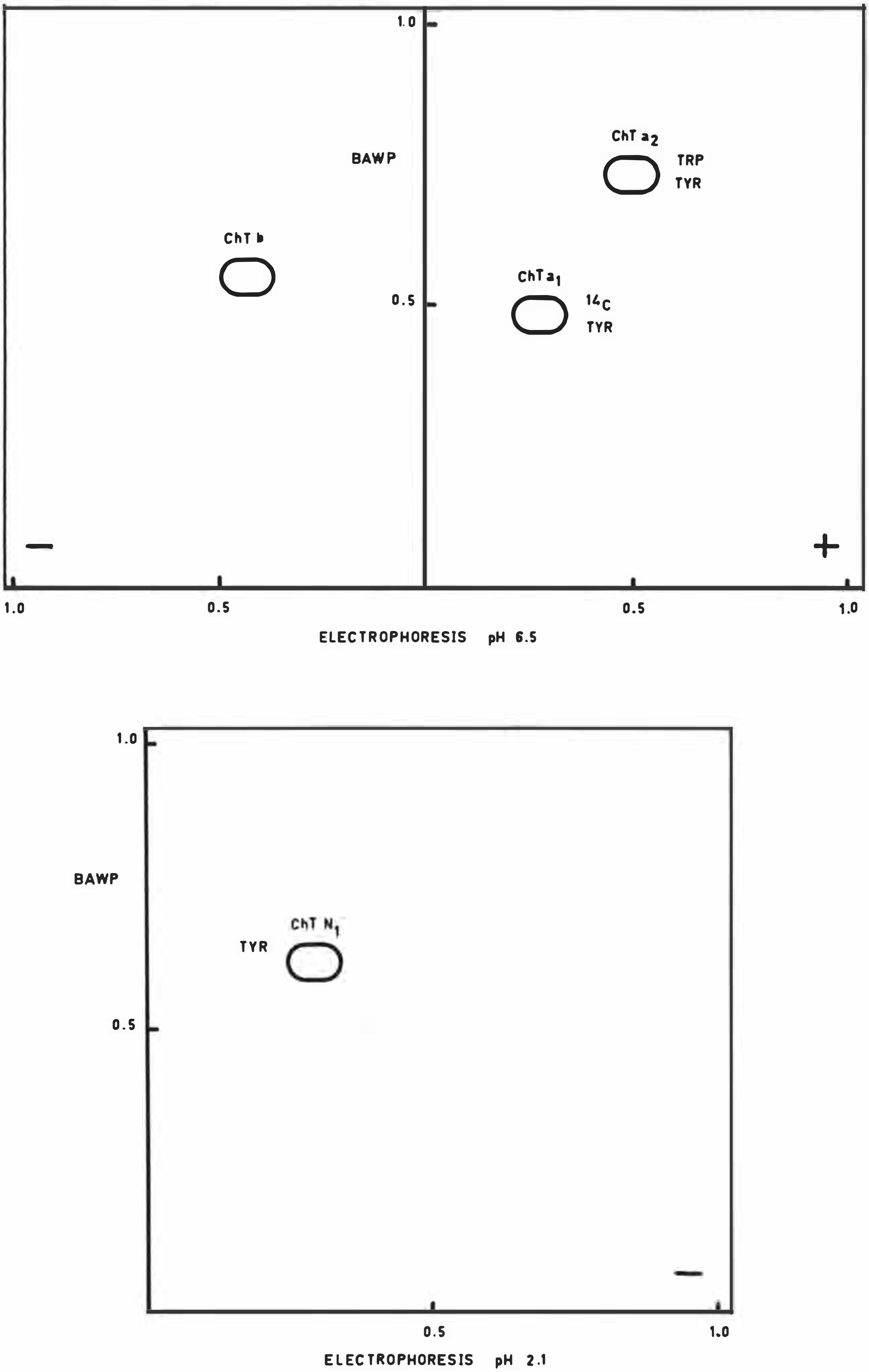


FIG. 3.21. CHYMOTRYPSIN DIGEST PEPTIDE MAPS OF PEPTIDE T_8.

S. aureus V8 protease digestion of peptide T_8:

Peptide T_8 (3 µmol) was digested with S. aureus V8 protease and the peptides were separated on a Sephadex G-25 Pharmacia K-16 column (Fig. 3.22.). The peptide eluting in fraction GE_1 was subjected to a rapid Edman degradation to provide the N-terminal sequence:

N-terminal	Residue 2	Residue 3
Glx	Tyr	Ala

Fraction GE_1 was further digested with pepsin and the peptic peptides were purified by paper electrophoresis to provide overlaps for the chymotryptic peptides. The peptide data and amino acid compositions of the peptic peptides are given in Table 3.22.. The peptide eluting in fraction GE_2 was further purified by paper methods. Peptide GE_2 was not mobile when subjected to electrophoresis at pH 6.5 or pH 2.1, but was mobile on paper in the BAWP chromatography solvent. The peptide was eluted from paper with BAWP, and the peptide purification data and amino acid composition of peptide GE_2 are given in Table 3.23..

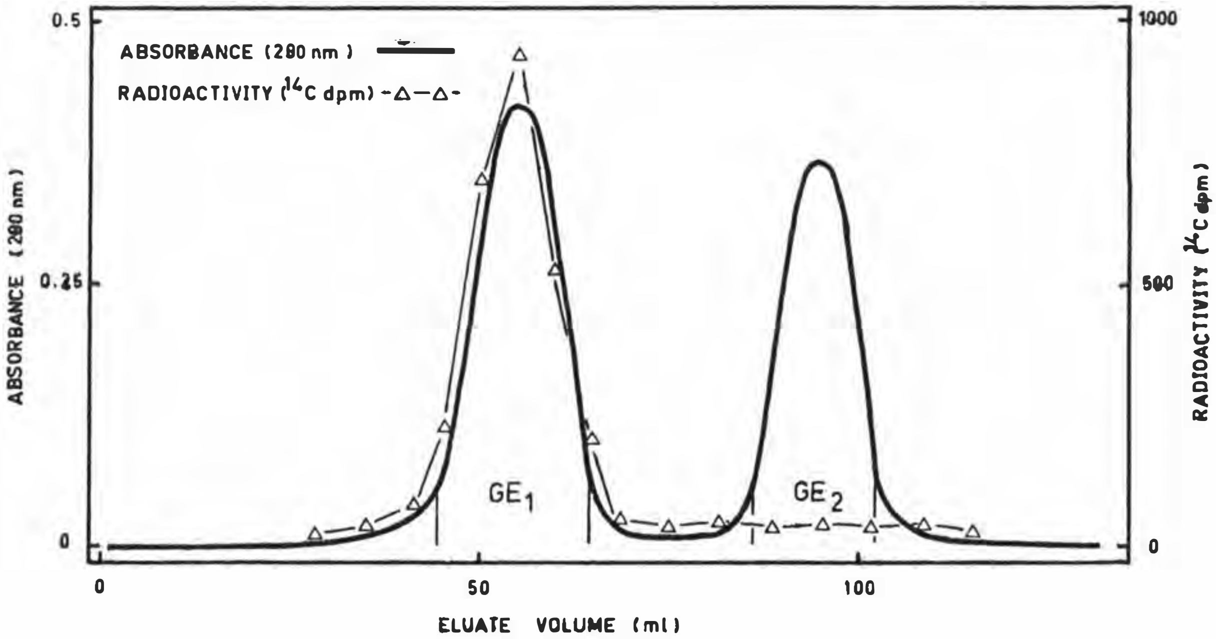


Fig. 3.22. Sephadex G-25 chromatography of peptides obtained by digestion of peptide T_8 with S. aureus V8 protease

Table 3.22. Peptide isolation data and amino acid compositions of the pepsin digest peptides from peptide GE_1 that was obtained from peptide T_8 by digestion with *S. aureus* V8 protease.

Peptide	GE_1Pa_1	GE_1Pa_2
Mobility:		
pH 6.5	-0.36	-0.53
pH 2.1	-	-
BAWP	0.74	0.74
Ninhydrin colour	R	R
N-terminal	Phe	Tyr
Fluorescence	-	-
^{14}C (dpm/µmol)	1.1×10^6	-
Yield (nmol)	270	200
Stained for:		
Tyr	-	+
Trp	-	-
Arg	-	-

Amino acid composition

Amino acid	No. of residues GE_1Pa_1	 GE_1Pa_2
His	1.0	-
Lys	-	-
Arg	-	-
CMCys Asp	1.8	-
Thr	1.9	1.0
Ser	-	-
Glu	-	1.0
Pro	1.0	-
Gly	2.0	1.1
Ala	1.9	-
Val	2.2	-
Met	-	-
Ile	1.0	-
Leu	-	-
Tyr	-	1.0
Phe	1.0	-

Table 3.23. Peptide isolation data and amino acid composition of peptide GE_2 that was obtained from peptide T_8 by digestion with *S. aureus* V8 protease.

Peptide	GE_2
Mobility:	
pH 6.5	-
pH 2.1	-
BAWP	0.83
Ninhydrin colour	Y
N-terminal	Gly
Fluorescence	+
^{14}C (dpm/µmol)	-
Yield (nmol)	300
Stained for:	
Tyr	+
Trp	+
Arg	-

Amino acid composition

Amino acid	No. of residues
His	-
Lys	0.9
Arg	-
CMCys Asp	1.0
Thr	-
Ser	-
Glu	-
Pro	-
Gly	2.0
Ala	-
Val	1.8
Met	-
Ile	1.1
Leu	-
Tyr	1.1
Phe	-

The peptides derived from peptide T_8 were sequenced and the T_8 peptide reconstructed. Two peptides isolated from the pepsin digest of peptide GE_1 were sufficient to provide overlaps for the chymotryptic peptides of peptide T_8 (Fig. 3. 23.).

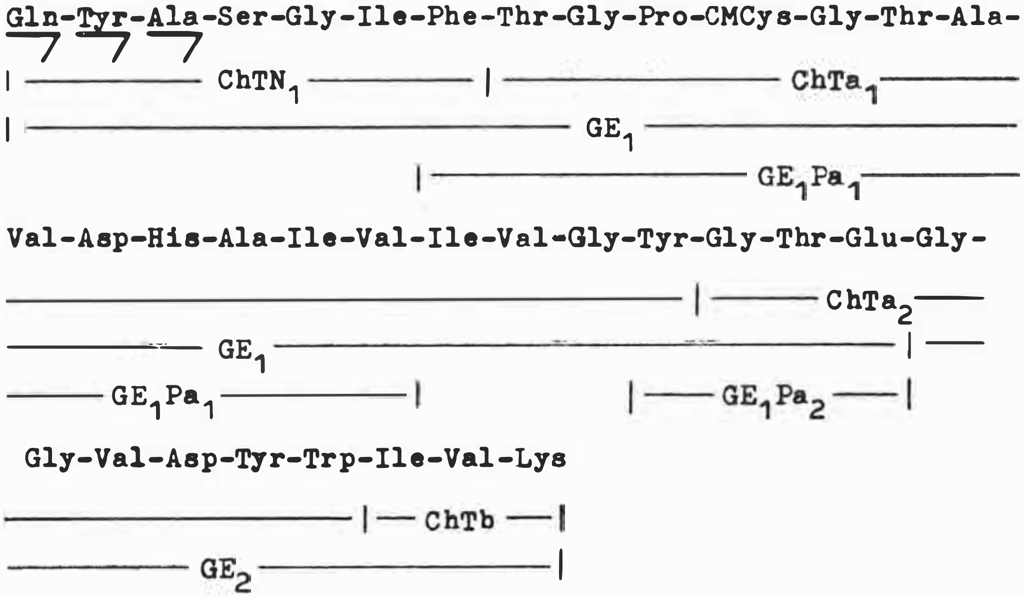


Fig. 3.23. Reconstruction of peptide T_8 from chymotrypsin, pepsin and S. aureus V8 protease digest peptides.

Amide and acid residues in peptide T_8 were confirmed by the Offord (1966) mobility procedure.

3.4.9. Peptide T_9:

Peptide T_9 was isolated from fraction VI of the Sephadex G-50 column elution profile (Fig. 3.2.). Peptide T_9 was purified by paper electrophoresis (Fig. 3.3.). The peptide purification data and amino acid composition of peptide T_9 are given in Table 3.24..

Table 3.24. Peptide isolation data and amino acid composition of peptide T_9.

Peptide	T_9	Amino acid	No. of residues
Mobility:		His	-
pH 6.5	-0.37	Lys	-
pH 2.1	-	Arg	1.0
BAWP	0.52	CMCys Asp	1.9
Ninhydrin colour	Y	Thr	1.9
N-terminal	Asn	Ser	1.0
Fluorescence	+	Glu	2.0
^{14}C (dpm/μmol)	-	Pro	-
Yield (μmol)	4.5	Gly	2.0
Stained for:		Ala	-
Tyr	+	Val	-
Trp	+	Met	0.9
Arg	+	Ile	-
		Leu	-
		Tyr	1.1
		Phe	-

Peptide T_9 was initially sequenced by the dansyl-Edman procedure.

Chymotrypsin digestion of peptide T_9:

Peptide T_9 (2 μmol) was digested with chymotrypsin and the peptides were purified by paper electrophoresis. The amino acid compositions and the sequences of the chymotryptic peptides were determined (Table 3.25.) to provide information on the location of the tryptophan residues and to further confirm the sequence.

Table 3.25. Peptide isolation data and amino acid compositions of the chymotrypsin digest peptides from peptide T_9.

Peptide	ChTa$_1$	ChTa$_2$	ChTN	ChTb
Mobility:				
pH 6.5	-0.43	-0.75	-	+0.48
pH 2.1	-	-	0.48	-
Ninhydrin colour	R	Y	Y	R
N-terminal	Asp	Gly	Asn	Met
Fluorescence	+	-	+	-
^{14}C (dpm/µmol)	-	-	-	-
Yield (nmol)	350	600	400	700
Stained for:				
Tyr	-	+	-	-
Trp	+	-	+	-
Arg	-	-	-	+

Amino acid composition:

Amino acid.	Residues.			
His	-	-	-	-
Lys	-	-	-	-
Arg	-	-	-	1.0
CMCys Asp	1.0	-	1.0	-
Thr	1.9	-	-	-
Ser	-	-	0.8	-
Glu	-	2.0	-	-
Pro	-	-	-	-
Gly	-	2.1	-	-
Ala	-	-	-	-
Val	-	-	-	-
Met	-	-	-	0.9
Ile	-	-	-	-
Leu	-	-	-	-
Tyr	-	1.0	-	-
Phe	-	-	-	-

The sequence of peptide T_9 was confirmed by the sequences of the chymotrypsin digest peptides to be:

Asn-Ser-Trp-Asp-Thr-Thr-Trp-Gly-Glu-Glu-Gly-Tyr-Met-Arg

|— ChTN —|— ChTa$_1$ —|— ChTa$_2$ —|-ChTb—|

Amide and acid residues in peptide T_9 were confirmed by the Offord (1966) mobility procedure.

3.4.10. Peptide T_{10}:

Peptide T_{10} was isolated from fraction VII of the Sephadex G-50 column elution profile (Fig. 3.2.). Peptide T_{10} was purified by paper electrophoresis (Fig. 3.3.). The peptide purification data and amino acid composition of peptide T_{10} are given in Table 3.26..

Table 3.26. Peptide isolation data and amino acid composition of peptide T_{10}.

Peptide	T_{10}
Mobility:	
pH 6.5	+0.50
pH 2.1	-
BAWP	0.82
Ninhydrin colour	R
N-terminal	Ile
Fluorescence	-
^{14}C (dpm/μmol)	-
Yield (μmol)	6.2
Stained for:	
Tyr	-
Trp	-
Arg	+

Amino acid composition	
Amino acid	No. of residues
His	-
Lys	-
Arg	1.0
CMCys Asp	-
Thr	-
Ser	-
Glu	-
Pro	-
Gly	-
Ala	-
Val	-
Met	-
Ile	1.0
Leu	1.0
Tyr	-
Phe	-

Peptide T_{10} was sequenced by the dansyl-Edman method to give the sequence:

$$\underset{\rightharpoondown}{\text{Ile}}\text{-}\underset{\rightharpoondown}{\text{Leu}}\text{-}\underset{\rightharpoondown}{\text{Arg}}$$

The charge on peptide T_{10} was confirmed by the Offord (1966) mobility procedure.

3.4.11. Peptide T_{11}:

Peptide T_{11} from fraction IV of the Sephadex G-50 column elution profile (Fig. 3.2.) was rechromatographed on DEAE cellulose. The first peak of the fraction IV material eluting from the DE-32 column was pooled (Fig. 3.4.). The peptide was mobile by paper electrophoresis and chromatography. The peptide data and amino acid composition are given in Table 3.27.. The specific radioactivity (1.3×10^6 dpm/µmol) of peptide T_{11} indicated that one cysteine residue was present in the peptide.

Table 3.27. Peptide isolation data and amino acid composition of peptide T_{11}.

Peptide	T_{11}
Mobility:	
pH 6.5	-
pH 2.1	0.47
BAWP	0.44
Ninhydrin colour	Y
N-terminal	Asn
Fluorescence	-
^{14}C (dpm/µmol)	1.3×10^6
Yield (µmol)	5.4
Stained for:	
Tyr	+
Trp	-
Arg	-

Amino acid composition	
Amino acid	No. of residues
His	-
Lys	1.0
Arg	-
CMCys	2.0
Asp	
Thr	1.8
Ser	0.9
Glu	-
Pro	1.9
Gly	4.3
Ala	2.3
Val	2.0
Met	0.9
Ile	1.2
Leu	-
Tyr	1.3
Phe	-

A rapid Edman degradation of peptide T_{11} provided the following N-terminal sequence:

N-terminal	Residue 2	Residue 3	Residue 4	Residue 5
Asn	Val	Gly	Gly	Ala

Thermolysin digestion of peptide T_{11}:

Peptide T_{11} was digested with thermolysin and the peptides purified by paper electrophoresis. The peptide isolation data and amino acid compositions of the peptides are given in Table 3.28. and Fig 3.24.. 2 µmol of the peptide was digested.

Table 3.28. Peptide isolation data and amino acid compositions of the thermolysin digest peptides from peptide T_{11}.

Peptide	Tha	ThN	Thb
Mobility:			
pH 6.5	-0.43	-	+0.62
pH 2.1	-	0.36	-
BAWP	0.46	0.87	0.47
Ninhydrin colour	Y	R	R
N-terminal	Asn	Ile	Val
Fluorescence	-	-	-
^{14}C (dpm/μmol)	1.3×10^6	-	-
Yield (nmol)	1400	1200	700
Stained for:			
Tyr	-	+	-
Trp	-	-	-
Arg	-	-	-

Amino acid composition:

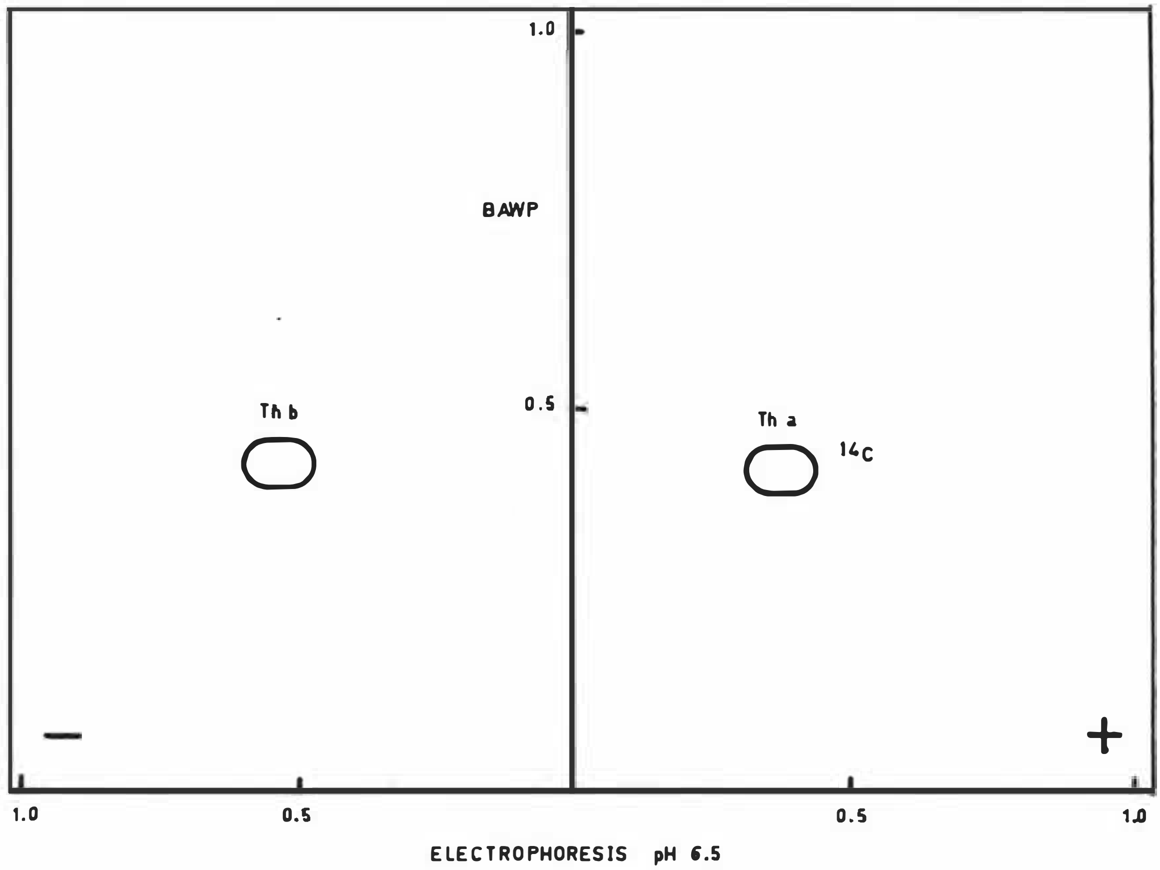
Amino acid.	Residues.		
His	-	-	-
Lys	-	-	1.0
Arg	-	-	-
CMCys Asp	1.9	-	-
Thr	0.8	0.9	-
Ser	-	0.8	-
Glu	-	-	-
Pro	-	1.8	-
Gly	4.3	-	-
Ala	1.1	1.0	-
Val	1.0	-	1.0
Met	-	0.9	-
Ile	-	1.1	-
Leu	-	-	-
Tyr	-	1.1	-
Phe	-	-	-

The thermolysin digest peptides were sequenced by the dansyl Edman method and the sequence of peptide T_{11} reconstructed:

Asn-Val-Gly-Gly-Ala-Gly-Thr-CMCys-Gly-Ile-Ala-Thr-Met-Pro-Ser-Tyr-Pro-Val-Lys

| —— Tha —— | —— ThN —— | -Thb — |

The absence of acid residues from peptide T_{11} was confirmed by the Offord (1966) mobility procedure.



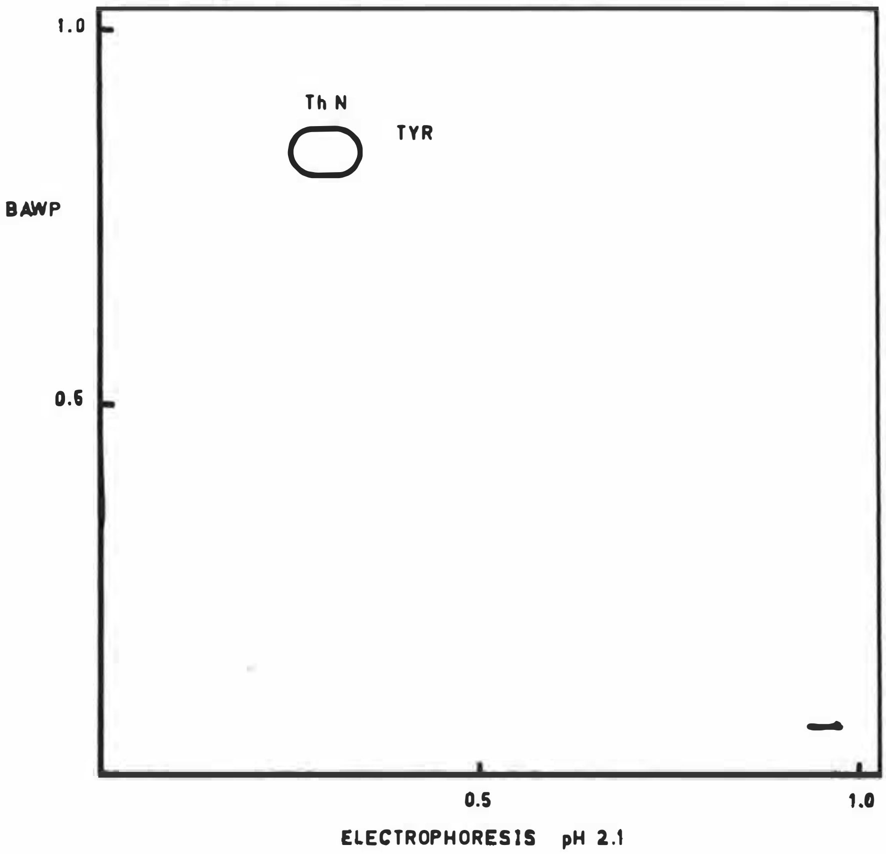


FIG.3.24. THERMOLYSIN DIGEST PEPTIDE MAPS OF PEPTIDE T_{11}.

3.4.12. Peptide T_{12}:

Peptide T_{12} was isolated from fraction VII of the Sephadex G-50 column elution profile (Fig. 3.2.). Peptide T_{12} was purified by paper electrophoresis (Fig. 3.3.). The peptide purification data and amino acid composition of peptide T_{12} are given in Table 3.29..

Table 3.29. Peptide isolation data and amino acid composition of peptide T_{12}.

Peptide	T_{12}
Mobility:	
pH 6.5	-
pH 2.1	0.50
BAWP	0.48
Ninhydrin colour	R
N-terminal	Tyr
Fluorescence	-
^{14}C (dpm/μmol)	-
Yield (μmol)	5.0
Stained for:	
Tyr	+
Trp	-
Arg	-

Amino acid composition

Amino acid	No. of residues
His	-
Lys	-
Arg	-
CMCys Asp	2.0
Thr	-
Ser	-
Glu	-
Pro	-
Gly	-
Ala	-
Val	-
Met	-
Ile	-
Leu	-
Tyr	1.0
Phe	-

Peptide T_{12} was sequenced by the dansyl-Edman method to give the sequence:

$$\underset{\rightharpoondown}{\underline{\text{Tyr}}}-\underset{\rightharpoondown}{\underline{\text{Asn}}}-\underset{\rightharpoondown}{\underline{\text{Asn}}}$$

The absence of acid residues from peptide T_{12} was confirmed by the Offord (1966) mobility procedure.

Section 3.5. Determination of the C-terminal region of actinidin:

3.5.1. Determination of the C-terminal region of actinidin from the cyanogen bromide fragments:

Carboxy[$^{14}C_2$]methyl actinidin (5 µmol) was reacted with cyanogen bromide and the fragments were subjected to gel filtration on a Sephadex G-50 Pharmacia K-26 column (Fig. 3.25.). Fraction CB-I was radioactive and eluted at the exclusion limit of the gel column. Fraction CB-II+III was radioactive and was pooled for rechromatography on a Sephadex G-25 Pharmacia K-26 column which resolved the material into two peaks, CB-II and CB-III (Fig. 3.26). Fraction CB-III was pure by electrophoresis at pH 6.5 and was not radioactive. Fraction CB-II resolved into two components (CB-IIa$_1$ and CB-IIa$_2$) on electrophoresis at pH 6.5, and both components were radioactive. The peptide purification data and amino acid compositions of peptides CB-IIa$_1$,CB-IIa$_2$ and CB-III are given in Table 3.30..

Table 3.30. Peptide isolation data and amino acid compositions of cyanogen bromide fragments from actinidin.

Peptide	CB-IIa$_1$	CB-IIa$_2$	CB-III
Mobility: pH 6.5	+0.15	+0.29	+0.27
Ninhydrin colour	R	R	R
N-terminal	Arg	Arg	Pro
Fluorescence	-	-	-
^{14}C (dpm/µmol)	1.1×10^6	1.2×10^6	-
Yield (µmol)	0.3	2.0	2.5
Stained for:			
Tyr	-	-	+
Trp	-	-	-
Arg	+	+	-

Amino acid composition

Amino acid	No. of residues CB-IIa$_1$	CB-IIa$_2$	CB-III
His	-	-	-
Hse	0.3	0.3	-
Lys	-	-	1.1
Arg	2.0	2.0	-
CMCys Asp	1.8	1.8	2.1
Thr	1.8	1.9	-
Ser	-	-	1.0
Glu	-	-	-
Hse	0.5	0.5	-
Pro	-	-	2.0
Gly	4.1	4.0	-
Ala	2.3	2.3	-
Val	1.0	1.0	1.2
Met	-	-	-
Ile	1.8	1.9	-
Leu	1.0	1.0	-
Tyr	-	-	1.9
Phe	-	-	-

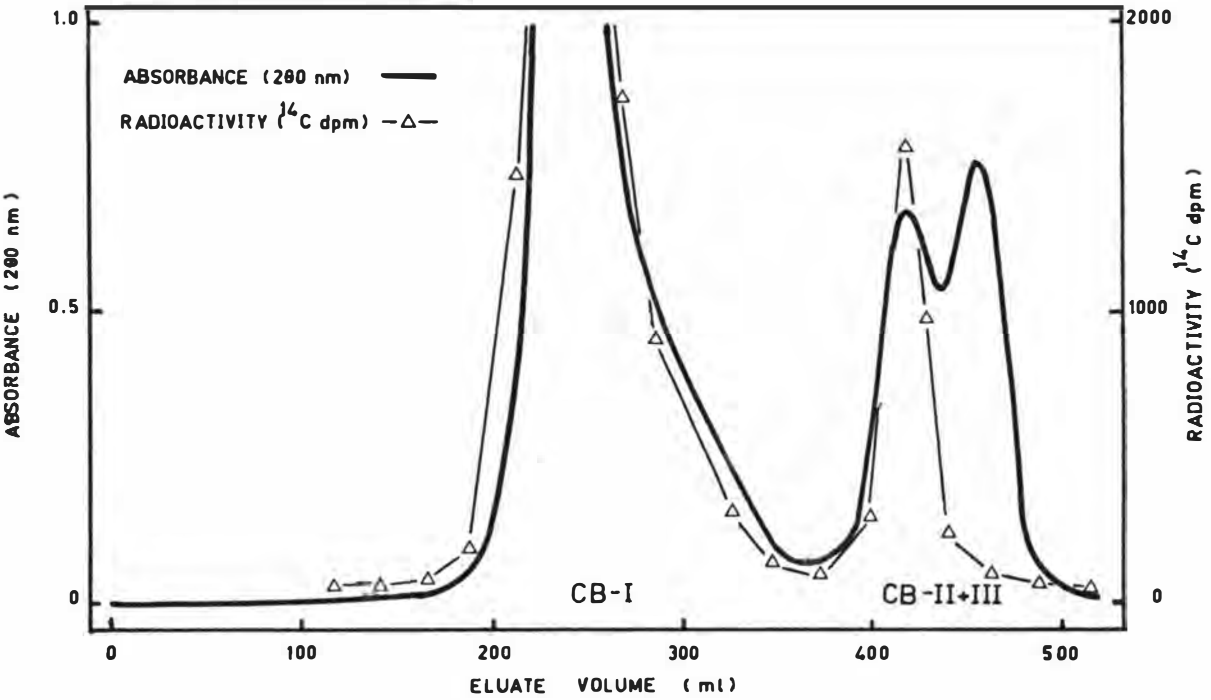


FIG.3.25. SEPHADEX G-50 CHROMATOGRAPHY OF THE CYANOGEN BROMIDE FRAGMENTS FROM CARBOXYMETHYL ACTINIDIN.

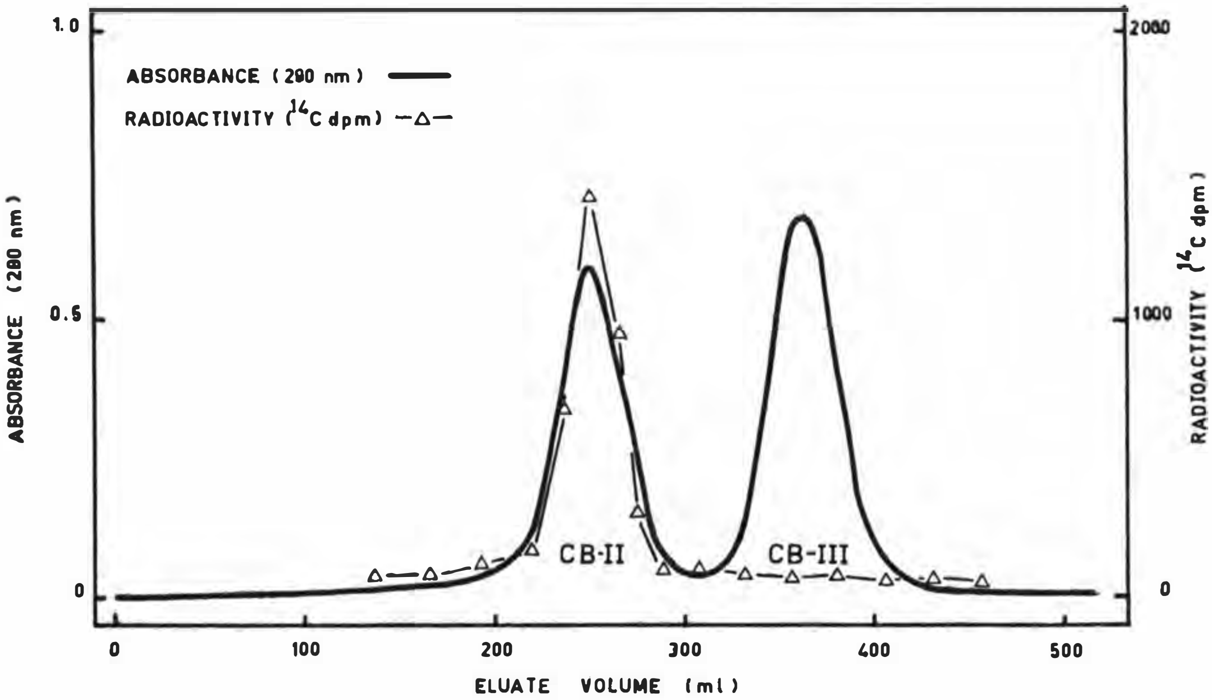


FIG.3.26. SEPHADEX G-25 CHROMATOGRAPHY OF THE CYANOGEN BROMIDE SEPHADEX G-50 FRACTION CB-II+III.

Cyanogen bromide fragment CB-III was sequenced by the dansyl Edman method to provide the sequence:

Pro-Ser-Tyr-Pro-Val-Lys-Tyr-Asn-Asn
⌝ ⌝ ⌝ ⌝ ⌝ ⌝ ⌝ ⌝ ⌝

Cyanogen bromide fragment CB-IIa$_2$ (200 nmol) was sequenced by the dansyl-Edman method for the first six residues which gave the sequence:

Arg-Ile-Leu-Arg-Asn-Val
⌝ ⌝ ⌝ ⌝ ⌝ ⌝

1.8 μmol of fragment CB-IIa$_2$ was digested with chymotrypsin and the peptides were separated by electrophoresis (Table 3.31.).

Table 3.31. Peptide isolation data and amino acid compositions of the chymotrypsin digest peptides from cyanogen bromide fragment CB-IIa$_2$.

Peptide	ChTa	ChTb
Mobility: pH 6.5	-0.30	+0.45
Ninhydrin colour	R	R
N-terminal	Arg	Arg
Fluorescence	-	-
^{14}C (dpm/μmol)	1.1x10^6	-
Yield (nmol)	500	600
Stained for:		
Tyr	-	-
Trp	-	-
Arg	+	+

Amino acid composition

Amino acid	No. of residues ChTa	 ChTb
His	-	-
$\overset{\frown}{\text{Hse}}$	0.2	-
Lys	-	-
Arg	1.0	1.0
CMCys Asp	1.9	-
Thr	1.8	-
Ser	-	-
Glu	-	-
Hse	0.5	-
Pro	-	-
Gly	4.2	-
Ala	2.1	-
Val	1.1	-
Met	-	-
Ile	0.9	0.9
Leu	-	0.9
Tyr	-	-
Phe	-	-

Peptides ChTa and ChTb from the cyanogen bromide fragment CB-IIa$_2$ were sequenced by the dansyl-Edman method to provide the sequence:

$$\underset{\rightharpoondown}{\text{Arg}}\text{-}\underset{\rightharpoondown}{\text{Ile}}\text{-}\underset{\rightharpoondown}{\text{Leu}}\text{-}\underset{\rightharpoondown}{\text{Arg}}\text{-}\underset{\rightharpoondown}{\text{Asn}}\text{-}\underset{\rightharpoondown}{\text{Val}}\text{-Gly-Gly-Ala-Gly-Thr-CMCys-Gly-Ile-Ala-Thr-Met}$$

|— ChTb —| ——————————— ChTa ——————————— |

Cyanogen bromide fragment CB-IIa$_1$ was found to have the same sequence.

Amide and acid residues in the cyanogen bromide fragments were confirmed by the Offord (1966) mobility procedure.

3.5.2. Confirmation of the C-terminal region of actinidin with carboxypeptidase A:

Three samples of actinidin (300 nmol per sample) were treated so that one sample acted as control, one sample was dithiothreitol activated and the other sample was acid denatured. An aliquot from each treated sample was digested with carboxypeptidase A.

The reaction products were analysed by polyacrylamide gel electrophoresis and by amino acid analysis.

The gel electrophoresis results showed a single band in the control and the dithiothreitol activated samples which indicated that actinidin did not autolyse under these conditions.

Amino acid analysis results showed no amino acids had been released from the control or dithiothreitol activated actinidin sample, but from the acid denatured actinidin sample, the amino acid residues released were: 2 asparagine, 1 tyrosine, 1 lysine and 1 valine.

Section 3.6. Determination of the amino acid sequence about the active site cysteine residue in actinidin:[a]

Carboxy[$^{14}C_2$]methyl actinidin (10 μmol)[b] that was radioactively labelled with ^{14}C only at the active site cysteine residue, was digested with trypsin and subjected to gel filtration on a Sephadex G-50 column to give an elution profile the same as that in Fig. 3.2. In this case, Fraction IV of the Sephadex G-50 column elution profile was the only radioactive fraction, which was pooled and rechromatographed on DEAE-cellulose to resolve the radioactive peptide, which corresponded to peptide T_3 (elution profile as for Fig. 3.4.).

The radioactive peptide was digested with chymotrypsin and the radioactive peptide from the chymotrypsin digest was purified by paper electrophoresis. This radioactive peptide had the same electrophoretic mobility and amino acid composition as peptide $ChTa_1$ (Section 3.4.3., peptide T_3, page 51), but had a specific radioactivity (dpm/μmol) that indicated only one radioactive cysteine residue was present in the peptide.

The peptide was sequenced by the dansyl-Edman procedure and the butyl acetate extract after each Edman cycle was analysed for radioactivity. In this way, the cysteine residue at the active site of actinidin and the sequence about the active site cysteine residue was shown to be:

$$\underrightarrow{\text{Ser}}\text{-}\underrightarrow{\text{Gln}}\text{-}\underrightarrow{\text{Gly}}\text{-}\underrightarrow{\text{Glu}}\text{-}\underrightarrow{\text{CMCys}}\text{-}\underrightarrow{\text{Gly}}\text{-}\underrightarrow{\text{Gly}}\text{-}\underrightarrow{\overset{*}{\text{CMCys}}}\text{-}\underrightarrow{\text{Trp}}$$

where * denotes the radioactively labelled residue.

---

[a] Dithiothreitol or β-mercaptoethanol do not reduce the disulphide bridges of actinidin in the absence of guanidine hydrochloride or urea at 4°C.

[b] 10 μmol actinidin of specific activity 1.4×10^6 dpm/μmol.

CHAPTER IV

DISCUSSION

Section 4.1. The purification of actinidin:

The molecular weights for actinidin of 15,400 and 12,800, obtained by gel filtration, for the enzyme prepared in an unblocked form (McDowall, 1970), were considerably lower than the value of 26,000 obtained by Boland and Hardman (1972) for actinidin prepared as the reversibly inactivated S-sulphenyl thiosulphate derivative. Actinidin was therefore prepared as the S-sulphenyl thiosulphate derivative to prevent any possibility of the enzyme undergoing autolysis.

Polyacrylamide gel electrophoresis of crystallized actinidin prepared according to the method given in Section 2.1. (page 16) provided a single band indicating that the purified enzyme was not contaminated by other proteins of a different molecular weight and charge.

Further evidence for the enzyme purity was provided by dansylation of crystallized actinidin, which indicated a single N-terminal leucine residue.

Section 4.2. The amino acid composition of actinidin:

Actinidin is reported to be an anionic protease (McDowall, 1970). The amino acid composition of actinidin, as determined by amino acid analysis and confirmed by the determination of the primary sequence in this study, shows that actinidin contains a high proportion of acidic amino acids relative to the number of basic amino acids (Table 3.2., page 39). The number of tryptophan residues in actinidin as determined colorimetrically by the method of Scoffone *et al* (1968) was found to be 6, which is in agreement with the number of tryptophan residues found in the sequences of the tryptic peptides.

In Table 4.1. the amino acid composition of actinidin is compared with the amino acid compositions of some plant thiol proteases. Papain and ficin contain a higher proportion of basic and a lower proportion of acidic residues than does actinidin, which is in agreement with actinidin being a more acidic protein than either papain or ficin. Although chymopapain and stem bromelain have higher molecular weights than papain, these enzymes have similar proportions of basic and acidic residues. Fruit bromelain is reported to be an acidic protein and has similar numbers of basic and acidic residues to actinidin, which may indicate that plant thiol proteases derived from latex sources are more basic than those derived from fruit sources. The proportions of hydrophobic and aromatic residues are similar in all of these proteins (Table 4.1.), but there is some variation in cysteine residue content, and it is therefore likely that there is a variation in the number of disulphide bridges present in these proteins.

Section 4.3. Purification of the tryptic peptides of actinidin:

The low proportion of basic amino acid residues in actinidin (6 lysine and 5 arginine) prompted the determination of the sequence of actinidin from the tryptic peptides. This approach was chosen because a tryptic digest of actinidin would produce a relatively small number of peptides (twelve) making the fractionation and purification of each peptide a less complex task. Digestion of actinidin with a less specific protease, such as chymotrypsin or thermolysin, would have resulted in a considerably more complex peptide mixture to fractionate due to the larger number of aromatic and hydrophobic residues.

Table 4.1. Amino acid composition of some plant thiol proteases.

Amino acid	Number of residues					
	Actinidin[a]	Papain[b]	Ficin[c]	Chymo-papain B[d]	Stem bromelain[e]	Fruit bromelain[e] [f]
His	1	2	1	5	2	1.4
Lys	6	10	5	25	23	7.8
Arg	5	12	10	10	12	8.6
Asp	17	6	17	27	29	29.8
Asn	12	13				
Thr	18	8	8	16	14	13.5
Ser	12	13	14	21	28	32.2
Glu	10	8	25	29	23	23.2
Gln	10	12				
Pro	7	10	11	14	14	11.6
Gly	28	28	28	39	35	32.6
Ala	18	14	20	19	35	23.8
Cys[g]	7	7	8	11	10	10.0
Val	17	18	18	25	22	19.8
Met	2	-	5	1	5	6.0
Ile	17	12	7	12	21	16.4
Leu	8	11	15	15	10	10.0
Tyr	14	19	15	20	21	22.4
Phe	5	4	5	7	9	7.6
Trp	6[h]	5	6	6	8	5.6
Total	220	212	218	302	321	263

[a] Values are based on the primary sequence (Fig. 4.1.)

[b] Values are based on the primary sequence (Fig. 4.4.)

[c] Data from Englund et al, 1968.

[d] Amino acid composition of chymopapain fraction IV taken to nearest integer (Kunimitsu and Yasunobu, 1967).

[e] Data from Ota et al, 1964.

[f] For comparison with stem bromelain, the data for fruit bromelain are expressed as mole ratios with leucine set at 10.

[g] Combined total of cysteine plus $\frac{1}{2}$-cystine.

[h] Determined independently by the method of Scoffone et al (1968), and confirmed by the primary sequence.

Sephadex G-50 gel chromatography of S-carboxymethylated actinidin that had been digested with trypsin, resolved the tryptic peptides into seven fractions (Fig. 3.2., pages 41,42). The first four fractions were found to contain peptides that were too large to enable purification by paper techniques. These peptides were purified on DEAE-cellulose (Fig. 3.4., pages 45,46). The last three fractions contained peptides that could be separated by paper techniques (Fig. 3.3., pages 43, 44) into six pure peptides which were sequenced directly by the dansyl Edman method (Section 2.8.2., page 31). The remaining six peptides that were purified by DEAE-cellulose required further degradation with either chymotrypsin, thermolysin, pepsin or S. aureus V8 protease to produce smaller peptides that could be sequenced by the dansyl-Edman method. These smaller peptides were then aligned to reconstruct the sequence of each tryptic peptide.

S. aureus V8 protease was particularly useful for the determination of amide residues because of the enzyme specificity for the carboxyl group of glutamic acid in ammonium bicarbonate buffer at pH 7.8 (Houmard and Drapeau, 1972). S. aureus V8 protease has been used in sequence studies more recently, and although cleavage next to some aspartic acid as well as glutamic acid residues was found, there was no cleavage next to glutamine residues (Emmens et al, 1976).

Section 4.4. Determination of the amino acid sequence of each tryptic peptide from actinidin:

Determination of the sequence of each tryptic peptide is discussed in turn and the peptides are aligned to form the primary sequence of actinidin.

Peptide T_1: (Section 3.4.1., page 49)

Peptide T_1 was purified by paper electrophoresis from peptides T_{10} and T_{12} which were present in fraction VII of the Sephadex G-50 column elution profile (Fig. 3.2., pages 41, 42). Peptide T_1 was sequenced by the dansyl-Edman method which showed that the 8 amino acid residues in the peptide were arranged in the following sequence:

Leu-Pro-Ser-Tyr-Val-Asp-Trp-Arg

The results of the amino acid analysis and staining for specific amino acid residues on paper confirmed that the sequence of peptide T_1 contained tryptophan, tyrosine and arginine (Table 3.3., page 49). The location of the tryptophan residue in the sequence was determined by staining a sample of the peptide for tryptophan after each cycle of the Edman degradation. The presence of an aromatic residue in peptide T_1 explained why this peptide was retarded on the Sephadex G-50 column (Fig. 3.2., pages 41, 42) and eluted in fraction VII (Porath, 1960).

There were no amide residues in peptide T_1 as this peptide was neutral at pH 6.5 and the amino acid composition indicated that only one aspartic acid residue was present in the peptide. Subsequently, peptide T_1 was found to be the only tryptic peptide with a leucine N-terminal, and as dansylation of a sample of crystallized actinidin gave a single N-terminal leucine residue, this suggested peptide T_1 was the N-terminal tryptic peptide of actinidin.

Peptide T_2: (Section 3.4.2., page 50)

Peptide T_2 was purified by paper electrophoresis from fraction V of the Sephadex G-50 column elution profile (Fig. 3.2., pages 41, 42). Peptide T_2 was sequenced by the dansyl-Edman method which showed that the 9 amino acid residues in the peptide were arranged in the following sequence:

```
Ser-Ala-Gly-Ala-Val-Val-Asp-Ile-Lys
 ⌝   ⌝   ⌝   ⌝   ⌝   ⌝   ⌝   ⌝   ⌝
```

There were no amide residues in peptide T_2 as this peptide was neutral at pH 6.5 and the amino acid composition indicated that only one aspartic acid residue was present in the peptide (Table 3.4., page 50).

<u>Peptide T_3</u>: (Section 3.4.3., page 51)

Peptide T_3 eluted from the Sephadex G-50 column in fraction IV (Fig. 3.2., pages 41, 42) and was purified by DEAE-cellulose chromatography from peptides T_{11} and T_8, that were present in this fraction (Fig. 3.4., pages 45, 46). Peptide T_3 had a specific radioactivity (dpm/µmol) that indicated there were 2 cysteine residues in the peptide out of a total of 22 residues. This peptide was further degraded by digestion with chymotrypsin (Table 3.6., page 52; Fig. 3.5., page 53) and thermolysin (Table 3.7., page 54; Fig. 3.6., page 55), and the sequence of each peptide was aligned in the following manner to reconstruct the sequence of peptide T_3:

```
Ser-Gln-Gly-Glu-CMCys-Gly-Gly-CMCys-Trp-Ala-Phe-Ser-Ala-Ile-Ala-
 ⌝   ⌝   ⌝
|--------------- ChTa -----------------|-ChTN2-|----------------
|----------------- Tha2 -------------------|-- ThN2 ---|- ThN1 -

Thr-Val-Glu-Gly-Ile-Asn-Lys

------- ChTN1 ------------|
---|-- Tha1 ---|-- Thb --|
```

A rapid Edman degradation of peptide T_3 gave the same N-terminal sequence as that of peptide ChTa. Peptide $ChTN_1$ was the only chymotryptic peptide containing lysine and was therefore the C-terminal sequence of peptide T_3. The location of peptide $ChTN_2$ was confirmed by determination of the sequences of the thermolysin digest peptides of peptide T_3. Peptides ChTa and Tha_2 both had a specific radioactivity (dpm/µmol) that indicated each peptide contained 2 cysteine residues. The location of the cysteine residues in these two peptides was determined by the dansyl-Edman method and confirmed by determination of the radioactivity after each cycle of the Edman degradation.

The mobilities of peptides ChTa and Tha_2 showed that they both had a net charge at pH 6.5 of -3 which indicated that both peptides contained one amide residue. To solve this problem as to which residue was glutamine and which was glutamic acid in the sequence, peptide ChTa was sequentially degraded by the Edman method. The peptide electrophoretic mobility after each cycle of the Edman degradation was plotted on an Offord peptide mobility diagram (Fig. 3.8., pages 57, 58). The results show that the fourth residue in the sequence of the peptide is glutamic acid, as a charge change of -1 occurred after removal of this residue from the peptide. The second residue is glutamine, as there was no charge change in the peptide on removal of the second residue.

Peptide T_4: (Section 3.4.4., page 59)

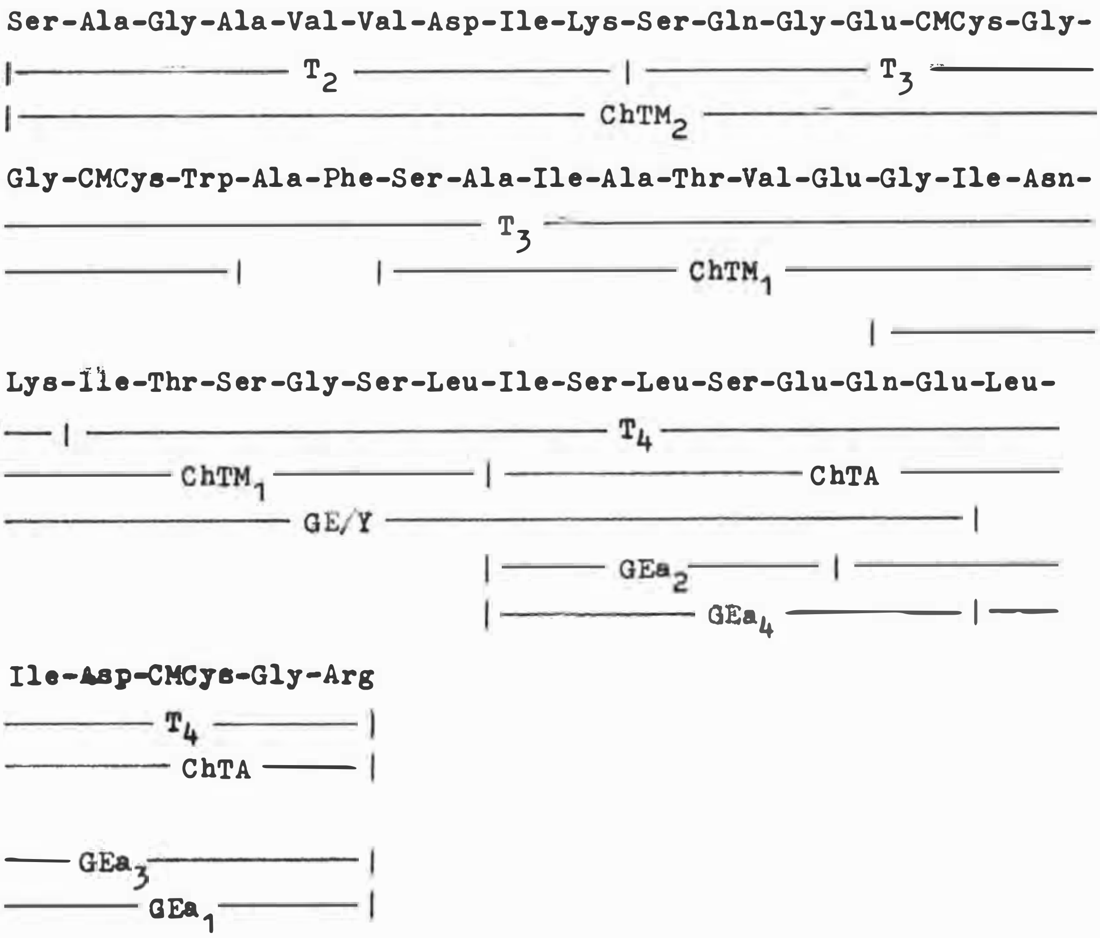
Peptide T_4 presented a problem in that it was the only tryptic peptide of S-carboxy$[^{14}C_2]$methyl actinidin that could not be located in either the Sephadex G-50 column elution profile (Fig. 3.2., pages 41, 42) or the DEAE-cellulose column elution profile (Fig. 3.4., pages 45, 46). Comparison of the amino acid composition of actinidin with the sequences of the eleven tryptic peptides isolated, indicated that peptide T_4 should contain in the peptide sequence 1 cysteine and 1 arginine, but no aromatic residues (no tyrosine, phenylalanine or tryptophan). Tentative alignment of the sequences of the eleven tryptic peptides to reconstruct the sequence of actinidin, using information from the three dimensional X-ray crystallographic structure of actinidin, indicated peptide T_4 was located between peptides T_3 and T_5. The location of peptide T_4 adjacent to peptide T_3 in the sequence of actinidin prompted the determination of the sequence of peptide T_4 by isolation of the maleyl tryptic peptide containing peptide T_4 from a tryptic digest of maleylated carboxymethyl actinidin. Peptide T_3 contained the active site cysteine residue (Section 3.6., page 103) and was a lysine C-terminal tryptic peptide. Maleylation of S-carboxy[$^{14}C_2$]methyl actinidin (labelled with ^{14}C only at the active site cysteine residue) followed by digestion with trypsin, would produce only one radioactive peptide which would contain peptide T_4 as part of the sequence.

The tryptic peptides from maleylated carboxymethyl actinidin were separated on a Sephadex G-75 column (Fig. 3.9., page 60). Two of the fractions were radioactive and the radioactive peptide in the second fraction was found to have the same sequence as peptide T_3, indicating that the maleylation of carboxymethyl actinidin had been incomplete.

Because peptide T_4 could not be obtained from a tryptic digest, the sequence of peptide T_4 was determined by analysis of the whole maleyl tryptic peptide that contained the sequence of peptide T_4. Half of the first radioactive fraction eluting from the Sephadex G-75 column was digested with chymotrypsin and a sample of the material was used to prepare two maleyl diagonal peptide maps. One map was stained with ninhydrin to reveal two peptides lying off of the diagonal ($ChTM_1$ and $ChTM_2$) of which peptide $ChTM_2$ was radioactive (Fig. 3.10., page 61). The other map was stained for arginine to reveal only one arginine containing peptide that was located on the diagonal (ChTA) (Fig. 3.10., page 61). Using the information from the maleyl diagonal, the three peptides (ChTA, $ChTM_1$ and $ChTM_2$) were purified by preparative electrophoresis at pH 6.5 (Table 3.8., page 62).

The remaining half of the first radioactive fraction from the Sephadex G-75 column was digested with S. aureus V8 protease. Samples of the digest subjected to electrophoresis at pH 6.5 and to BAWP chromatography, showed that a nonradioactive peptide which stained yellow with ninhydrin remained at the origin (peptide GE/Y). This peptide was separated from the other peptides in the digest by precipitation at pH 3.5 during demaleylation of the digest peptides in solution. The amino acid composition

(Table 3.10., page 65) and the N-terminal (glycine) indicated that peptide GE/Y was sufficiently pure to sequence.

Peptides $ChTM_1$, GE/Y and one third of peptide ChTA were sequenced directly by the dansyl-Edman method. The remainder of peptide ChTA was digested with S. aureus V8 protease and the peptides produced were purified by paper electrophoresis and sequenced. The sequences of peptides $ChTM_1$, GE/Y, ChTA and the S. aureus V8 protease digest peptides of ChTA were aligned to reconstruct the sequence of the maleyl tryptic peptide that contained peptide T_4, and therefore determine the sequence of peptide T_4:

Ser-Ala-Gly-Ala-Val-Val-Asp-Ile-Lys-Ser-Gln-Gly-Glu-CMCys-Gly-

Gly-CMCys-Trp-Ala-Phe-Ser-Ala-Ile-Ala-Thr-Val-Glu-Gly-Ile-Asn-

Lys-Ile-Thr-Ser-Gly-Ser-Leu-Ile-Ser-Leu-Ser-Glu-Gln-Glu-Leu-

Ile-Asp-CMCys-Gly-Arg

The amino acid composition, N-terminal, specific radioactivity and tryptophan content of peptide $ChTM_2$ indicated that this peptide corresponded to peptide T_2 and the N-terminal sequence of peptide T_3. Peptide $ChTM_2$ was confirmed to contain lysine because it was located off of the diagonal in the peptide map

(Fig. 3.10., page 61). The sequence of peptide $ChTM_1$ indicated that the N-terminal sequence of the peptide contained the C-terminal sequence of peptide T_3, while the C-terminal sequence of peptide $ChTM_1$ contained a new six residue sequence. Peptide ChTA was the C-terminal peptide of the maleyl tryptic peptide as peptide ChTA was the only peptide containing arginine. The peptides obtained by S. aureus V8 protease digestion of peptide ChTA (GEa_1, GEa_2, GEa_3 and GEa_4) indicated the location of the two glutamic acid residues and the single glutamine residue.
The sequence of peptide GE/Y provided the overlapping sequence for peptides $ChTM_1$, ChTA, GEa_2 and GEa_3, to complete the reconstruction of the maleyl tryptic peptide and provide the sequence of peptide T_4.

Peptide T_5: (Section 3.4.5., page 67)

Peptide T_5 was purified by paper electrophoresis from peptide T_9 which was present in fraction VI of the Sephadex G-50 column elution profile (Fig. 3.2., pages 41,42). Peptide T_5 was sequenced by the dansyl-Edman method which showed that the 5 amino acid residues in the peptide were arranged in the following sequence:

$$\underset{\rightharpoondown}{\mathrm{Thr}}-\underset{\rightharpoondown}{\mathrm{Gln}}-\underset{\rightharpoondown}{\mathrm{Asn}}-\underset{\rightharpoondown}{\mathrm{Thr}}-\underset{\rightharpoondown}{\mathrm{Arg}}$$

The results of the amino acid analysis and staining for specific amino acids on paper confirmed peptide T_5 contained arginine (Table 3.11., page 67). There were two amide residues in peptide T_5, as from the mobility this peptide had a net charge of +1 at pH 6.5.

Peptide T_6: (Section 3.4.6., page 68)

Peptide T_6 eluted from the Sephadex G-50 column in fraction I (Fig. 3.2., pages 41, 42) and was purified by DEAE-cellulose chromatography from peptide T_7 in fraction II (Fig. 3.4., pages 45, 46). Peptide T_6 had a specific radioactivity (dpm/µmol) that indicated there were 2 cysteine residues present in the peptide, out of a total of 43 residues.

This peptide was further degraded by digestion with chymotrypsin (Table 3.13., page 69;Fig. 3.12., page 70) and S. aureus V8 protease (Table 3.14., page 72; Table 3.15., page 73; Fig. 3.14., page 74). The sequence of each peptide was aligned in the following manner to reconstruct peptide T_6:

```
Gly-CMCys-Asp-Gly-Gly-Tyr-Ile-Thr-Asp-Gly-Phe-Gln-Phe-Ile-Ile-
 ⇀   ⇀     ⇀
|-------- ChTa5 ---------|----- ChTa1 -------|-ChTN1-|---------
|----------------------- ChTa4 ----------------------|
|--------------------------- GEb -----------------------------
|---- GEbChTa3 ----------|--- GEbChTa1 ------|GEbChTN1|--------
                                                     |--------
Asn-Asp-Gly-Gly-Ile-Asn-Thr-Asp-Glu-Asn-Tyr-Pro-Tyr-Thr-Ala-

-------------------- ChTa2 ------------------------|--------

------------ GEb ------------------|---------- GEa ---------
------------- GEbChTa2 ------------|---- GEaChTN --|--------
--- a1 --------|------- a2 --------|
Gln-Asp-Gly-Asp-CMCys-Asp-Val-Ala-Leu-Gln-Asp-Gln-Lys

----------- ChTa3 -------------------|--- ChTN2 -----|

-------------------- GEa ----------------------------|
---------------------- GEaChTa ----------------------|
```

Digestion of peptide T_6 with chymotrypsin produced six peptides that were sequenced by the dansyl-Edman method (Table 3.13., page 69; Fig. 3.12, page 70). In peptide $ChTa_2$, the Tyr-Pro peptide bond was not cleaved by chymotrypsin as the enzyme does not always cleave peptide bonds formed by the imino group of proline (Kasper, 1975b). Due to the large number of aromatic and hydrophobic residues present in peptide T_6, digestion of the peptide with thermolysin resulted in a peptide mixture too complex to enable the determination of overlapping sequence for the alignment of the chymotryptic peptides. The presence of five glutamic acid residues in peptide T_6, as indicated by the amino acid composition, prompted the digestion of peptide T_6 with

S. aureus V8 protease to determine which of these residues were present as amides, and also to provide sequence overlaps for alignment of the chymotryptic peptides.

Sephadex G-25 chromatography of the S. aureus V8 protease digest of peptide T_6 produced a single broad peak (Fig. 3.13., page 71). Peptide mapping at pH 6.5 and pH 3.5 showed only two peptides were present, one of which was insoluble at pH 3.5. This difference in solubility between the two peptides was utilised to purify each peptide by selective precipitation of one peptide from solution at pH 3.5. The acid soluble peptide (GEa) showed a single N-terminal aspartic acid, and as the peptide contained lysine, it was therefore the C-terminal sequence of peptide T_6. The acid insoluble peptide (GEb) showed a single N-terminal glycine and did not contain lysine. Both peptides had a specific radioactivity (dpm/µmol) that indicated 1 cysteine residue was present in each peptide. The large number of acidic residues in peptide T_6 (11 aspartic acid and 5 glutamic acid) could have made the determination of amide residues a considerable task. However, digestion of peptide T_6 with S. aureus V8 protease showed that there was only one glutamic acid residue present in the peptide, and four glutamine residues.

Chymotrypsin digestion of peptide GEa produced two peptides (Table 3.15., page 73; Fig. 3.14., page 74): peptide GEaChTa contained lysine and overlapped peptides $ChTa_3$ and $ChTN_2$; peptide GEaChTN had an asparagine N-terminal as it was neutral at pH 6.5, and corresponded to the C-terminal sequence of peptide $ChTa_2$. With the knowledge that peptide $ChTN_2$ was neutral at pH 6.5 and contained two glutamine residues, the peptide therefore contained one aspartic acid residue.

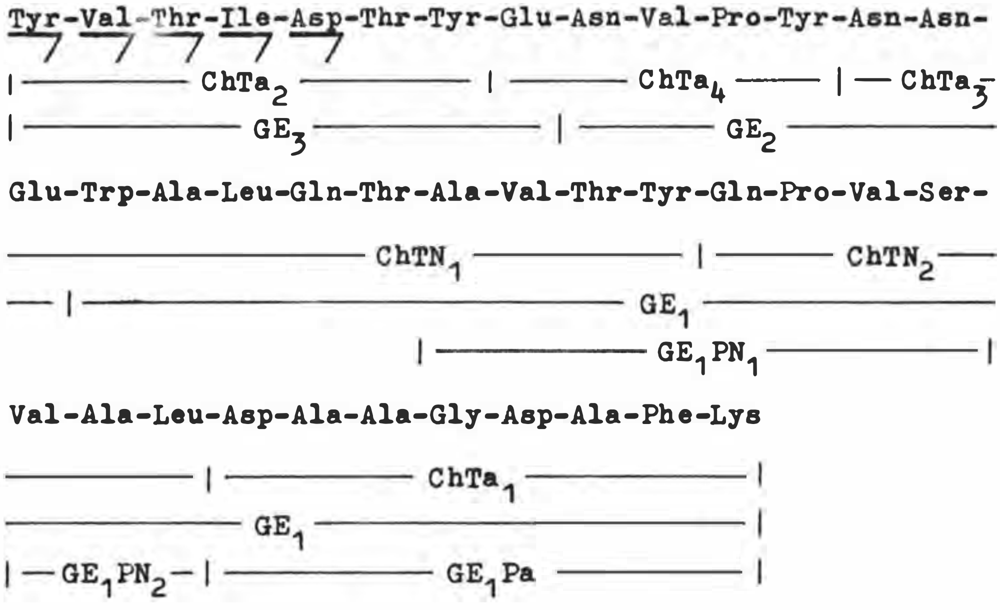
Chymotrypsin digestion of peptide GEb produced four peptides (Table 3.15., page 73; Fig. 3.14., page 74). Peptides $GEbChTa_3$, $GEbChTa_1$ and $GEbChTN_1$ corresponded to peptides $ChTa_5$, $ChTa_1$ and $ChTN_1$ respectively, and these peptides were overlapped by peptide $ChTa_4$ which was obtained in low yield and was an incomplete chymotrypsin digest product of peptide T_6. Peptide $GEbChTa_2$, together with peptide GEaChTN, corresponded to the sequence of peptide ChTa$_2$. Peptides ChTa$_5$, ChTa$_4$, GEbChTa$_3$, ChTa$_3$ and GEaChTa each had a specific radioactivity that indicated 1 cysteine residue was present in each peptide, and the location of the cysteine residue in each peptide was confirmed by determination of the radioactivity after each cycle of the Edman degradation.

The electrophoretic mobility of peptide GEbChTa$_2$ showed that the peptide had a net charge at pH 6.5 of -3, which indicated that the peptide contained two amide residues. To solve this problem, peptide GEbChTa$_2$ was digested with thermolysin to produce two peptides: a$_1$ and a$_2$. These two peptides were sequentially degraded by the Edman procedure. The peptide mobilities for both peptides after each cycle of the Edman degradation were plotted on an Offord peptide mobility diagram (Fig. 3.16., pages 76, 77). The results show that the fourth residue in both peptides a$_1$ and a$_2$ is an aspartic acid , as a charge change of -1 occurred after removal of this residue from each peptide. The third residue in peptide a$_1$ and the second residue in peptide a$_2$ are asparagine as there was no charge change in the peptide on removal of the second residue.

<u>Peptide T$_7$</u>: (Section 3.4.7., page 78)

Peptide T$_7$ eluted from the Sephadex G-50 column in fraction II (Fig. 3.2., pages 41, 42) and was purified by DEAE-cellulose chromatography from peptides T$_6$ and T$_8$ in fractions I and III respectively (Fig. 3.4., pages 45, 46). Peptide T$_7$ was not radioactive indicating that the peptide did not contain cysteine. Peptide T$_7$ contained 39 residues.

This peptide was further degraded by digestion with chymotrypsin (Table 3.17., page 79; Fig. 3.17., page 80) and <u>S. aureus</u> V8 protease (Table 3.18., page 82; Table 3.19., page 83; Fig. 3.19., page 84). The sequence of each peptide was aligned in the following manner to reconstruct peptide T$_7$:



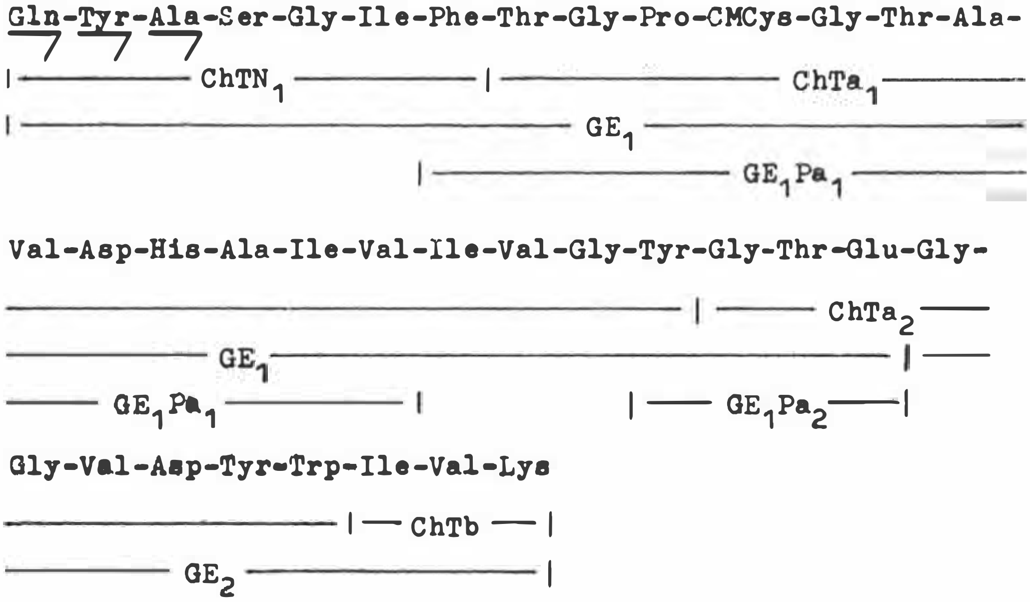
Peptide T_7 was digested with chymotrypsin producing six peptides that were sequenced by the dansyl-Edman method. S. aureus V8 protease was used in preference to thermolysin to obtain overlapping sequence for the alignment of the chymotryptic peptides, because like peptide T_6, a complex peptide digest was obtained from peptide T_7 with thermolysin. S. aureus V8 protease produced three peptides (GE$_1$, GE$_2$ and GE$_3$) which were initially separated on a Sephadex G-25 column (Fig. 3.18., page 81). Peptides GE$_2$ and GE$_3$ could be purified by paper techniques and were sequenced directly by the dansyl-Edman method. Peptide GE$_3$ had the same N-terminal sequence as peptide T_7 and overlapped peptides ChTa$_2$ and ChTa$_4$. Peptide GE$_2$ overlapped peptides ChTa$_4$ and ChTa$_3$. A rapid Edman degradation of peptide GE$_1$ confirmed the N-terminal sequence of this peptide and provided overlapping sequence for peptides ChTa$_3$ and ChTN$_1$. The location of the tryptophan residue that was present as the N-terminal residue of peptide GE$_1$ and present as the C-terminal residue of peptide ChTa$_3$, was confirmed by staining samples of each peptide for tryptophan after each cycle of the Edman degradation. Digestion of peptide GE$_1$ with pepsin produced three peptides: peptide GE$_1$PN$_1$ overlapped peptides ChTN$_1$ and ChTN$_2$; peptide GE$_1$PN$_2$ contained the C-terminal sequence of peptide ChTN$_2$; and peptide GE$_1$Pa provided the C-terminal region of peptide T_6 confirming peptide ChTa$_1$.

Examination of the net charge, as determined from the electrophoretic mobilities at pH 6.5, on each of the peptides from the chymotrypsin, S. aureus V8 protease and pepsin digestions, enabled the determination of all of the amide and acid residues in peptide T_7.

Peptide T_8: (Section 3.4.8., page 86)

Peptide T_8 eluted from the Sephadex G-50 column in fraction III (Fig. 3.2., pages 41, 42) and was purified by DEAE-cellulose chromatography from peptide T_7 in fraction II, and peptides T_3 and T_{11} in fraction IV (Fig. 3.4., pages 45, 46). Peptide T_8 had a specific radioactivity (dpm/μmol) that indicated 1 cysteine residue was present in the peptide, out of a total of 36 residues.

This peptide was further degraded by digestion with chymotrypsin (Table 3.21., page 87; Fig. 3.21., page 88) and S. aureus V8 protease (Table 3.22., and Table 3.23., page 90). The sequence of each peptide was aligned in the following manner to reconstruct peptide T_8:



Peptide T_8 was digested with chymotrypsin producing four peptides that were sequenced by the dansyl-Edman method.

S. aureus V8 protease was used in preference to thermolysin to obtain overlapping sequence for the alignment of the chymotryptic peptides. Digestion of peptide T_8 with *S. aureus* V8 protease produced two peptides (GE_1 and GE_2) which were separated on a Sephadex G-25 column (Fig. 3.22., page 89). Peptide GE_2 was purified by paper techniques using the BAWP chromatography solvent. The peptide did not migrate on paper by electrophoresis at pH 6.5 or pH 2.1, or in the BAW chromatography solvent. Peptide GE_2 contained tryptophan and tyrosine as well as lysine, which showed it was the C-terminal sequence of peptide T_8. Peptide GE_2 overlapped peptide $ChTa_2$, which contained tryptophan, and peptide ChTb, which was the C-terminal chymotryptic peptide. The location of the tryptophan residue in peptides $ChTa_2$ and GE_2 was confirmed by staining a sample of each peptide for tryptophan after each cycle of the Edman degradation. A rapid Edman degradation of peptide GE_1 provided an N-terminal sequence the same as that obtained for peptide T_8 confirming that peptide GE_1 contained the N-terminal sequence of peptide T_8. Digestion of peptide GE_1 with pepsin produced two peptides: peptide GE_1Pa_1 overlapped peptides $ChTN_1$ and $ChTa_1$; peptide GE_1Pa_2 overlapped peptides $ChTa_1$ and $ChTa_2$.

Examination of the net charge as determined from the mobility at pH 6.5 of each of the peptides from the chymotrypsin, *S.aureus* V8 protease and pepsin digestions, enabled the determination of all of the amide and acid residues in peptide T_8.

Peptide T_9: (Section 3.4.9., page 92)

Peptide T_9 was purified by paper electrophoresis from peptide T_5 which was present in fraction VI of the Sephadex G-50 column elution profile (Fig. 3.2., pages 41, 42). Peptide T_9 was sequenced by the dansyl-Edman method which showed that the residues in the peptide were arranged in the following sequence:

Asn-Ser-Trp-Asp-Thr-Thr-Trp-Gly-Glu-Glu-Gly-Tyr-Met-Arg
⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁ ⇁

|— ChTN —|—— $ChTa_2$ ——|——— $ChTa_1$ ———|-ChTb -|

The results of the amino acid analysis showed that the peptide contained methionine, and together with the results of staining for specific amino acids on paper, confirmed that the sequence of peptide T_9 contained tryptophan and arginine (Table 3.24., page 92). No amino acid could be identified for either the third or seventh residues in the sequence as determined by the dansyl-Edman method, which suggested that these positions were occupied by tryptophan. The location of the two tryptophan residues was confirmed by digestion of peptide T_9 with chymotrypsin and analysis of the chymotryptic peptides (Table 3.25., page 93). Peptides ChTN and $ChTa_2$ were both found to contain tryptophan by staining a sample of each peptide for tryptophan after each cycle of the Edman degradation. The presence of two aromatic residues in peptide T_9 explained why this peptide was retarded on the Sephadex G-50 column (Fig. 3.2., pages 41, 42) and eluted in fraction VI (Porath, 1960).

The amide and acid residues in peptide T_9 were determined from the electrophoretic mobilities of the chymotryptic peptides, which agreed with a net charge of -2 for peptide T_9 as determined from the electrophoretic mobility of the whole peptide.

Peptide T_{10}: (Section 3.4.10., page 94)

Peptide T_{10} was purified by paper electrophoresis from peptides T_1 and T_{12} which were present in fraction VII of the Sephadex G-50 column elution profile (Fig. 3.2., pages 41, 42). Peptide T_{10} was sequenced by the dansyl-Edman method which showed that the 3 amino acid residues in the peptide were arranged in the following sequence:

$$\underset{\rightharpoondown}{\text{Ile}}\text{-}\underset{\rightharpoondown}{\text{Leu}}\text{-}\underset{\rightharpoondown}{\text{Arg}}$$

The results of the amino acid analysis and staining for specific amino acids on paper confirmed that the sequence of peptide T_{10} contained arginine (Table 3.26., page 94). There were no amide residues in peptide T_{10}.

Peptide T_{11}: (Section 3.4.11., page 95)

Peptide T_{11} eluted from the Sephadex G-50 column in fraction IV (Fig. 3.2., pages 41, 42) and was purified by DEAE-cellulose chromatography from peptides T_3 and T_8 that were present in this fraction (Fig. 3.4., pages 45, 46). Peptide T_{11} had a specific radioactivity (dpm/µmol) that indicated there was 1 cysteine residue present in the peptide, out of a total of 19 residues (Table 3.27., page 95).

This peptide was further degraded by digestion with thermolysin (Table 3.28., page 96; Fig. 3.24., page 97) and the sequence of each of the three peptides obtained was aligned in the following manner to reconstruct peptide T_{11}:

Asn-Val-Gly-Gly-Ala-Gly-Thr-CMCys-Gly-Ile-Ala-Thr-Met-Pro-
⇁ ⇁ ⇁ ⇁ ⇁
|———————— Tha ————————|———— ThN ————

Ser-Tyr-Pro-Val-Lys
——————|—Thb—|

A rapid Edman degradation of peptide T_{11} gave the same N-terminal sequence as that of peptide Tha . Peptide Tha had a specific radioactivity (dpm/µmol) which indicated that it contained 1 cysteine residue. The location of the cysteine residue in the peptide was determined by the dansyl-Edman method, and confirmed by determining at which stage of the Edman degradation the radioactive residue was removed. Peptide Thb was the only peptide containing lysine and was therefore the C-terminal sequence of peptide T_{11}.

Although peptide T_{11} contained one tyrosine residue, the peptide was not digested by chymotrypsin because this residue was present in the sequence ; Tyr-Pro, and peptide bonds involving the imino group of proline are not always cleaved by chymotrypsin (Kasper, 1975b).

There was one amide residue in peptide T_{11} as this peptide was neutral at pH 6.5. The single amide residue, asparagine, was located at the N-terminus of peptide T_{11} which was consistent with the peptide staining yellow on paper with ninhydrin.

Peptide T_{12}: (Section 3.4.12., page 98)

Peptide T_{12} was purified by paper electrophoresis from peptides T_1 and T_{12} which were present in fraction VII of the Sephadex G-50 elution profile (Fig. 3.2., pages 41, 42). Peptide T_{12} was sequenced by the dansyl-Edman method which showed that the residues in the peptide were arranged in the following sequence:

$\underrightarrow{\text{Tyr}}$-$\underrightarrow{\text{Asn}}$-$\underrightarrow{\text{Asn}}$

The results of the amino acid analysis and staining for specific amino acids on paper confirmed that the sequence of peptide T_{12} contained tyrosine (Table 3.29., page 98). There were two amide residues in peptide T_{12}, as this peptide was neutral at pH 6.5.

Peptide T_{12} was the only tryptic peptide that did not contain a basic residue (no lysine or arginine) which suggested that this peptide was the C-terminal tryptic peptide of actinidin. This suggestion was confirmed by analysis of cyanogen bromide fragments of actinidin and a carboxypeptidase A experiment, both of which are discussed in the next section.

Section 4.5. The C-terminal region of actinidin:

(Section 3.5., page 99)

Carboxypeptidase A digestion of the S-sulphenyl thiosulphate derivative of actinidin, or actinidin that had been activated with dithiothreitol, did not release any amino acids from the C-terminus. Acid denaturation of actinidin, followed by digestion with carboxypeptidase A, released the following amino acid residues: 2 asparagine, 1 tyrosine, 1 lysine and 1 valine, which were determined by amino acid analysis. From the three dimensional structure of actinidin (Baker, 1976b), it appears likely that the protein molecule requires some denaturation to make the C-terminal region of the primary sequence accessible to an exopeptidase.

To confirm the C-terminal region, carboxy[$^{14}C_2$]methyl actinidin was reacted with cyanogen bromide and the peptide fragments produced were initially separated on a Sephadex G-50 column in two fractions (CB-I and CB-II+III) (Fig. 3.25., page 100). Fraction CB-I was radioactive and eluted at the exclusion limit of the gel column. The front portion of the peak corresponding to fraction CB-II+III was radioactive and this fraction was rechromatographed on Sephadex G-25 which separated the material into fractions CB-II and CB-III, of which CB-II was radioactive (Fig. 3.26, page 100). Fraction CB-III was shown to contain one pure peptide when subjected to paper electrophoresis. The 9 residues in this peptide were determined to be arranged in the following sequence by the dansyl-Edman method:

$$\underset{\rightharpoondown}{\text{Pro}}\text{-}\underset{\rightharpoondown}{\text{Ser}}\text{-}\underset{\rightharpoondown}{\text{Tyr}}\text{-}\underset{\rightharpoondown}{\text{Pro}}\text{-}\underset{\rightharpoondown}{\text{Val}}\text{-}\underset{\rightharpoondown}{\text{Lys}}\text{-}\underset{\rightharpoondown}{\text{Tyr}}\text{-}\underset{\rightharpoondown}{\text{Asn}}\text{-}\underset{\rightharpoondown}{\text{Asn}}$$

Peptide CB-III did not contain homoserine or homoserine lactone suggesting that it was the C-terminal cyanogen bromide fragment. The C-terminal region of peptide CB-III corresponded to the sequence of the tryptic peptide T_{12} (Section 3.4.12., page 98), and contained all of the residues released by carboxypeptidase A (Section 3.5.2., page 102). Peptide CB-III provided an overlap between the tryptic peptides T_{11} and T_{12}.

Fraction CB-II separated into two components (CB-IIa$_1$ and CB-IIa$_2$) when subjected to paper electrophoresis. These two components had the same amino acid composition and N-terminal, and both had a similar specific radioactivity (dpm/μmol), indicating that there was one cysteine residue present in each of the peptides, out of a total of 17 residues. The yield of peptide CB-IIa$_2$ was six times that of peptide CB-IIa$_1$ (Table 3.30., page 99). Peptide CB-IIa$_1$ was digested with chymotrypsin (Table 3.31., page 101) and the peptides were sequenced by the dansyl-Edman method to provide the following sequence for peptide CB-IIa$_1$:

Arg-Ile-Leu-Arg-Asn-Val-Gly-Gly-Ala-Gly-Thr-CMCys-Gly-Ile-Ala-Thr-Met

|— ChTb —|————————— ChTa —————————|

Peptide CB-IIa$_1$ was initially sequenced by the dansyl-Edman method for the first six residues which provided sequence that overlapped peptides ChTb and ChTa. The location of the cysteine residue in peptide ChTa was determined from the sequence and confirmed by determining at which stage of the Edman degradation the radioactive group was removed. The amino acid composition of peptide ChTa showed the peptide contained homoserine and homoserine lactone, which indicated that the peptide was not the C-terminal cyanogen bromide fragment of actinidin. Peptide CB-IIa$_2$ was found to have the same sequence as peptide CB-IIa$_1$.

Two peptide fragments were obtained because the C-terminal methionine of fragment CB-II is converted to homoserine lactone (a neutral residue) during the cyanogen bromide reaction, and homoserine lactone is in equilibrium with homoserine (an acidic residue), even under acidic conditions (Armstrong, 1949). As a result, a proportion of fragment CB-II contained homoserine lactone (CB-IIa$_2$) and was more basic than peptide CB-IIa$_1$ which contained homoserine. On acid hydrolysis, peptides CB-IIa$_1$ and CB-IIa$_2$ both contained homoserine lactone and homoserine because homoserine can cyclise to homoserine lactone under strong acid conditions (Gross, 1967).

Section 4.6. Alignment of the tryptic peptides of actinidin:

The amino acid compositions of each tryptic peptide as determined from the sequences of the peptides (Section 3.4., pages 47 to 98) are tabulated and compared (Table 4.2.) to the total amino acid composition obtained for actinidin (Section 3.2., page 39). The results of the tryptic peptide sequences are in agreement with the total amino acid composition of actinidin.

The alignment of the tryptic peptides to reconstruct the primary sequence of actinidin (Fig. 4.1.) was determined, in part, with information from cyanogen bromide fragments, and information from the tryptic peptides of maleylated carboxymethyl actinidin. The alignment of the tryptic peptides was completed with information from the three dimensional X-ray crystallographic structure of actinidin, which also confirmed the sequence overlaps obtained by chemical methods.

Reaction of carboxymethyl actinidin with cyanogen bromide produced three fragments: CB-I (194 residues), CB-II (17 residues) and CB-III (9 residues). Fragment CB-III contained the C-terminal sequence of actinidin because it was the only cyanogen bromide fragment not containing homoserine lactone or homoserine, and fragment CB-III contained all of the amino acids that were released by carboxypeptidase A in the C-terminal sequence of the fragment. Fragment CB-III overlapped tryptic peptides T_{11} and T_{12}.

The N-terminal sequence of fragment CB-II overlapped tryptic peptides T_9, T_{10} and T_{11}. Peptide T_9 was the only tryptic peptide with a Met-Arg sequence and T_{10} was present in the sequence of fragment CB-II. The N-terminal region of peptide T_{11} containing a radioactively labelled cysteine residue was present in the C-terminal sequence of fragment CB-II.

Tryptic digestion of maleylated carboxymethyl actinidin that was radioactively labelled at the active site cysteine residue, produced a radioactive peptide containing 50 residues, from which the sequence of peptide T_4 was determined (Section 3.4.4., page 59). The sequences of the peptides obtained from a chymotrypsin digestion of the radioactive maleyl tryptic peptide, and the peptide obtained by digestion of the maleyl tryptic peptide with S. aureus V8 protease, aligned peptides T_2, T_3 and T_4. The location of peptide T_4 was confirmed by it being the only

Table 4.2. Comparison of the amino acid composition of actinidin with the amino acid compositions of the tryptic peptides as determined from from the amino acid sequence of each peptide.

Amino acid	**Tryptic peptide** (Number of residues)												**Amino acid composition of actinidin**
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{11}	T_{12}	
His	-	-	-	-	-	-	-	1	-	-	-	-	1
Lys	-	1	1	-	-	1	1	1	-	-	1	-	6
Arg	1	-	-	1	1	-	-	-	1	1	-	-	5
Asp	1	1	1	1	1	11	6	2	2	-	1	2	29
Thr	-	-	1	1	2	3	4	3	2	-	2	-	18
Ser	1	1	2	4	-	-	1	1	1	-	1	-	12
Glu	-	-	3	3	1	5	4	2	2	-	-	-	20
Pro	1	-	-	-	-	1	2	1	-	-	2	-	7
Gly	-	1	4	2	-	7	1	7	2	-	4	-	28
Ala	-	2	3	-	-	2	6	3	-	-	2	-	18
Cys	-	-	2	1	-	2	-	1	-	-	1	-	7
Val	1	2	1	-	-	1	5	5	-	-	2	-	17
Met	-	-	-	-	-	-	-	-	1	-	1	-	2
Ile	-	1	2	3	-	4	1	4	-	1	1	-	17
Leu	1	-	-	3	-	1	2	-	-	1	-	-	8
Tyr	1	-	-	-	-	3	4	3	1	-	1	1	14
Phe	-	-	1	-	-	2	1	1	-	-	-	-	5
Trp	1	-	1	-	-	-	1	1	2	-	-	-	6
Total	8	9	22	19	5	43	39	36	14	3	19	3	220

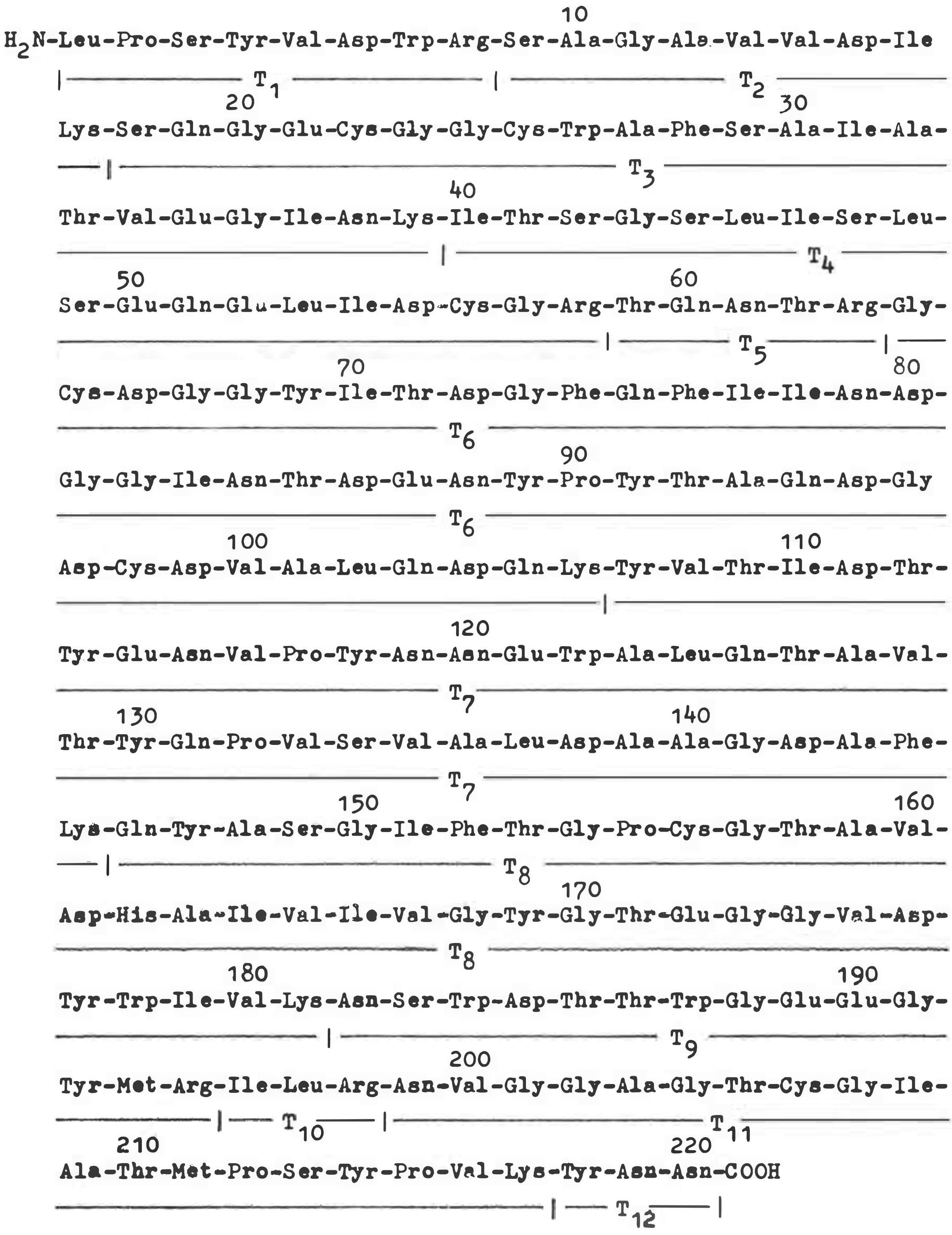


Fig. 4.1. Alignment of the tryptic peptides of actinidin.

peptide of these three that contained arginine; peptides T_2 and T_3 both contained one lysine residue which was maleylated, thus preventing cleavage of the peptide chain with trypsin at these positions. The remaining tryptic peptides of actinidin were aligned with the aid of the three dimensional X-ray crystallographic structure of actinidin determined by Dr E.N. Baker (1976b), and this information was also used to confirm the overlaps of the tryptic peptides that were determined from cyanogen bromide fragments and the maleyl tryptic peptides.

Section 4.7. The degree of homology between the active site amino acid sequences from actinidin and some other plant thiol proteases:

There is considerable homology between the amino acid sequences about the essential cysteine residue of the plant thiol proteases (Fig. 1.2., page 4). Comparison of the amino acid sequence about the essential cysteine residue of actinidin, with the sequences available for other plant thiol proteases (Fig. 4.2.), shows that this sequence homology is also extended to actinidin.

	15	16	17	18	19	20	21	22	23	24	25	26[g]
actinidin[a]	Asp	Ile	Lys	Ser	Gln	Gly	Glu	Cys	Gly	Gly	CySH[f]	Trp
papain[b]	Pro	Val	Lys	Asn	Gln	Gly	Ser	Cys	Gly	Ser	CySH	Trp
ficin[c]	Pro	Ile	Arg	Gln	Gln	Gly	Gln	Cys	Gly	Ser	CySH	Trp
bromelain[d]				Asn	Gln	Asp	Pro	Cys	Gly	Ala	CySH	Trp
chymopapain[e]			Lys	Arg	Val	Pro	Asp	Ser	Gly	Glu	CySH	Tyr

Fig. 4.2. Amino acid sequences containing the essential cysteine in some thiol proteolytic enzymes.

[a] As determined in this study
[b] Light *et al*, 1964.
[c] Wong and Liener, 1964; Husain and Lowe, 1970b.
[d] Husain and Lowe, 1970c.
[e] Tsunoda and Yasunobu, 1966.
[f] CySH represents the essential cysteine residue.
[g] Numbering of residues as for the sequence of papain and actinidin.

From Fig. 4.2., it appears likely that glutamine 19, cysteine 22, glycine 23, cysteine 25 and tryptophan 26 are residues that are conserved in the active site region.

As well as the high degree of homology among the amino acid sequences about the essential cysteine residue in the plant thiol proteases, comparison of the amino acid sequences about the active site histidine residue also show considerable homology. Involvement of a histidine residue in the active site of plant thiol proteases was demonstrated by the bifunctional reagent crosslinking experiments of Husain and Lowe (1968a, 1968b, 1970b, 1970c). Comparison of the amino acid sequence about the histidine residue of actinidin, with the corresponding sequences of other plant thiol proteases, demonstrates a considerable degree of sequence homology (Fig. 4.3.).

The studies of the active site three dimensional structure of actinidin (Baker, 1976b) show that it is very similar to the active site structure of papain (Drenth et al, 1968b), indicating that similar residues are involved both at the hydrophobic binding site and in the region containing the essential cysteine, histidine and tryptophan residues (Fig. 1.3., page 5). Furthermore, the amino acid residues involved in the active site of actinidin come from widely different regions of the primary amino acid sequence, in agreement with the findings for papain (Drenth et al, 1968b). This indicates that a large proportion of the protein molecule is required to adopt a particular conformation that is controlled, to some extent, by the active site structure of these enzymes.

actinidin[a]	Thr-Gly-Pro-Cys-Gly-Thr-Ala-Val-Asp-His[e]-Ala-Ile-Val-Ile-Val-Gly-Tyr
papain[b]	Val-Gly-Pro-Cys-Gly-Asn-Lys-Val-Asp-His-Ala-Val-Ala-Ala-Val-Gly-Tyr
ficin[c]	Thr-Gly-Pro-Cys-Gly-Thr-Ser-Leu-Asp-His-Ala-Val-Ala-Leu
stem bromelain[d]	His-Ala-Val-Thr-Ala-Ile-Gly-Tyr

Fig. 4.3. Amino acid sequences about the histidine residue in the active site of some plant thiol proteases.

[a] As determined in this study.

[b] Husain and Lowe, 1968a, 1968b; Light _et al_, 1964.

[c] Husain and Lowe, 1970b.

[d] Husain and Lowe, 1970c.

[e] Active site histidine.

Section 4.8. The degree of homology between the primary amino acid sequence of actinidin and other plant thiol proteases:

For a group of proteolytic enzymes such as the plant thiol proteases to have evolved from a common ancestral protein, as for example the cytochrome c proteins appear to, it could be expected that there would be a considerable degree of homology among the primary sequences of these thiol proteases. The high degree of homology in the active site region among the plant thiol proteases already discussed, supports the finding that these enzymes have similar kinetic properties (Glazer and Smith, 1971a; Boland and Hardman, 1973). From the similarity between the three dimensional structure of actinidin and papain, it is likely that regions within the primary sequences of the molecules, such as the disulphide bridges, would show considerable sequence homology. Comparison of the available amino acid sequences about the cysteine residues of actinidin and other plant thiol proteases (Fig. 4.4.), does in fact show that there is a considerable degree of homology. The three disulphide bridges in papain were found to be between cysteine residues: 22 and 63, 56 and 95, 153 and 200 . It is therefore likely that there are three disulphide bridges in actinidin as this protein also contains seven cysteine residues, and by homology with papain, the disulphide bridges are likely to be formed between the corresponding cysteine residues.

There is also a substantial amount of homology among the plant thiol proteases in other regions of the sequences, as indicated by the boxed regions in Fig. 4.4.. The degree of similarity among the sequences is even greater when it is considered that many of the amino acid substitutions in the primary sequences of the plant thiol proteases are for similar residues (hydrophobic residues for hydrophobic, charged residues for charged). As a result, the evolution of a number of protein molecules from a common ancestral protein might therefore be less complex as there would be little need for a major change in general protein three dimensional conformation.

```
                                               10                                      20
Actinidin: Leu-Pro-Ser-Tyr-Val-Asp-Trp-Arg-Ser-Ala-Gly-Ala-Val-Val-Asp-Ile-Lys-Ser-Gln-Gly-Glu-Cys-Gly-Gly-Cys-
Papain:    Ile-Pro-Glu-Tyr-Val-Asp-Trp-Arg-Gln-Lys-Gly-Ala-Val-Thr-Pro-Val-Lys-Asn-Gln-Gly-Ser-Cys-Gly-Ser-Cys-
Bromelain: Val-Pro-Gln-Ser-Ile-Asp-Trp-Arg-Asp-Tyr-Gly-Ala-Val-Thr-Ser-Val-Lys-Asn-Gln-Asn-Pro-Cys-Gly-Ala-Cys-
Ficin:                                                         Pro-Ile-Arg-Gln-Gln-Gly-Gln-Cys-Gly-Ser-Cys-

                           30                                      40                                      50
A          Trp-Ala-Phe-Ser-Ala-Ile-Ala-Thr-Val-Glu-Gly-Ile-Asn-Lys-Ile-Thr-Ser-Gly-Ser-Leu-Ile-Ser-Leu-Ser-Glu-
P          Trp-Ala-Phe-Ser-Ala-Val-Val-Thr-Ile-Glu-Gly-Ile-Ile-Lys-Ile-Arg-Thr-Gly-Asn-Leu-Asn-Gln-Tyr-Ser-Glu-
B          Trp-Gly-Phe-Ala-Ala-Ile-Ala-Thr-Val-Glu-Ser-Val-Ala-Ser-Ile-Tyr-Lys-Gly-Ile-Leu-Gln-Pro-Leu-Ser-Gln-
F

                                               60                                      70
A          Gln-Glu-Leu-Ile-Asp-Cys-Gly-Arg-Thr-Gln-Asn-Thr-Arg-Gly-Cys-Asp-Gly-Gly-Tyr-Ile-Thr-Asp-Gly-Phe-Gln-
P          Gln-Glu-Leu-Leu-Asp-Cys-Asp-Arg- - - - -Arg-Ser-Tyr-Gly-Cys-Asn-Gly-Gly-Tyr-Pro-Trp-Ser-Ala-Leu-Gln-
B          Gln-Gln-Val-Asp-Asp-Cys-Ala-Lys-                    Gly-Cys-Lys
F

                           80                                      90                                      100
A          Phe-Ile-Ile-Asn-Asp-Gly-Gly-Ile-Asn-Thr-Asp-Glu-Asn-Tyr-Pro-Tyr-Thr-Ala-Gln-Asp-Gly-Asp-Cys-Asp-Val-
P          Leu-Val-Ala-Gln-Tyr-Gly- - -Ile-His-Tyr-Arg-Asn-Thr-Pro-Tyr-Tyr-Glu-Gly-Val-Gln-Arg-Tyr-Cys-Arg-Ser-
B
F

                                               110                                     120
A          Ala-Leu-Gln-Asp-Gln-Lys-Tyr-Val-Thr-Ile-Asp-Thr-Tyr-Glu-Asn-Val-Pro-Tyr-Asn-Asn-Glu-Trp-Ala-Leu-Gln-
P          Arg-Glu-Lys-Gly-Pro-Tyr-Ala-Ala-Lys-Thr-Asp-Gly-Val-Arg-Gln-Val-Gln-Pro-Tyr-Asn-Gln-Gly-Ala-Leu-Leu-
B                                                              Lys-Ala-Arg-Val-Pro-Arg-Asn-Asn-Glu-Ser-Ser-Met-
F
```

```
                     130                                      140                                       150
A   Thr-Ala-Val-Thr-Tyr-Gln-Pro-Val-Ser-Val-Ala-Leu-Asp-Ala-Ala-Gly-Asp-Ala-Phe-Lys-Gln-Tyr-Ala-Ser-Gly-
P   Tyr-Ser-Ile-Ala-Asn-Gln-Pro-Val-Ser-Val-Val-Leu-Gln-Ala-Ala-Gly-Lys-Asp-Phe-Gln-Leu-Tyr-Arg-Gly-Gly-
B   Tyr-Ala-Val-Ser-Lys-Gln-Pro-Ile-His-Ala-Val-Thr-Ala-Ile-Gly-Tyr
F

                                         160                                       170
A   Ile-Phe-Thr-Gly-Pro-Cys-Gly-Thr-Ala-Val-Asp-His-Ala-Ile-Val-Ile-Val-Gly-Tyr-Gly-Thr-Glu-Gly-Gly-Val-
P   Ile-Phe-Val-Gly-Pro-Cys-Gly-Asn-Lys-Val-Asp-His-Ala-Val-Ala-Ala-Val-Gly-Tyr-Asn- - - - - - - - -Pro-
B
F           Thr-Gly-Pro-Cys-Gly-Thr-Ser-Leu-Asp-His-Ala-Val-Ala-Leu

                     180                                      190                                       200
A   Asp-Tyr-Trp-Ile-Val-Lys-Asn-Ser-Trp-Asp-Thr-Thr-Trp-Gly-Glu-Glu-Gly-Tyr-Met-Arg-Ile-Leu-Arg-Asn-Val-
P   Gly-Tyr-Ile-Leu-Ile-Lys-Asn-Ser-Trp-Gly-Thr-Gly-Trp-Gly-Glu-Asn-Gly-Tyr-Ile-Arg-Ile-Lys-Arg-Gly-Thr-
B                                               Arg-Trp-Gly-Glu-Ala-Gly-Tyr-Ile-Arg-Met-Ala-Arg-Asp-Val-
F

                                             210                                       220
A   Gly-Gly-Ala- - -Gly-Thr-Cys-Gly-Ile-Ala-Thr-Met-Pro-Ser-Tyr-Pro-Val-Lys-Tyr-Asn-Asn-COOH
P   Gly-Asn-Ser-Tyr-Gly-Val-Cys-Gly-Leu-Tyr-Thr-Ser-Ser-Phe-Tyr-Pro-Val-Lys-Asn-COOH
B   Ser-Ser-Ser-Ser-Gly-Ile-Cys-Gly-Ile-Ala-Ile-Asp-Pro-Leu-Tyr-Pro-Thr-Glu-Gly
F
```

Fig. 4.4. Homologies among the available sequences of some plant thiol proteolytic enzymes.

Spaces in the sequences indicate possible deletions or insertions of amino acids in the respective protein sequences.
Sequence numbering as for the sequence of actinidin.

The determination of the primary amino acid sequence of actinidin from the tryptic peptides has provided the first complete amino acid primary sequence of a plant thiol protease for comparison with the sequence of papain. The degree of homology among the sequences of papain and actinidin, and the fragments of sequence available for other plant thiol proteases, demonstrates that the structural characteristics of actinidin are very similar to those determined so far for other plant thiol proteases, thus providing further evidence for these enzymes having evolved from a common ancestral protein.

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