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STUDIES ON THE STRUCTURE AND
FUNCTION OF SOME ENZYME
INHIBITORS FROM PLANTS

A THESIS
SUBMITTED IN ACCORDANCE WITH
THE REQUIREMENTS OF THE UNIVERSITY
OF DURHAM FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY

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DEPARTMENT OF BOTANY



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I went to the woods
because I wished to live deliberately,
to front only the essential facts of life
and see if I could not
learn what it had to teach,
and not,
when I came to die,
discover that I had not lived.

—THOREAU

In loving memory of the greatest friend I ever had,
my father, to the woman who bears the warmest and
sweetest name, my mother.
And also to all my brothers and sisters, especially
Afif, Asem & Nabil.

(i)

SUMMARY

One of the numerous α -amylase isoinhibitors found in wheat flour was extracted and purified to homogeneity by heat treatment, ethanol precipitation, gel-filtration and ion-exchange chromatography. The complete amino acid sequence of the isoinhibitor was determined by the use of the conventional dansyl-Edman degradation technique, in conjunction with the recently introduced micro-sequence method employing a 4-*NN*-dimethylamino-azobenzene-4-isothiocyanate and phenylisothiocyanate double coupling.

The complete amino acid sequence, which is the first to be reported for any α -amylase inhibitor, contains 123 amino acids and is completely lacking in histidine and phenylalanine. A low level of microheterogeneity was observed in 5 positions. The sequence of the 24 amino acids at the N-terminus of the protein was found to be identical with that reported previously, except for an amide replacement at position 8.

In addition, the complete amino acid sequence of a protease inhibitor from soybean seeds was also determined. This trypsin inhibitor (PI-IV) which was provided by Dr. D.E. Foard (Oak Ridge, Tennessee) was shown to be identical to the DII inhibitor of Odani and Ikenaka (1976).

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INTRODUCTION

The protein inhibitors of enzymes, which are found to occur naturally in the animal and plant kingdom, were first discovered by Weinland (1903) who used the term 'anti-enzyme' to describe the effects of the compounds responsible for the resistance of certain nematodes to digestion by enzymes of the alimentary canal. The first inhibitory factors discovered in plants were the protease inhibitors recognised by Read and Hass (1938) and an insoluble α -amylase inhibitor 'sisto-amylase', described by Chrzaszcz and Janicki (1933, 1934).

The presence of these inhibitors in plants was not confirmed until the mid 1940's when Kunitz (1945, 1946) isolated and purified a trypsin inhibitor from soybean seeds, and Kneen and Sandstedt (1943, 1946) and Militzer et al. (1946 a,b) described an α -amylase inhibitor from wheat seeds, while Bowman (1945) extracted a heat-labile fraction from navy beans capable of inhibiting human pancreatic α -amylase. Since that time protein inhibitors of many different enzymes have been discovered in a wide variety of plant tissues. Table 1 shows the range of enzymes which are known to be inhibited by protein inhibitors found in plants.

The inhibitors which have been most widely studied are the proteinase inhibitors. The extensive literature dealing with these proteins has been the subject of many recent reviews: Puzstai 1967; Vogel et al. 1968; Liener and Kakade 1969; Dechary 1970; Kassell 1970; Laskowski and Sealock 1971; Fritz and Tschesche 1971; Werle and Zickgraf-Rudel 1972; Ryan 1973; Whitaker and Feeney 1973; Fritz et al. 1974; Means et al. 1974; Tschesche 1974; Peeters 1975; Reich et al. 1975; Royer 1975; Umezawa 1976;

Table 1 Range of Enzymes known to be Inhibited by Proteins
from Plants

Proteinases

A. Serine Proteinases

Chymotrypsin (EC. 3.4.21.1)^{a, b}

Trypsin (EC 3.4.21.4)^{a, b}

Thrombin (EC. 3.4.21.5)^{a, b}

Plasmin (EC. 3.4.21.7)^{a, b}

Kallikrein (EC. 3.4.21.8)^{a, b}

Acrosin (EC. 3.4.21.10)^{a, b}

Elastase (EC. 3.4.21.11)^{a, b}

Aspergillus oryzae alkaline proteinase (EC. 3.4.21.15)^{a, b}

Enterokinase (EC. 3.4.21.9)^c

B. Sulphydryl Proteinases

Papain (EC. 3.4.22.2)^{a, b}

Ficin (EC. 3.4.22.3)^{a, b}

Staphylococcal thiol proteinase (EC. 3.4.22.13)^d

C. Acidic Proteinases

Pepsin (EC. 3.4.23.1)^{a, b}

Rennin (EC. 3.4.23.4)^{a, b}

Cathepsin (EC. 3.4.23.5)^{a, b}

D. Metalloproteinases and others

Carboxypeptidase A (EC. 3.4.17.2)^{a, b}

Table 1 cont'd

Carboxypeptidase B (EC. 3.4.17.3)^e

Bacillus subtilis neutral proteinase (EC. 3.4.24.4)^{a,b}

Bacillus nato proteinase (EC. 3.4.4.16)^{a,b}

Other Enzymes

α -amylase (1,4- α -D-glucan glucanhydrolase)(EC. 3.2.1.1)^f

Acid invertase (EC. 3.2.1.26)^g

Endopolygalacturonase (endo- β -1,3-glucanase) (EC. 3.2.1.39)^{h,i}

Phospho-inositol kinase (EC. 2.7.1.67)^j

Pectate lyase (EC. 4.2.2.2)^k

Endopectin lyase (EC. 4.2.2.10)^l

Catalase (EC. 1.11.1.6)^m

Iso-citrate lyase (EC. 4.1.3.1)ⁿ

NADH-Nitrate reductase (EC. 1.6.6.1)^o

L-Phenylalanine ammonia lyase (EC. 4.3.1.5)^p

Lipase (EC. 3.1.1.3)^q

Guanylate cyclase (EC. 4.6.1.2)^{r,s}

Cinnamic-4-hydroxylase (EC. 1.14.13.11)^p

Trehalase (EC. 3.2.1.28)^t

Urease (EC. 3.5.1.5)^u

Peroxidase (EC. 1.11.1.7)^v

Ornithine aminotransferase (EC. 2.6.1.13)^w

Footnotes to Table 1

- a. Richardson (1977)
- b. Ryan (1979)
- c. Lau et al. (1980)
- d. Abe et al. (1980)
- e. Beitz et al. (1978)
- f. Buonocore et al. (1977)
- g. Matsushita and Uritani (1976)
- h. Fisher et al. (1973)
- i. Gomez et al. (1979)
- j. Majunder and Biswas (1973)
- k. Göbel and Bock (1978)
- l. Bock et al. (1975)
- m. Sorenson and Scandalios (1976)
- n. Surendranathan and Nair (1978)
- o. Jolly and Tolbert (1978)
- p. Billett et al. (1978)
- q. Kim et al. (1977)
- r. Vesely et al. (1977)
- s. Takemoto et al. (1980)
- t. Murao and Miyata (1978)
- u. Malhotra and Rani (1978)
- v. Vanlelyveld and Bester (1978)
- w. Dulloo (1980)

Birk 1976; Richardson 1977; Ryan 1979, and will not be covered in detail in this introduction as the work on the soybean trypsin inhibitor only forms a minor part of this thesis.

The other major group of plant inhibitors which have been widely investigated are the proteins affecting the activity of α -amylase enzymes from a wide spectrum of animals. Much of the previous research on these plant α -amylase inhibitors which form the major subject of this thesis has been reviewed by Saunders (1975), Marshall (1975) and Buonocore et al. (1977).

The α -amylase inhibitors are best known in polyploid wheat (Triticum aestivum) seeds, (Deponte et al. 1976; Petrucci et al. 1974; O'Donnell and McGeeney 1976; Redman 1975; Granum and Whitaker 1977; and Pace et al. 1978). These inhibitors are also found in many other cereal species such as rye (Secale cereale), (Strumeyer 1972, Marshall 1977 and Granum 1978), oat seeds (Avena sativa) (Elliot and Leopold 1953), barley seeds (Hordeum vulgare) (Bedetti et al. 1974) whereas no detectable amount of inhibitory activity was reported in rice (Oryza sativa), split peas (Pisum sativum), potatoes (Solanum tuberosum), carrot (Daucus carota), swede seeds (Brassica napus) (Granum 1979), sweet corn (Zea mais) and diploid wheat species (Bedetti et al. 1974). A more detailed examination of diploid species of both Aegilops and Triticum by Vitozzi and Silano (1976) has shown that whilst the former genus contains several members with high levels of α -amylase inhibitors, only T. urartu contained them amongst the diploid Triticum species.

Inhibitory activity has also been reported in species other than the cereals such as kidney beans (Phaseolus vulgaris) (Marshall 1975; Jaffé et al. 1973; Marshall and Lauda 1975b and

Pick and Wöber 1978), field bean seeds (Vicia sativa) and soybean (Glycine max) (Bedetti et al. 1974), but not in other legume seeds e.g. pea seeds (Pisum sativum) and lentil (Lens esculenta) (Bedetti et al. 1974). Other reports have indicated amylase inhibitors in taro root (Colocasia esculenta) (Rao et al. 1967, 1970) in acorns (Quercus spp.) (Stankovic and Markovic 1963) and in unripe mango fruit (Mangifera indica) (Mattoo and Modi 1970).

In addition to the protein inhibitors described above, other less specific α -amylase inhibitors include non-proteinaceous low molecular weight compounds such as the inhibitor β complex from potatoe tubers, transcinnamic and salicylic acid (Hemberg and Larsson 1961), synthetically prepared abscisic acid (Hemberg 1967, 1975) the antibiotic nojirimycin (5-amino-5-deoxy-D-glycopyranose, Niwa et al. 1970), a peptide-like compound (Ueda and Koba 1973), and an oligo-saccharide with a molecular weight of 1500 produced by certain strains of Streptomyces (Murao and Ohyama 1975). A number of oligo- and poly-saccharide derivatives with molecular weights in the range of 500-8000 which are very active in inhibiting α -amylases are produced by several Ampullariella and Actinoplanes species (Frommer et al. 1972). The numerous reports of different amylase inhibitors found amongst members of the Actinomycetes have been reviewed by Frommer et al. (1979). These inhibitors all appear to have a chain of α -1,4-linked D-glucopyranose units with an integral core structure consisting of an unsaturated cyclitol unit bound to 4, 6-dideoxy-4-amino-D-glucopyranose. There are also higher molecular weight inactivators (Miller and Kneen 1947; Chrzaszcz and Janicki 1933, 1934; Strumeyer and Malin 1969).

It has been found that all the protein α -amylase inhibitors from wheat described so far are present in the endosperm part of the seed and their inhibitory activity towards yellow mealworm amylase steadily increases during seed maturation and reaches its maximum value at the stage of full maturity (Pace et al. 1978). These findings are basically in agreement with the qualitative results that Sandstedt and Beckord (1946) reported for the inhibitory activity of the wheat protein on human salivary α -amylase. The localization of these inhibitors in the endosperm of wheat was initially shown by Kneen and Sandstedt (1943) and was confirmed microscopically by Sandstedt and Beckord (1946). These results have been further confirmed by the assay of the inhibitor content in different wheat milling fractions by Saunders (1975) and also by measurement of the inhibitory activity in both wheat brans and whole ground wheat (Marshall 1975). It has been reported that the amount of these inhibitors decreases as soon as the seed starts to germinate, and no further inhibitory activity can be detected in the newly formed tissues, such as coleoptiles and roots (Pace et al. 1978), while in the meantime α -amylase becomes active during germination due to its release from the complex formed with the inhibitors (Warchalewski 1976; Greenwood and McGregor 1965a, 1965b; Geddes 1946; Kruger 1972 and Wierzbowski and Skupin 1965).

These inhibitors make up about 2/3 of the total albumin content of wheatseeds, that is, about 0.2% of the seed weight (Petrucci et al. 1974 and Silano et al. 1975). Some other workers have described the level of these inhibitors in terms of units of inhibitory activity/mg seed dry weight. For example, in wheat flour Granum (1979) found that the content of α -amylase inhibitors

was in the range of 351-590 units/g dry weight, whilst it was only 180 units/g in rye flour. Others such as O'Donnell and McGeeney (1976) found that the level was 635 units/g. Lower values of only 280 units/g have been reported by Shainkin and Birk (1970). In legume seeds these inhibitors are present at a level of 1.5mg/g dry weight in white beans (Phaseolus vulgaris), (Pick and Wóber 1978) and 3-4mg/g dry weight in kidney beans (Marshall and Lauda 1975b). The level in beans has also been expressed in units/g seed dry weight by Jaffé and Lette (1968) who reported their levels to be 770 units/g dry weight of red beans, 650 units/g dry weight of black beans and 2440 units/g dry weight of white beans.

α -amylases isolated from different sources show significant differences in susceptibility to inhibition by the specific proteinaceous inhibitors of α -amylase obtained from plants. Table 2 shows the wide range of different α -amylase inhibitors which have been tested against many sources of α -amylases. In general summarizing this table it can be seen that the inhibitors from wheat can be classified in three different families on the basis of their molecular weights, electrophoretic mobilities (see later section of introduction) and inhibitory specificities. Most of the inhibitors which belong to the 0.28 family (molecular weight 12,000) are active towards insect α -amylase and inactive towards both mammalian and bacterial α -amylase. Others belonging to the 0.19 family (molecular weight 24,000) were mostly active towards both mammalian and insect α -amylase and inactive against bacterial α -amylase. There is still some controversy over the activity of the compounds of molecular weight 60,000; the third family towards mammalian α -amylase. Perhaps the most detailed studies

Footnotes to Table 2

- a. Petrucci et al. (1974).
- b. Saunders and Lang (1973)
- c. Shainkin and Birk (1970)
- d. Granum and Whitaker (1977)
- e. Silano et al. (1973)
- f. Deponte et al. (1976)
- g. Silano et al. (1975)
- h. Granum (1978)
- i. Petrucci et al. (1976)
- j. O'Donnell and McGeeney (1976)
- k. Bedetti et al. (1974)
- l. Strumeyer (1972)
- m. Rao et al. (1970)
- n. Warchalewski (1977)
- o. Marshall and Lauda (1975b)
- p. Pick and Wöber (1978)
- q. Marshall (1977)

(✓) = Inhibitory activity positive

(0) = Inhibitory activity negative

- = Inhibitory activity not determined

(w) = Inhibitory activity weak

were made by Silano et al. (1975) who reported a quantitative survey of the three families of wheat albumins and their inhibitory action on α -amylases from eighteen insect species, from twenty three marine species and from seventeen different species of birds and mammals. A peculiar and interesting specificity of some inhibitors extracted by Warchalewski (1977) from winter wheat was that they showed a positive reaction against the endogenous amylase of the same plant. The α -amylase inhibitors from legumes were found to be mostly active against mammalian salivary and pancreatic amylases and not against plant amylases (Marshall and Lauda 1975b; Pick and Wöber 1978 and Marshall 1977).

One of the main reasons why the protease and α -amylase inhibitors have received the greatest share of the attention given to inhibitors from plants is their possibly deleterious role in the nutrition of animals and man. Both groups of inhibitors tend to occur particularly in those plants which make a major daily contribution to man's source of foodstuffs. We have already seen how the α -amylase inhibitors are particularly concentrated in the seeds of cereals and some legumes. Similarly, the protease inhibitors have been best studied in the nutritionally important seeds of the Leguminosae, the Gramineae and the tubers and fruits of the Solanaceae (Richardson 1977).

In their early studies Miller and Kneen (1947) suggested that the α -amylase inhibitor of a polyphenolic nature in sorghum was unlikely to have an effect in vivo, because it is rather easily inactivated as a result of its ability to combine with many proteins besides α -amylase. However, brown sorghums, which contain high levels of tannins have been shown to retard the growth of chicks and to interfere with dry matter digestibility

in rats more than the white (low tannin) sorghum (Connor et al. 1969; Maxson and Rooney 1972 and Maxson et al. 1973).

Applebaum (1964) and Applebaum et al. (1964, 1965) were considered as pioneers in studying the nutritional aspect of the proteinaceous α -amylase inhibitors. The inhibitors were extracted from wheat bran as crude protein and added to the synthetic diets on which the larvae of Tenebrio molitor L. were fed. Such addition of the α -amylase inhibitors adversely affected development and greatly increased the mortality of the larvae, leading these workers to conclude that α -amylase inhibitors were active both in vitro and in vivo.

This work led to more interest in the same field from various workers. Silano et al. (1975) reported that insects attacking wheat grain and wheat products usually have higher α -amylase activity and are more susceptible to wheat α -amylase inhibitors than those insects which do not normally attack these plants. Also a most interesting characteristic of these inhibitors was reported by Kneen and Sandstedt (1946); Shainkin and Birk (1970) and Silano et al. (1975) who stated that wheat albumin α -amylase inhibitors do not inhibit the endogenous wheat α and β amylases. However, Warchalewski (1975, 1976, 1977) reported the presence of three thermo-stable native inhibitors of protein nature which were active against the endogenous α -amylases in malted Canadian durum wheat. Thus it is not clear yet whether the inhibitors in wheat do serve directly as regulatory agents in wheat seed starch metabolism. (Marshall and Lauda 1975b and Silano et al. 1973).

The most likely function proposed in the literature (Marshall and Lauda 1975b and Silano et al. 1975) for these inhibitors is that they act as natural defence mechanisms against predatory

insects by rendering inactive the susceptible digestive α -amylases of the attacking animal. The results obtained from studies on the effects of these inhibitors on the larval pests (rice weevil and yellow mealworm) of stored grains indicate that the α -amylase inhibitors in wheat could be involved in the resistance of wheat to postharvest infestation (Yetter et al. 1979). It has also been concluded (Lang et al. 1974 and Saunders 1975) that the biological activity of these inhibitors is due to their ability to interfere with starch metabolism.

The digestion of starch is initiated by salivary and pancreatic α -amylases which break down the starch, primarily into maltose. This maltose is then broken down into glucose by maltases in the brush border of the intestinal mucosa cells. The inhibition of α -amylase activity could cause decreased starch digestion resulting in decreased glucose absorption and lowered blood glucose level, and therefore reduced caloric availability which would result in a reduced growth rate.

When the wheat α -amylase inhibitors were fed with a starch-enriched basal diet to rats by Lang et al. (1974) a significant decrease in fecal carbohydrate content and consequently starch availability was noticed. When the inhibitors were destroyed by autoclaving prior to their addition to the diet, starch availability was more or less similar to that obtained by feeding the rats on a basal diet only. When sucrose replaced starch in the diet there was no change in the daily weight gain in the presence of the inhibitors.

Glucose and insulin levels in the blood of human volunteers, rats and dogs have been described by Puls and Keup (1973), who found that postprandial hyperglycaemia and hyperinsulinaemia in

human volunteers, rats and dogs resulting from loading with raw starch could be progressively reduced by the addition of amylase inhibitor preparations to the food intake and that the inhibitors could possibly be deliberately employed as therapeutic agents in patients suffering from diabetes mellitus, obesity or hyperlipoproteinemia. Moreover, pancreatic hypertrophy, increase in α -amylase secretion and growth depression were noticed in young chickens (Macri et al. 1977) when they were given a continuous intake of purified α -amylase inhibitor enclosed in cellulose-coated microgranules, resistant to the peptic action in the chickens' gizzard, but the native inhibitors added to the diet in an unprotected form had no effect on the growth rates of the young chickens. Other work done by Savaiano et al. (1977) has shown that the addition of the purified inhibitors from red kidney beans (Phaseolus vulgaris) to various diets also did not affect the availability of starch or alter the growth rates of weanling rats. These results which point to the possible differential susceptibility of the inhibitors to peptic digestion depending on whether they are fed as an integral component of solid food, or as a purified (crystalline) supplement to a diet may help to explain the apparently contradictory results of other workers.

Various treatments of plant foods such as germination, fermentation and controlled heat treatment can lead to an improvement in their nutritive value which is often presumed to result from a reduction in the level of the inhibitors. During seed germination there is a decrease in the protein content of the seeds and consequently an increase in the free amino acids and amides (Chibnall 1939). At the same time it appears certain that the proteinaceous α -amylase inhibitors decrease and maybe

almost disappear. This has been confirmed recently by Pace et al. (1978) who found no inhibitory activity in germinated wheat seeds.

It has been recognised for many years that the nutritive value of many plant proteins, particularly legumes and cereals is very poor unless subjected to cooking or some other form of heat treatment (Bessho and Kurosawa 1967 and Liener and Kakade 1969). Certainly some of the nutritional improvement is due to partial destruction of the α -amylase inhibitors and other enzyme inhibitors which can be achieved by a 95-121^oC heat treatment (Hernandez and Jaffe 1968, Marshall 1975 and Granum 1978a), but there is also good evidence that levels of several other anti-nutritional factors such as the phytohaemagglutinins, goitrogens, cyanogenic glucosides, lathyrogens, and substances causing favism are reduced by similar treatment (Liener 1974). Also because of their relatively high thermostability some fraction of the α -amylase inhibitors can survive in an active form during the baking processes involved in the manufacture of bread and breakfast cereals (Marshall 1975 and Buonocore et al. 1977). Bessho and Kurosawa (1967) tested the heat inactivation of the α -amylase inhibitors in flour by using pancreatic α -amylase, and found that the inhibitory activity decreased with length of baking time, also that there was no α -amylase inhibitory activity in most of the outer part of the loaf. On the other hand some inhibitory activity was detected in the central part of loaves and in pancakes. This tendency has been confirmed by Granum (1979) who showed by testing on human salivary α -amylase, that baking at 210^oC for 36 minutes removed 80-90% of the inhibitory activity in white bread, rye bread and whole wheat bread, as well as leading to a significant depression of the inhibitory activity

in boiled spaghetti and red beans. Marshall (1975) reported a complete loss of inhibitory activity when the inhibitors were autoclaved at 121°C for 30 minutes. Another of the benefits of cooking was illustrated by the results of Puls and Keup (1973) who found that the inhibitor had much less effect on the digestion of cooked starch as compared to raw starch.

Methods of assaying the activity of α -amylase inhibitors have been investigated by various workers (Nelson 1944; Hopkins and Bird 1954; Bernfeld 1955; Fossum and Whitaker 1974 and Marshall and Lauda 1975a). It seems that all of these workers agree that the inhibition of α -amylase by the protein inhibitors from plants is a time-dependant reaction. Thus, in all investigated assay methods, pre-incubation of the α -amylase with the inhibitors prior to the addition of the soluble starch is necessary, rather than simply to incorporate the inhibitor directly into a digest containing a mixture of enzyme and substrate (Buonocore et al. 1977 and Marshall 1975). The required pre-incubation time to achieve maximum inhibition was found to be in the range of 30-40 minutes (Bernfeld 1955 and Marshall and Lauda 1975a). No inhibition was detected when α -amylase and the inhibitor were pre-incubated at 0°C. The optimum temperature, ionic strength and pH values of the buffer used were experimentally determined by Bernfeld (1955) and Marshall and Lauda (1975a) and were found to be 35-37°C with a 0.02M buffer system in the pH range 4.8 - 7.0.

The activity of the α -amylase remaining after incubation with the inhibitors was determined by measuring the increase of reducing sugar resulting from starch hydrolysis, either by using an alkaline copper-reagent (Nelson 1944) or by dinitrosalicylate reagent (Bernfeld 1955; see Materials and Methods section II.1).

Another method of measuring α -amylase inhibitor activity depends on the determination of the α -amylase activity remaining after the interaction of an aliquot of the inhibitor preparation with a known amount of α -amylase. An iodine staining method was used to determine the amount of starch hydrolysed (Hopkins and Bird 1954 and Marshall and Lauda 1975a). However, it has been reported that this method cannot be used for a wide range of starch concentration (Strumeyer and Romano 1966) and may be affected by inactive proteins if they are present (Wilding 1963).

A number of semi-quantitative screening techniques are available for the detection of α -amylase inhibitors in biological material. For example there is a starch-agar gel, described by Fossum and Whitaker (1974) in which 3mm wide cellulose strips saturated with the solution to be examined for inhibitory activity are placed on a substrate slab gel consisting of 0.25% starch in 1.5% agar for 2h. at 37°C. After this time the strips were removed and another 3mm wide cellulose strip saturated with the amylase solution was placed at right angles across the first strip. The system was incubated for 6h. at 37°C. Amylase activity was shown by clear lysis-zones on a deep purple background after adding an iodine containing solution. The presence of inhibitors was indicated by interruption or narrowing of the lysis-zone where the inhibitor-containing and amylase-containing strips were crossed. A recent report (Vitozzi et al. 1976) has described a Technicon Autoanalyzer adapted to carry out a continuous assay of α -amylase activity to detect the presence of α -amylase inhibitors. This technique was based upon the Bernfeld reducing sugar procedure and could detect α -amylase inhibitors which act slowly.

The early studies of Kneen and Sandstedt (1943, 1946) on the α -amylase

inhibitors in wheat indicated that these inhibitors were proteins in nature, soluble in water, precipitated in ethanol and ammonium sulphate, very sensitive to both oxidizing agents (e.g. chlorine, bromine, sodium chlorite and hydrogen peroxide) and reducing agents (e.g. sodium sulphate, hydrogen sulphide and ascorbic acid) and relatively stable to heat treatment. This last property has been very advantageous in permitting several workers to employ a heat treatment stage in their purification methods.

The separation and purification of the components of the three albumin families from wheat has largely depended on their physico-chemical properties. Thus in different laboratories various workers have tended to employ gel-filtration and ion-exchange chromatography most frequently, but the bio-chemical properties of the inhibitors are such that the technique of affinity chromatography would appear to be a particularly useful tool for their purification (Marshall 1977), in the same way as it has been used for the purification of some protease inhibitors (Mosolov et al. 1979).

Marshall (1977) reported the purification of two α -amylase inhibitors from wheat and rye flour. Hog pancreatic α -amylase was immobilized by coupling to cyanogen bromide-activated Sepharose 4B and the heat-treated extracts of wheat and rye were applied to the column which was thoroughly washed to remove unbound protein. Washing the column with 66% aqueous ethanol, reversed the interaction of the inhibitors with the immobilized α -amylase and resulted in the elution of a sharp peak of the inhibitor. This technique was applied in reverse by Buonocore et al. (1975) to purify a large number of amylases inhibited by the albumin from wheat kernel (e.g. human saliva, chick pancreas, octopus digestive gland and

Tenebrio molitor larvae). The procedure involved linking the protein inhibitors from wheat to Sepharose and then specifically eluting the amylase absorbed to the gel with a high concentration of maltose.

Originally it was also reported that the gliadin fractions which occur in wheat seeds and were thought to cause coeliac disease, contained inhibitory activity when tested on both human salivary and hog pancreatic α -amylases (Strumeyer 1972 and Strumeyer and Fisher 1973). A number of pure gliadin fractions have since been tested for anti-amylase activity by Pace et al. (1978) who were unable to detect any inhibition on human salivary, yellow mealworm and pig pancreatic α -amylases. These workers also showed that α -amylase inhibitors, normally occurring in albumin fractions of wheat, can be extracted with solvents (70% ethanol, dilute acid) that were considered typical for wheat gliadin.

The molecular weights of the α -amylase inhibitors from plants lie in the range between 12,500-60,000 daltons. The molecular weights of the inhibitors from wheat have been variously reported as 14,500, 29,000 and 30,000 (Granum and Whitaker 1977), 21,000 (Saunders and Lang 1973 and O'Donnell and McGeeney 1976), 18,000 and 26,000 (Shainkin and Birk 1970) 12,500; 24,000 and 60,000 (Deponte et al. 1976). Many of the larger molecular weight inhibitors exhibit a reversible dissociation and association reaction in the presence or absence of dissociating agents such as urea, guanidine HCl or sodium didecyl sulphate leading to the formation of a number of sub-units with molecular weights close to 12,000 daltons (Petrucci et al. 1974 and Deponte et al. 1976). Similarly the α -amylase inhibitor extracted from beans (Phaseolus vulgaris) is an albumin of about 43,000 daltons molecular weight which

dissociated into sub-units with molecular weights of either 15,500 or 11,000 daltons in the presence of a dissociating agent (Marshall and Lauda 1975b and Pick and Wöber 1978).

The inhibitors from the cereals appear to be simple proteins like the protease inhibitors with no carbohydrate molecules attached to them (Saunders and Lang 1973 and Granum and Whitaker 1977) or with at most a single reducing sugar per mole (Petrucci et al. 1978) but the α -amylase inhibitors isolated from various beans are glycoproteins in nature with as much as 15% of carbohydrate in their structure (Marshall and Lauda 1975b and Pick and Wöber 1978).

As a result of the investigations carried out by several groups of workers (Shainkin and Birk 1970; Feillet and Nimmo 1970; Cantagalli et al. 1971; Silano et al. 1973; Saunders and Lang 1973 and Deponte et al. 1976) it soon became evident that in wheat seeds the α -amylase inhibitors exist in multiple molecular forms.

Petrucci et al. (1974) and Deponte et al. (1976) have classified all of the albumin α -amylase inhibitors in wheat kernels into three major inhibitor groups separable by Sephadex G-100 gel chromatography with molecular weights of 60,000 (Peak II), 24,000 (Peak III) and 12,000 (Peak IV). The smallest of these, the 12,000 molecular weight group (Peak IV), showed heterogeneity in polyacrylamide gel electrophoresis at pH 8.5 with a main component of 0.28 mobility (with respect to bromophenol blue) and a pI of 6.2 (Silano et al. 1973) and was called the 0.28 family from the electrophoretic mobility of the main component.

Cantagalli et al. (1971) purified three fractions which appeared to belong to the 0.28 family (coded 0.28, 0.34 and 0.39 according to their mobilities in polyacrylamide gel electrophoresis).

Further separation of fraction 0.34 into fractions 0.32, 0.35 and 0.49 was achieved by Silano et al. (1973) who described them as being very closely related as seen from their identical conformational structures indicated by circular dichroism spectra, amino acid compositions and their common activity against yellow mealworm (Tenebrio molitor) larval mid-gut α -amylase and their inactivity towards both human salivary and pancreatic α -amylases.

The close homology of the 0.28 α -amylase inhibitor family in wheat has been confirmed recently by Redman (1975, 1976) who purified three isoinhibitor albumins from wheat flour (Triticum aestivum), each composed of approximately 120 amino acids but lacking in phenylalanine and histidine. These isoinhibitors showed a complete homology in their 24 N-terminal amino acids, and slight differences in the amino acid compositions of the whole proteins and their CNBr fragments.

Some other research workers (Shainkin and Birk 1966 and 1970 and Granum and Whitaker 1977) have obviously investigated α -amylase inhibitors which were very similar to those of the 0.28 family in their molecular weight and specificity, but have not always described them as members of this group.

The 0.28 family is not the only isoinhibitor family amongst the wheat flour albumins to show heterogeneity, since Petrucci et al. (1974) and Deponte et al. (1976) have also shown that the proteins in Peak III (molecular weight of 24,000 daltons) have a high heterogeneity on polyacrylamide gel electrophoresis at pH 8.5 with a main component of 0.19 mobility and a pI of 7.1. This group was first described by Ewart (1969), then by Sodini et al. (1970), and extensively studied and characterized by

Petrucci et al. (1976). All of the 0.19 components were active against human and insect (Tenebrio molitor) α -amylases and showed a great similarity in their amino acid compositions and their circular dichroic spectra in the far and near U.V.

Close examination of the properties of the AmI₂ inhibitor (Shainkin and Birk 1970), the I3A inhibitor (Feillet and Nimmo 1970) the 0.20 inhibitor of O'Donnell and McGeeney (1976) and the inhibitors I and II of Saunders and Lang (1973) strongly suggest that they are all probably components of the 0.19 family.

Peak III (24,000 M.W.) and Peak II (60,000 M.W.) undergo a reversible dissociation and association behaviour in the presence or absence of dissociating agents e.g. guanidine HCl (GuHCl) or sodium dodecylsulphate (SDS) yielding a number of sub-units close to 12,000 molecular weight (Deponete et al. 1976). The scheme shown in figure 1 (after Deponete et al. 1976) summarizes diagrammatically all of the information available on the albumin α -amylase inhibitors from wheat as well as the known and inferred relationships among different inhibitor groups.

This association and dissociation phenomena observed in the albumin α -amylase inhibitors prompted some workers to suggest that these inhibitors are coded for by a small number of structurally related genes, possibly derived by mutation from a common ancestor, (Vitozzi and Silano 1976) and that some mutant genes have produced albumins which are able to associate, thus giving rise to polymer components (Deponete et al. 1976 and Buonocore et al. 1977).

Although differing in their molecular weights, inhibitors 0.28 and 0.19 appear to be related (Shainkin and Birk 1970; Silano et al. 1973; Silano et al. 1977 and Petrucci et al. 1978).

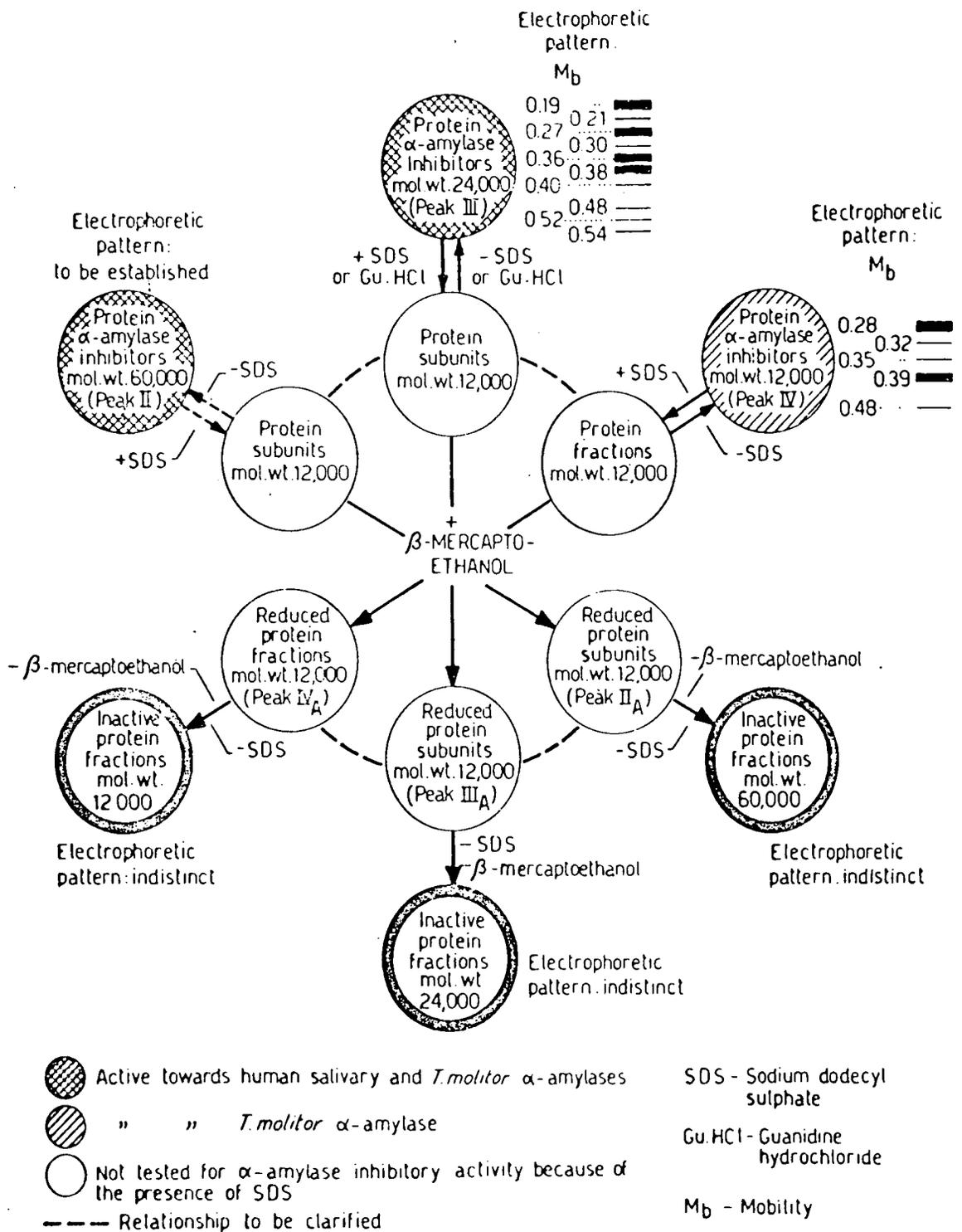


Fig.1 Scheme summarizing the information available on albumin α-amylase inhibitors from wheat and the known and inferred relationships among different inhibitor groups. (Deponete et al. 1976)

The differences in the molecular weights and specificity of the two α -amylase inhibitors AmI_1 and AmI_2 of Shainkin and Birk (1970) were thought to be due to the presence of an additional fragment with an active site against human α -amylase which when added to AmI_1 formed AmI_2 . This view was supported by Silano et al. (1973) who reported that the inhibitors of the 0.28 family might actually be contained in inhibitor 0.19 as one of the two sub-units.

There is also some evidence for this view from the similarity in their denaturation temperatures (Silano and Zahnley 1978) and for a relationship between the 0.28 and 0.19 inhibitors from the homology shown in the preliminary sequence analyses which have been carried out by Petrucci et al. (1978). These workers sequenced the 24 N-terminal amino acids of the 0.19 inhibitor without separating its two sub-units and compared their sequence with that of the 0.28 inhibitor partially sequenced by Redman (1976). Nine of the first twenty-four N-terminal residues were found to be identical, these being the first, fourth, seventh, ninth, fifteenth, seventeenth, eighteenth, twenty-first and twenty-second positions in the sequence.

The third family of α -amylase inhibitors amongst the wheat albumin fractions is the Peak II of Petrucci et al. (1974) and Deponte et al. (1976). These investigators showed that the proteins in this peak possessed an inhibitory activity against both human salivary α -amylase and insect larval mid-gut α -amylase. The Peak II proteins (molecular weight 60,000) also undergo a reversible dissociation into five sub-units, each of an equal molecular weight to Peak IV (the smallest molecular weight inhibitors of 12,000 daltons). The large molecular weight Peak II inhibitor showed five components when subjected to gel electrophoresis

under both alkaline and acidic buffered conditions (Deponte et al. 1976). However, this family of wheat α -amylase inhibitors may need more characterization and further investigation to know whether this inhibitory activity does belong to each sub-unit individually or all of them as a cluster together.

It is important to mention here that some of the wheat albumins extracted by various workers in different laboratories have not been tested against any source of α -amylase (Ewart 1969; Feillet and Nimmo 1970; Sodini et al. 1970 and Redman 1975, 1976). Generally as their amino acid compositions and most of their physico-chemical properties were very similar they were assumed to be identical with the inhibitors.

Several different studies on the α -amylase:inhibitor interaction using the classical methods of Lineweaver-Burk and Dixon plots have shown in all cases that it was non-competitive in nature (Marshall 1975; Buonocore et al. 1976; Silano et al. 1977 and Pick and Wöber 1978). This finding is in accord with the different specificities of inhibition noted earlier for a range of different α -amylases (Table 2). Marshall (1975) has pointed out that if the inhibitors acted by competing with starch for the active site of the enzyme, one would expect all amylases to be affected. This is not the case. It would appear that certain amylases have groupings which can combine with some of the inhibitors while others do not, although all the amylases have the catalytic structures necessary for starch hydrolysis.

There is now good evidence that several of the inhibitors act by binding to the α -amylase to form stable stoichiometric complexes. The amylase inhibitors from various legume seeds appear

to form 1:1 molar complexes (Marshall 1975, Marshall and Lauda 1975b and Pick and Wöber 1978). In the case of the wheat 0.19 (Mw 24,000 dimeric) forms interacting with yellow mealworm amylase there is also a molar stoichiometric ratio of one mole of enzyme per mole of inhibitor (dimer) whereas under the same conditions the wheat 0.28 (Mw 12,000 monomer) forms give a complex with one mole of enzyme per two moles of inhibitor (Buonocore et al. 1976 and Silano et al. 1977). These stoichiometric ratios have been confirmed by kinetic, spectral, gel-filtration and differential-calorimetric studies (Buonocore et al. 1980).

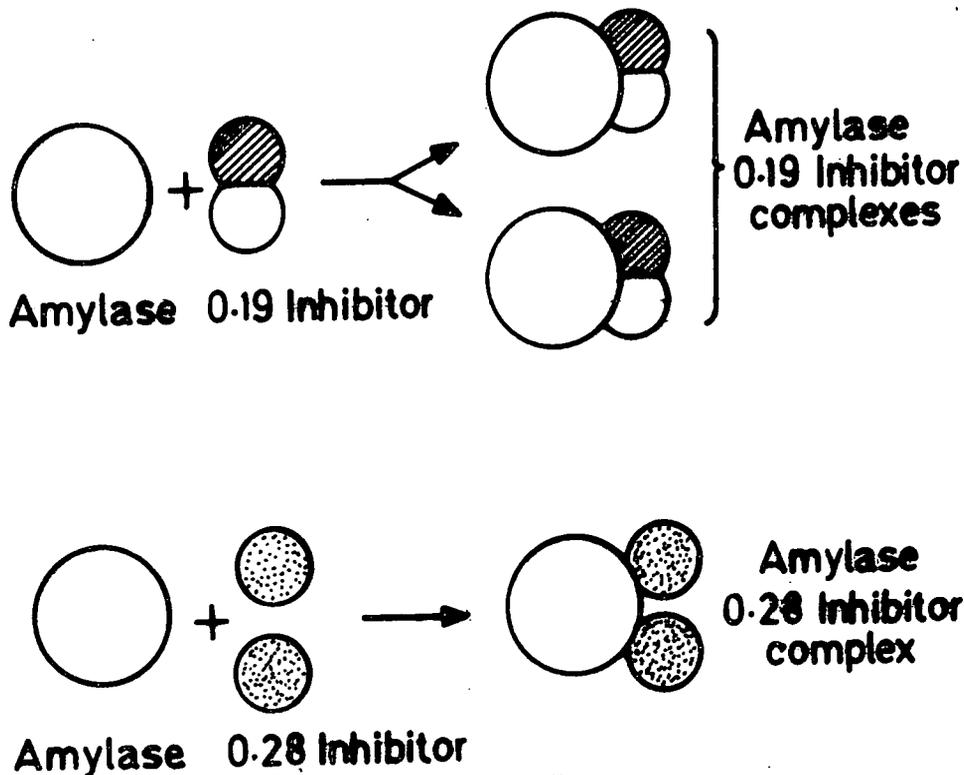


Fig. 2 (after Silano et al. 1977). "Hypothetical model of the complexes formed between inhibitor 0.19 or 0.28 and α -amylase"

They have been explained by means of a hypothetical model (Fig. 2) in which the enzyme molecule has two binding sites for the inhibitors (one for each protomer of molecular weight

12,000) and where each inhibitor 0.19 sub-unit and each 0.28 molecule have a single binding site for the amylase (Silano et al. 1977 and Buonocore et al. 1978). As the two 0.19 sub-units (protomers) are not identical and both can interact with each binding site on the amylase the formation of two non-identical amylase 0.19 complexes, containing one mole of enzyme per mole of inhibitor is to be expected. This has in fact been observed during their elution from Bio-Gel columns by Buonocore et al. (1977, 1980). Two molecules of 0.28 inhibitor should have more freedom than two sub-units linked in a 0.19 molecule, of adjusting themselves to complementary parts of the amylase molecule. Again this appears to be the case, as the 0.28 inhibitor appears to form a more stable complex with the yellow mealworm enzyme than the 0.19 inhibitor. The model does not imply significant structural differences between the binding sites of each 0.19 sub-unit and the 0.28 molecule and is in line with the close structural similarity of the 0.28 and 0.19 forms indicated by the preliminary sequence studies of Redman (1976) and Petrucci et al. (1978).

However, the molar absorption coefficient difference of α -amylase in the presence of inhibitor 0.28 is twice that observed in the presence of inhibitor 0.19 (Buonocore et al. 1980). This suggests that either one of the two binding sites of the amylase molecule for inhibitor 0.19 is silent, or the reactive tryptophan residues involved in the enzyme-inhibitor binding are located on the inhibitors and one of the two inhibitor 0.19 sub-units lacks such residues. Actually the 0.28 inhibitor does appear to have a higher content of tryptophan residues than does the inhibitor 0.19 (Shainkin and Birk 1970). It is clear however that a better understanding of the inhibitor-enzyme binding reaction requires a

more detailed knowledge of the structure of the inhibitor molecule.

The major aim of this investigation was to determine the complete primary structure of one of the α -amylase inhibitors found in wheat (Triticum aestivum) seeds. An important and necessary pre-requisite of such an aim, in view of the complex heterogeneity displayed by this group of inhibitors in wheat, was the ability to purify one of the isoinhibitors to homogeneity in sufficient quantities to permit detailed sequence analysis. This was achieved with one of the isoinhibitors of the CIII/0.28 (12,000 molecular weight) group. This thesis can be considered as a pioneer work in this field as it is the first in which the complete amino acid sequence of an α -amylase inhibitor from plants is reported. In addition the complete amino acid sequence of a protease inhibitor (PI IV) purified from soybean seeds by Dr. D.E. Foard (Oak Ridge, Tennessee) was also determined.

MATERIALS AND METHODS

I. GENERAL

1. Biological Materials

(a) Wheat (Triticum aestivum var. Flanders) seeds were obtained from West Cumberland Farmers Ltd., Seed Division, Head Office, Burlene, Hexham, Northumberland.

(b) Protease inhibitor PI IV purified from soybean seed (Glycine max var. Tracy) was kindly provided by Dr. D.E. Foard, University of Tennessee, Biology Division, Oak Ridge National Laboratory, Tenn., 37830, U.S.A.

(c) Fraction I, small sub-unit partially purified from pea seeds (Pisum sativum) was kindly provided by Mr. J. Gilroy, University of Durham, Botany Department, South Road, Durham, DH1 3LE, England.

2. Chemicals and Reagents

All chemicals, except the ones mentioned below, were obtained from B.D.H. Ltd., Dorset, and were of analytical grade when available.

Hog pancreatic α -amylase, E.C. 3.2.1.1 (DFP treated, twice crystallized) was obtained from Sigma Chemical Co., St. Louis, Mo., P.O. Box 14508, U.S.A.

Trypsin, E.C. 3.4.21.4. (twice recrystallized, DPCC treated) was obtained from Sigma Chemical Co., P.O. Box 14508, St. Louis, Mo., U.S.A.

Chymotrypsin, E.C. 3.4.21.1. (three times recrystallized) was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.

Carboxypeptidase A, E.C. 3.4.17.1. (di-isopropylphosphoro-fluoridate-treated, Crystalline, suspended in water) was obtained

from Sigma Chemical Co., St. Louis, Mo., P.O. Box 14508, U.S.A.

Thermolysin, E.C. 3.4.24.4. (crystalline) was obtained from Daiwa Kasei K.K., Osaka, Japan.

Staphylococcus aureus V8 protease (E.C. 3.4.21.19.) was obtained from Miles Laboratories Ltd., England.

CM52 Cellulose Cation Exchanger and DE52 Cellulose Exchanger were obtained from Whatman Ltd., Springfield Mill, Maidstone, Kent, England.

Sephadex G-10

Sephadex G-50

Sephadex G-75

were obtained from Pharmacia Ltd., Uppsala, Sweden.

Bio-gel P2

Bio-gel P4

were obtained from Bio-Rad Laboratories Ltd., London.

Guanidine Hydrochloride, Sequenal grade was obtained from Pierce Chemicals Ltd., Box 117, Rockford, Illinois 61105, U.S.A.

Ninhydrin (Indantrione hydrate) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

Pyridine (Sequencer grade) was obtained from Rathburn Chemicals, Walkerburn, Peebleshire, Scotland.

3,5-Dinitrosalicylic acid (crystalline) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

4-NN-Dimethylaminoazobenzene 4-isothiocyanate (crystalline) was prepared by Mr. J. Gilroy, as described by Chang et al. (1976) and Chang (1977)

p-Dimethylaminobenzaldehyde was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Arginylarginine was obtained from Cyclo Chemical Corporation, Los Angeles, Cal., U.S.A.

Coomassie brilliant blue, was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Cyanogen Bromide (CNBr) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

3. Other Materials

Polyamide sheets were obtained from:-

- (a) Cheng Chin Trading Co., Ltd., Tapei, Taiwan, through B.D.H. Chemicals Ltd.
Schleicher & Schuell F 1700 from
- (b) Pierce Chemicals Ltd., Box 117, Rockford, Illinois 61105, U.S.A.

Visking tubing was obtained from:
Medicell International Ltd., 239 Liverpool Road, London N1 1LX.

II. PURIFICATION METHODS

(A) A generalized scheme for the purification of α -amylase inhibitor is shown in Fig. 3.

(B) The protease inhibitor PI IV was purified from Tracy soybean seeds as described by Hwang et al. (1977).

1. α -Amylase Inhibitor Assay

α -amylase inhibitor was assayed quantitatively according to the method of Bernfeld (1955).

(a) Dinitrosalicylic acid solution was prepared by dissolving 1g of 3,5-dinitrosalicylic acid in 20ml of 2N NaOH and 50ml water. Then 30g of potassium sodium-tartrate (Rochelle Salt) were added. The volume was made up to 100ml with distilled water. The prepared solution was covered by parafilm to protect it from atmospheric CO_2 .

(b) Starch solution (substrate solution) was made by dissolving 1g of soluble starch in 50ml of boiling water and the volume was made up to 100ml by adding more cold distilled water.

0.5ml of Enzyme solution (0.25mg of hog pancreatic α -amylase/50mM Tris-HCl buffer pH 7) was added to 1 ml of inhibitor solution, shaken and incubated at room temperature for 30 minutes. 0.5ml of the starch solution (1% w/v with H_2O) was added and the mixture was incubated for 10 minutes with occasional shaking at room temperature. 2ml of dinitrosalicylate solution was added to stop the reaction and to give colouration. Then the mixture was heated in a water bath at 100°C for 10 minutes, 10ml of distilled water was added, mixed, and the absorption at 530nm was measured. The change in the absorption represents the production of the reducing

sugar liberated by starch digestion, and indirectly, the extent of α -amylase inhibitor activity.

Two control experiments were carried out during the assaying procedure, in the first experiment the enzyme solution was incubated with the substrate without adding inhibitor solution, while in the second experiment the inhibitor was added to the substrate solution without any addition of the enzyme. These two control experiments were carried out to illustrate the maximum and the minimum digestion of starch under the same experimental conditions.

2. Extraction of Wheat Proteins

Extraction was carried out at room temperature, wheat seeds were ground in a mill using the 0.1mm sieve (Brook Laboratory Mill, size 8 inches), supplied by Christy and Morris Ltd., Chelmsford, England.

1kg of wheat flour was homogenized with 2l. of distilled water, using a Waring blender at its maximum speed for 3 minutes. The macerate was then centrifuged at 2000g for 15 minutes in an MSE 4L centrifuge (Measuring and Scientific Equipment Ltd., London, England). The supernatant was recentrifuged at 9000g for 20 minutes (M.S.E. high speed 18).

3. Concentration of Protein Solutions

Owing to the relatively large volume of liquid (1.6l.) encountered during preliminary extraction and purification procedures, attempts were made to determine the most effective and least time consuming method for the concentration of the extracts. Rotary evaporation (Buchi rotavapour, Orme Scientific Ltd., Middleton, Manchester, England) was found to be the most effective

method, due to the high temperature resistance of the inhibitor.

1.61. of the clear supernatant was concentrated to 300ml solution using the rotary evaporation technique in vacuo at 55°C.

4. Heat Fractionation

The 300ml sample of concentrated extract solution was heated to 70°C in a boiling water bath. The sample temperature was maintained at 70°C for 10 minutes. The copious precipitate of protein which formed during this heating process was removed by centrifugation at 6000g for 20 minutes at 10°C and discarded.

5. Ethyl Alcohol Fractionation

A calculated volume of absolute ethyl alcohol was added slowly to the clear supernatant to give a 70% (v/v) alcohol solution. The solution was stirred using a magnetic stirrer for 2h. at room temperature, centrifuged, and the pellet was discarded. Then a calculated volume of absolute alcohol was added to the 70% (v/v) alcohol sample solution to bring up the concentration to 90% (v/v) solution. This solution was stirred for 8h. at 4°C and the precipitate which formed was collected by centrifuging at 9000g. The pellet of protein thus obtained was dissolved in a minimum volume of 50mM pyridine-acetate buffer pH 6.5 and dialysed extensively against the same buffer at 4°C.

6. Gel-Filtration Chromatography

A column (140cm x 6.8cm) of Sephadex.G-75 was prepared by pouring a slurry of the gel equilibrated in 50mM pyridine-acetate buffer pH 6.5. Aliquot (80ml) samples of the inhibitor extract were loaded onto the column, which was then eluted with the equilibrating buffer at a flow rate of 120ml/h. Fractions of 14ml were collected using a (L.K.B. Ltd., Box 305, S-161 26

Bromma, Sweden) fraction collector. The column eluate was monitored at 280nm for the presence of proteins. Inhibitor assays were carried out on every second fraction (Fig. 9). Fractions showing inhibitory activity were pooled and concentrated by rotary evaporation on in vacuo at 55°C and then lyophilized (Fig. 9).

7. Ion-Exchange Chromatography

A column (23cm x 2.5cm) of DE 52 Cellulose (preswollen by the manufacturer) was equilibrated in 50mM Tris-HCl buffer pH 8.4. The sample of crude inhibitor was dissolved in 30ml of the same buffer and loaded onto the column which was developed at a flow rate of 60ml/h. Fractions of 6ml were collected using an L.K.B. (L.K.B. Ltd., Box 305 S-161 26 Bromma, Sweden) fraction collector. The column was then washed with about 4 x column volumes of the starting equilibrating buffer, during which period one of the inhibitors was eluted. The other two inhibitors were eluted by using a linear gradient of NaCl from 0-0.1M NaCl (1L. of each)(Fig. 10.)

III. CRITERIA OF HOMOGENEITY

1. N-terminal Analysis

N-terminal analyses were made using 30 Mg samples of both native and S-carboxymethylated protein according to the method of Gray and Hartley (1963b) and will be described in detail in section (IV 9).

2. Polyacrylamide Gel Electrophoresis

Homogeneity was determined by subjecting the protein samples to polyacrylamide disc gel electrophoresis on 7% gels, as described

by Ornstein (1964) and Davis (1964). The gels were buffered with Tris-HCl at pH 8.9, and ammonium persulphate was used as a catalyst, with N,N,N,N tetramethyl ethylene-diamine (TEMED) as an accelerator for polymerization.

The gels were prepared from standard stock solution as follows:-

Solution (A)	1N HCl	48ml
	Tris	36.56g
	N,N,N,N tetramethyl ethylene-diamine (TEMED)	0.24ml

The volume was brought up to 100ml with distilled water

Solution (B)	Acrylamide	28g
	N,N,N Methylenebis-acrylamide (Bis)	0.652g
	Distilled water to 100ml	

Solution (C)	Ammonium Persulphate	0.14g
	in 100ml of distilled water (freshly prepared)	

The final gels were prepared by mixing these stock solutions in a ratio of:

$$1 \text{ (A)} : 2 \text{ (B)} : 4 \text{ (C)} : 1 \text{ (H}_2\text{O)}$$

by volume.

The gel solution was poured into perspex cylinders (0.6cm x 8cm) which were firmly closed at one end with a piece of plastic cap to give flat bottoms to the gels. Sufficient gel was poured into each tube to give an equal length in all the tubes. The gel was then carefully over-layered with 3mm of distilled water. The tubes were exposed to daylight for 1-2h. After polymerization had occurred the water layer at the top and the plastic caps at the bottom were removed and the tubes were

fitted into the electrophoresis apparatus. Both reservoir compartments of the electrophoresis apparatus were filled with the following reservoir buffer which was adjusted to pH 8.3.

Tris	6.0g	} diluted 10x; used once only
Glycine	28.8g	
Made up to 1000ml with distilled water		

10 μ l aliquots of the sample solution which contained 2mg protein/ml dissolved in a 50% (w/v) sucrose solution were loaded on top of the gels. Bromophenol blue (50 μ l) was added as a marker and the power supply was connected. The cathode was attached to the upper reservoir, and the current was adjusted to 2mA/gel for the first 15 minutes and then increased to 4mA/gel. Electrophoresis was carried out until the front marker reached the bottom of the gel. The power supply was turned off and the gels were removed from the tubes by rimming under water using a needle syringe to introduce water to separate the surfaces of tube and gel. The gels were cut at the front marker and dipped in the staining solution (0.25% (w/v) Coomassie brilliant blue in methanol-acetic acid-water, 25:7:68 by volume) for 3-4h. Destaining was carried out by immersing the gels in several changes of the above solvent mixture for 24h.

IV. QUANTITATIVE AMINO ACID COMPOSITION OF PROTEINS AND FRAGMENTS

The amino acid composition of proteins and fragments were determined using a Locarte amino acid analyser. Proteins (0.03-0.05 μ M^{mols}) and fragments (0.05-0.15 μ M^{mols}) were hydrolysed with 0.5ml 6N HCl at 105°C in evacuated, sealed Pyrex tubes.

Duplicate samples were prepared to perform 24h. and 72h.

hydrolyses so that zero time values might be obtained for threonine and serine, and maximum values of valine, isoleucine and leucine (Moore and Stein 1963).

After the hydrolysis was completed the samples were dried in vacuo and kept at 4°C until required for analysis.

Cysteine was determined as CM-cysteine following S-carboxymethylation as described by Crestfield et al. (1963).

Tryptophan was determined qualitatively by using the spectrophotometric absorption at 280nm and chemically by the use of the Ehrlich reagent (section 5 IV) and the DABITC method of Chang et al. (1978).

V. DETERMINATION OF AMINO ACID SEQUENCE

1. Reduction and S-Carboxymethylation

The method used was a modification of the method used by Crestfield et al. (1963). Samples of the protein (10-30mg) were dissolved in 2ml of 6M guanidine hydrochloride - 1M Tris, pH 8.5, and kept under nitrogen. 30 μ l of 2-mercaptoethanol were added and the mixture left under nitrogen for 4h. at room temperature. 0.3ml of a freshly prepared solution of iodoacetic acid (0.268g/ml) in 0.1M NaOH was added and the solution was kept in the dark for 15-20 minutes.

The reaction products were then separated in the dark by chromatography on a column of Sephadex G-10 (30cm x 1.5cm), equilibrated with 50mM pyridine-acetate buffer at pH 5.6. The column was developed at a flow rate of 30ml/h., and 1 ml fractions were collected.

The reduced and S-carboxymethylated protein was located spectrophotometrically at 280nm and the protein peak was pooled and lyophilized.

2. Cleavage by Cyanogen Bromide (CNBr)

CNBr cleavage was carried out by the method of Steers et al. (1965). Lyophilized samples of the reduced and S-carboxymethylated protein (100mg) were dissolved in 0.5ml of 70% (v/v) formic acid. An 8-fold excess (w/w) of CNBr was added, mixed, and kept in the dark for 24h. at room temperature.

The mixture was diluted 10x with distilled water and the reaction was terminated by lyophilization. The fragments were then separated by:

(a) Gel-Filtration Chromatography

A column (3.2cm x 200cm) of Sephadex G-75 equilibrated and eluted with 50mM pyridine-acetate buffer pH 5.6 was used for gel chromatography of the CNBr fragment dissolved in the same buffer. 10ml fractions were collected at a flow rate of 55ml/h (Fig. 14). Fragments and peptides were located in the eluting buffer both by spectrophotometry at 280nm and by examining small samples (10-40 μ l) of selected examples of the eluted fractions for the presence of N-terminal amino acids by the dansyl technique of Gray and Hartley, (1963b). Fractions containing particular proteins or fragment peaks were pooled and freeze-dried.

(b) Chromatography on CM 52 Cellulose

A column (1.6cm x 30cm) was packed by pouring a slurry of CM 52 Cellulose (preswollen by the manufacturer), equilibrated in 5mM sodium acetate buffer, adjusted to pH 3.5 with acetic acid into the column. Packing was achieved by passing through 400ml of the starting buffer. Samples of the CNBr generated

fragments after gel filtration were dissolved in 10ml of the starting buffer and loaded on the top of the column which was then washed by passing through 60-65ml of the starting buffer and then further developed using a linear gradient of sodium chloride from 0-0.2M (300ml of each).

3ml fractions were collected and the fragments were located spectrophotometrically at 280nm (Fig. 15 a,b). Fractions corresponding to each of the resulting peaks were pooled and their N-terminal amino acids were determined according to Gray and Hartley (1963b).

3. Digestion with Proteolytic Enzymes

(A) Chymotrypsin and Trypsin

The reduced and carboxymethylated protein (20-30mg) was dissolved in 250 μ l of 0.2M N-ethylmorpholine-acetate buffer, pH 8.5. A calculated weight of the enzyme solution (1mg/ml) in the same buffer was added to the buffer containing the protein sample to give a 2% (w/w) enzyme/substrate ratio. The enzyme-substrate mixture was then incubated for 2-4h. at 37°C. The reaction was terminated by loading the mixture onto the top of a P2 gel filtration column for chromatography.

(B) Thermolysin

The digest conditions and enzyme concentration were as for chymotrypsin and trypsin except that the buffer contained 5mM CaCl₂ to activate the enzyme.

(C) Staphylococcus aureus V8 Protease

The 2% (w/w) enzyme-substrate proportion was maintained, but the buffer 50mM Na₂HPO₂-NaH₂PO₄, pH 7.8 system was as described by Houmard and Drapeau (1972).

4. Purification of Peptides

(A) Gel Filtration

Peptides resulting from tryptic, chymotryptic, thermolysin and Staphylococcus aureus V8 digestion were subjected to gel chromatography on a column (0.8cm x 190cm) of Bio-gel P2 equilibrated with 50mM pyridine-acetate buffer pH 5.6. The presence of peptides in the eluted fractions was detected by N-terminal analysis.

The first major peak of peptides eluted from the P2 column chromatography was pooled, freeze dried and subjected to a second gel chromatography on a column (1.0cm x 160cm) of Sephadex G-50 (fine) equilibrated with 50mM pyridine-acetate buffer, pH5.6.

(B) High Voltage Paper Electrophoresis

Impure peptides after gel chromatography were subjected to a high voltage paper electrophoresis as described by Thompson et al. (1970) at pH 6.5 (pyridine-acetic acid-water, 25:1:225 by volume) on a flat-plate apparatus (107cm x 15cm) (The Locarte Co., London, SW3). The separation was achieved on Whatman 3mm paper (107cm x 15cm) at a voltage of 9KV to give a current of 30-50mA for 120-150 minutes at 7 p.s.i. Peptides requiring further separation were purified by the same electrophoretic technique performed at pH 1.9 (acetic acid-formic acid-water; 4:1:45 by volume).

A standard marker solution was added to the peptides before running in electrophoresis to enable the calculation of mobilities. This marker solution was made by dissolving Arginylarginine in 1M NaHCO₃ to give a 0.1M solution and this was heated with an equal volume of 0.2M dansylchloride in acetone. After 1h. at 37°C the mixture was diluted 1000-fold and dansylarginine dis-

solved in ethanol was added to give a final concentration of 0.1mM.

(C) Paper Chromatography

Peptides needing further purification were separated by descending paper chromatography using the BAWP solvent system (n-butanol-acetic acid-water-pyridine; 75:15:60:50 by volume). Samples were mixed with 50 μ l of a marker solution containing 1mg/ml of dansyl-arginine in 95% (v/v) ethanol and chromatographed on Whatman 3mm (35cm x 50cm) paper for 24h. at room temperature in Gallenkamp frames and bags.

5. Location of Peptides

(i) Peptides in solution (fractions from column chromatography) were located spectrophotometrically by measuring the absorbance at 280nm on a Perkin-Elmer, Model 402, Spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, Bucks., England).

(ii) N-terminal analysis (as described later) was also applied to selected fractions after column chromatography.

(iii) Cadmium/ninhydrin reagent (Heilmann et al. 1957).

The location of peptides on paper was determined by using 10% strips cut from the electrophoretogram or chromatogram. These guide strips were dipped in a solution containing the following reagents:

Cadmium Acetate	100mg
Water	10ml
Acetic Acid (Glacial)	5ml
Acetone	100ml
Ninhydrin	1g

After dipping the guide strip was allowed to dry at room

temperature for 2-3 minutes and then heated at 105°C for 1-2 minutes. Guide strips from paper chromatography were allowed to dry at room temperature for 10h., during which time the pink/red colour of the peptides appeared. Heating at 105°C was found to be inadvisable as it tended to lead to an overall pink colouration of the paper.

(iv) Ehrlich Reagent

2% (w/v) p-dimethylaminobenzaldehyde in a 20% solution (v/v) of HCl in acetone was prepared just before required. The paper was dipped in the above solution and allowed to dry at room temperature. A purple colour showed a positive reaction indicating the presence of tryptophan. Greater sensitivity was obtained when this test followed the ninhydrin staining method (Easley 1965). The pink ninhydrin spots on the guide strips became colourless and a positive colour appeared, wherever tryptophan containing peptides were located.

6. Mobilities of Peptides

(A) Electrophoresis

The mobilities of the peptides at pH 6.5 were measured from a true neutral point determined as 4/11 of the distance between the standard dansyl-arginylarginine and the 1 - dimethylaminophthalene -5- sulphonic acid measured ^{relative to} the dansyl-arginylarginine.

At pH 1.9 the mobilities were measured from the 1 - dimethylaminonaphthalene -5- sulphonic acid and expressed relative to the distance of the dansyl-arginine standard.

(B) Chromatography

The mobilities of the peptides were measured from the origin and expressed relative to the distance moved by the

dansyl-arginine standard.

7. Elution of Peptides

Peptides were eluted with 20% (v/v) pyridine into Pyrex screw-cap tubes (1cm x 6cm). In each case 0.5ml was collected by elution and then freeze dried. The dried peptides were stored at 4°C.

8. Semi-quantitative Amino Acid Analysis of Peptides by Thin Layer Chromatography of the Dansyl-derivatives

An aliquot of the peptide (10 n mol) in a Durham tube (6mm x 30mm, A. Gallenkamp Ltd., London) was dried in vacuo over solid NaOH. 50 μ l of 6M HCl was added and the tube sealed and heated at 105°C for 18h. After this period the tube was opened and the acid was removed by drying in vacuo over solid NaOH. The free amino acids in the hydrolysate were labelled by the dansyl method of Gray and Hartley (1963a), but without the final hydrolysis. The dansyl derivatives of the amino acids were identified by chromatography on thin layer sheets of polyamide. (See Methods IV 9).

9. N-terminal Analysis

The dansyl procedure as described by Gray and Hartley (1963b) was used. A sample (1-10 n mol) of the peptide was transferred to a clean Durham tube (30mm x 6mm, A. Gallenkamp Ltd., London) and dried in vacuo over NaOH and concentrated H₂SO₄. The residue was dissolved in 10 μ l of 0.1M sodium bicarbonate and dried again. 5 μ l of water and 5 μ l of a solution containing 5mg of dansylchloride/ml in acetone were pre-mixed and added. The tube was sealed by parafilm and the reaction stopped after 1h. at 45°C by drying in vacuo over NaOH and concentrated H₂SO₄. Then 40-50 μ l of 6M HCl was added, and the tube sealed using an oxygen flame.

The dansyl labelled peptide was hydrolysed in the sealed tube at 105°C for 6-18h., and then after opening the tube the hydrolysate was dried in vacuo over NaOH. The contents of the tube were dissolved in 10 μ l of 20% (v/v) pyridine and spotted on both sides of the polyamide sheet in a 4:1 ratio and dried under a hot air draught. 1 μ l of a chromatography marker solution containing 0.1mg/ml of each of the following; dansyl-proline, dansyl-isoleucine, dansyl-phenylalanine, dansyl-glycine, dansyl-glutamic acid, and dansyl-serine in 95% (v/v) ethanol was applied to the reverse of the sheet. Frames of the type described by Smith (1958) were used to support up to five polyamide sheets for simultaneous separations.

The solvents used to separate the dansyl-derivatives of amino acids by thin layer chromatography were:

Solvent A 1.5% (v/v) formic acid with distilled water
(Woods and Wang 1967)

Solvent B Toluene-acetic acid 9:1 (v/v)

Solvent C Butylacetate-methanol-acetic acid (30:20:1 by volume)
Ramshaw et al. (1970)

The dansyl-derivatives were identified by running the sheets in Solvent A for 45 minutes, drying and then running at right angles in Solvent B. After drying the sheets were examined under a U.V. light (350nm) and the result recorded (Fig. 4). The chromatograms were then developed in Solvent C in the same direction as Solvent B for 45 minutes. The dansyl-derivatives resolved by this system were then examined and recorded (Fig. 5). The co-chromatography of unknown samples with known standards was used to help in the identification of close pairs of derivatives.

FIGURE 4

Chromatography of dansyl-amino acids on polyamide thin layers.

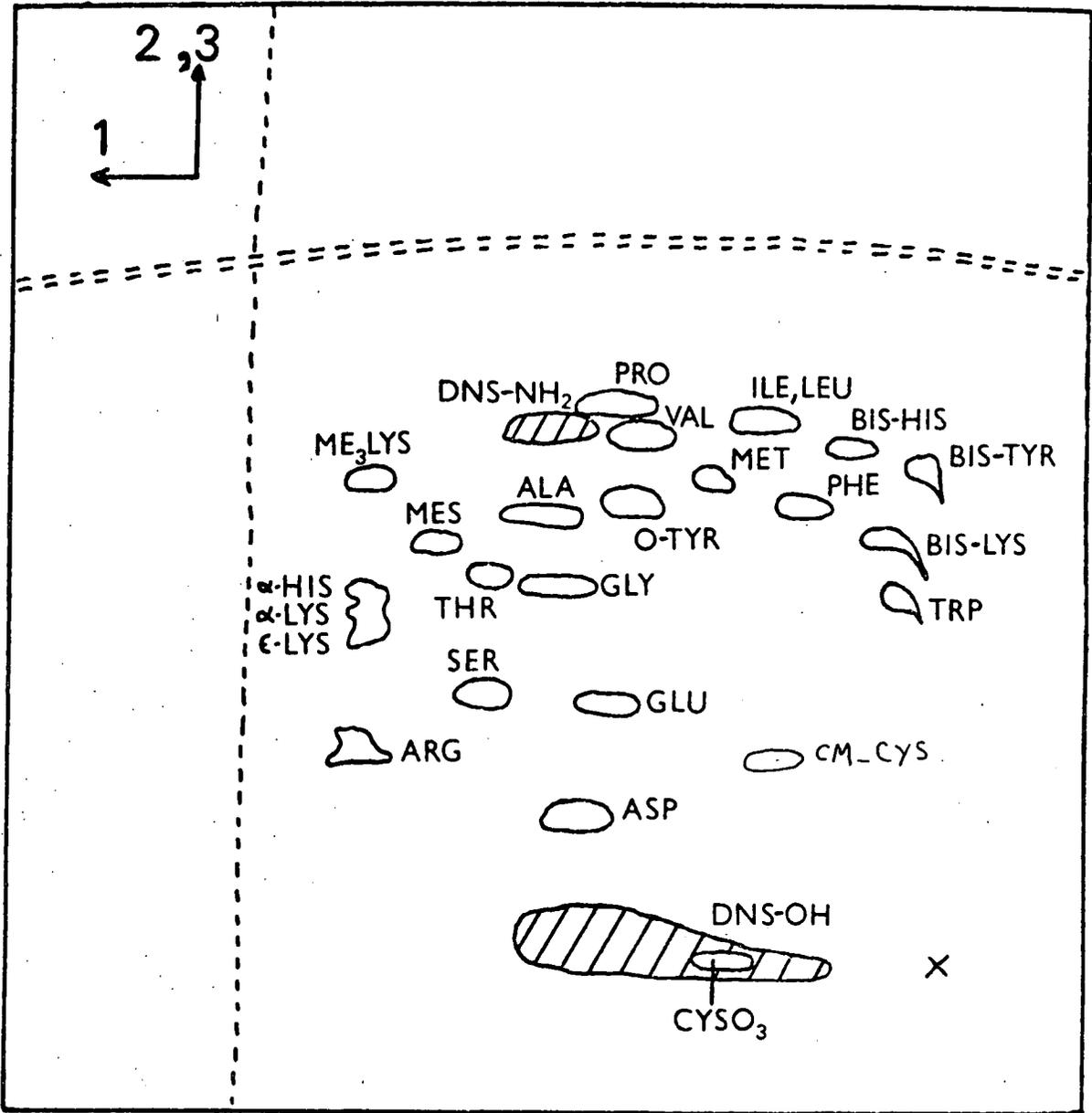
Development was by Solvent A 1.5% (v/v) formic acid in the first dimension and Solvent B Toluene-acetic acid 9:1 (v/v) in the second dimension

FIGURE 5

Chromatography of dansyl-amino acids on polyamide thin layers.

Development was by Solvent A 1.5% (v/v) formic acid in the first dimension and Solvent B Toluene-acetic acid 9:1 (v/v) followed by Solvent C Butylacetate-methanol-acetic acid 30:20:1 (v/v) in the second dimension.

FIG. 5



The polyamide sheets were re-used after washing for 1h. in a washing solution consisting of acetone-water-ammonia, (50:46:4 by volume).

10. Edman Degradation Procedure using Phenylisothiocyanate (PITC)

The Edman degradation procedure used was based on the methods of Edman (1956) and Blömbäck et al. (1966). The peptide (0.1-0.5 μ mol) was dissolved in 150 μ l of 20% (v/v) aqueous pyridine. Then 150 μ l of a 5% (v/v) solution of re-distilled phenylisothiocyanate (PITC) in pyridine were added. The tube was immediately flushed with oxygen-free nitrogen for 10 secs. (Ilse and Edman 1963) and capped quickly. The samples were allowed to react at 45°C for 1h. The excess reagents and volatile reaction by-products were then removed by drying in vacuo over solid NaOH and P₂O₅ at 60°C. When the samples were completely dry, 200 μ l of anhydrous trifluoroacetic acid (TFA) was added (Elmore and Toseland 1956). The tubes were sealed with parafilm and incubated at 45°C for 30 minutes. Excess reagent was removed by drying in vacuo over solid NaOH at 60°C. The degraded peptides were dissolved in 200 μ l of distilled water and extracted twice with 1.5ml of butylacetate (Gray 1967) and then dried in vacuo over concentrated H₂SO₄ and solid NaOH.

Following each of these cycles of the Edman degradation procedure, approximately 5-10% of the peptide material was removed and used to identify the newly released N-terminal amino acid by the application of the dansyl labelling method of Gray and Hartley (1963b), described above. When using this method of peptide sequencing amide residues were determined from calculations based on the peptide's mobilities after electrophoresis at pH 6.5.

(Fig. 6). (Offord 1966.)

11. Microsequence Analysis using 4-NN-Dimethylaminoazobenzene-4'-isothiocyanate (DABITC)/Phenylisothiocyanate (PITC) Double Coupling Method of Chang et al. (1978)

Samples of the peptides or proteins (5-9 n mol) were placed in a clean tube (0.5cm x 3cm) fitted with a ground glass stopper. They were dissolved in 80 μ l of 50% (v/v) aqueous pyridine and then 40 μ l of a freshly prepared DABITC solution containing 10 n mol DABITC/ μ l pyridine (2.82mg/ml) was added. DABITC was found to be unstable in pyridine, so solutions in this solvent were prepared just prior to a degradation cycle from stock solutions of DABITC in acetone (1.4mg/ml). The tube was flushed with nitrogen for 10 secs., the contents mixed and then incubated at 52°C for 50 minutes. A second coupling was carried out by adding 10 μ l of PITC and heating at 52°C for 30 minutes.

The excess of reagent and by-products were removed by extracting three times with 0.5ml of a solvent mixture containing heptane-ethylacetate; 2:1 by volume. In each extraction the mixture was agitated vigorously for 10 secs. on a vortex mixer and then centrifuged for 3 minutes. The organic layer was removed with a fine pipette attached to a vacuum line and discarded. The extracted mixture was dried down in vacuo over NaOH.

50 μ l of anhydrous trifluoroacetic acid was added to the well dried mixture and the tube was flushed with nitrogen for 10 secs. and allowed to react for 15 minutes at 52°C. The sample was evaporated to dryness in a vacuum desiccator and then dissolved in 50 μ l of distilled water. Extraction of the cleaved 4-NN-dimethylamino²⁰benzene 4-thiazolinone (DABTZ) - amino acids (and PTZ-amino acids) was achieved by adding 200 μ l of butylacetate

FIGURE 6

The mobility of peptides on pH 6.5 electrophoresis. The electrophoretic mobility of peptides relative to dansyl-arginylarginine at pH 6.5 is plotted against their molecular weight for charges (E) of ± 1 to ± 3 .

Peptides containing cysteic acid do not conform directly to this diagram (after Offord 1966).

FIG.6

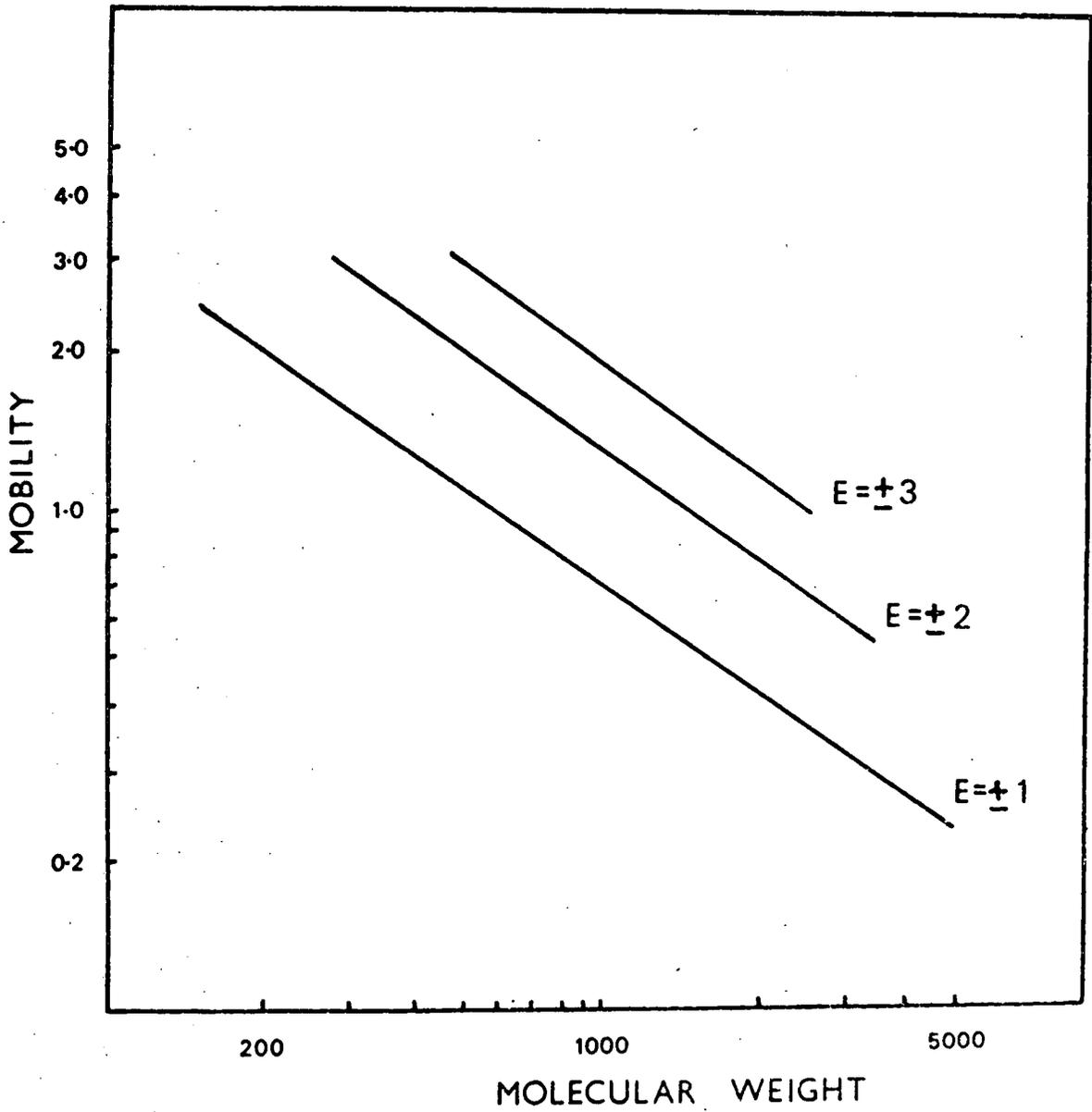
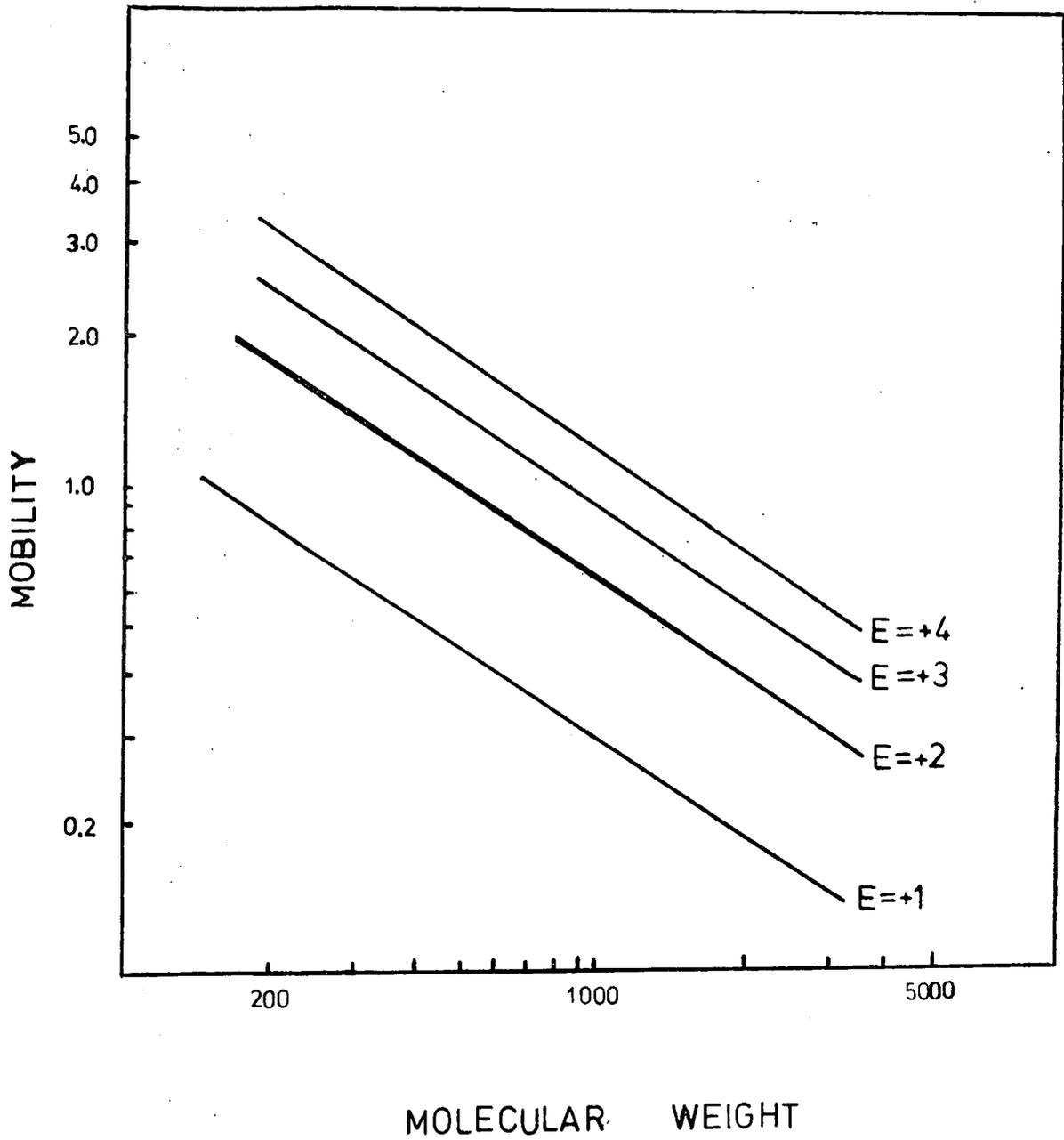


FIGURE 7

The mobility of peptides on pH 1.9 electrophoresis.

The Electrophoretic mobility of peptides at pH 1.9 is plotted against their molecular weight for charges (E) of +1 to +4. Peptides containing cysteic acid do not conform to this diagram (after Bailey and Ramshaw 1973).

Fig. 7



to the peptides in the water phase, mixing on a vortex mixer, followed by centrifugation, and careful removal of the butylacetate phase containing the amino acid thiazolinone.

After the removal of butylacetate, the peptides in the water phase were dried down in vacuo over NaOH ready for the next degradation cycle, while the butylacetate extract was dried down in a Pyrex screw-cap tube (0.8cm x 6cm). The conversion of the thiazolinones of the amino acids into thiohydantoin was performed by adding 20 μ l of distilled water and 40 μ l of acetic acid saturated with HCl to the well dried residue and incubating at 52°C for 50 minutes. The sample was dried down in vacuo over NaOH and re-dissolved again in a suitable volume of ethanol (10-40 μ l). 1/40-1/5 of the ethanolic solution was used for thin layer chromatography to identify the 4-NN-dimethylaminoazobenzene 4 - thiohydantoin (DABTH) - amino acid on small (2.5cm x 2.5cm) polyamide sheets using a solvent A (acetic acid-water 2:1 by volume) in the first dimension separation and a solvent B (toluene:n-hexane:acetic acid 2:1:1 by volume) in the second dimension. The sheet was dried for 2 minutes in a hot air draught after each solvent run and then ^{finally} exposed to HCl vapour. This exposure resulted in the appearance of red, blue and purple spots of the DABTH amino acid derivatives, the two dimensional separation of which is represented in Figure 8.

12. Determination of C-terminal Amino Acids using Carboxypeptidase A Digestion

10 μ l of carboxypeptidase A (treated with diisopropyl-phosphorofluoridate) were washed three times with 2ml of distilled water in a screw-cap tube. The enzyme was then suspended in 150 μ l of 0.2M NaHCO₃ at 0-2°C and dissolved using 100-150 μ l 0.1M

FIGURE 8

Chromatography of DABTH-amino acids on polyamide sheets. Development was by solvent A (acetic acid-water; 2:1 by volume) in the first dimension and solvent B (toluene:n-hexane:acetic acid; 2:1:1 by volume) in the second dimension.

The colours (after exposure to HCl vapour) of the derivatives are represented by solid areas (red), dotted areas (blue) and hatched areas (purple). (e) is the blue synthetic marker DABTC-diethylamine. (U) is a blue coloured thiourea formed by the coupling of PITC with hydrolyzed DABITC.

(S) is DABTH-Ser (S), a dehydro-product (S^A), a polymerized product, (S^B) a polymerized-product (S \bar{O}) hydrated dehydro-product with an OH group in the position

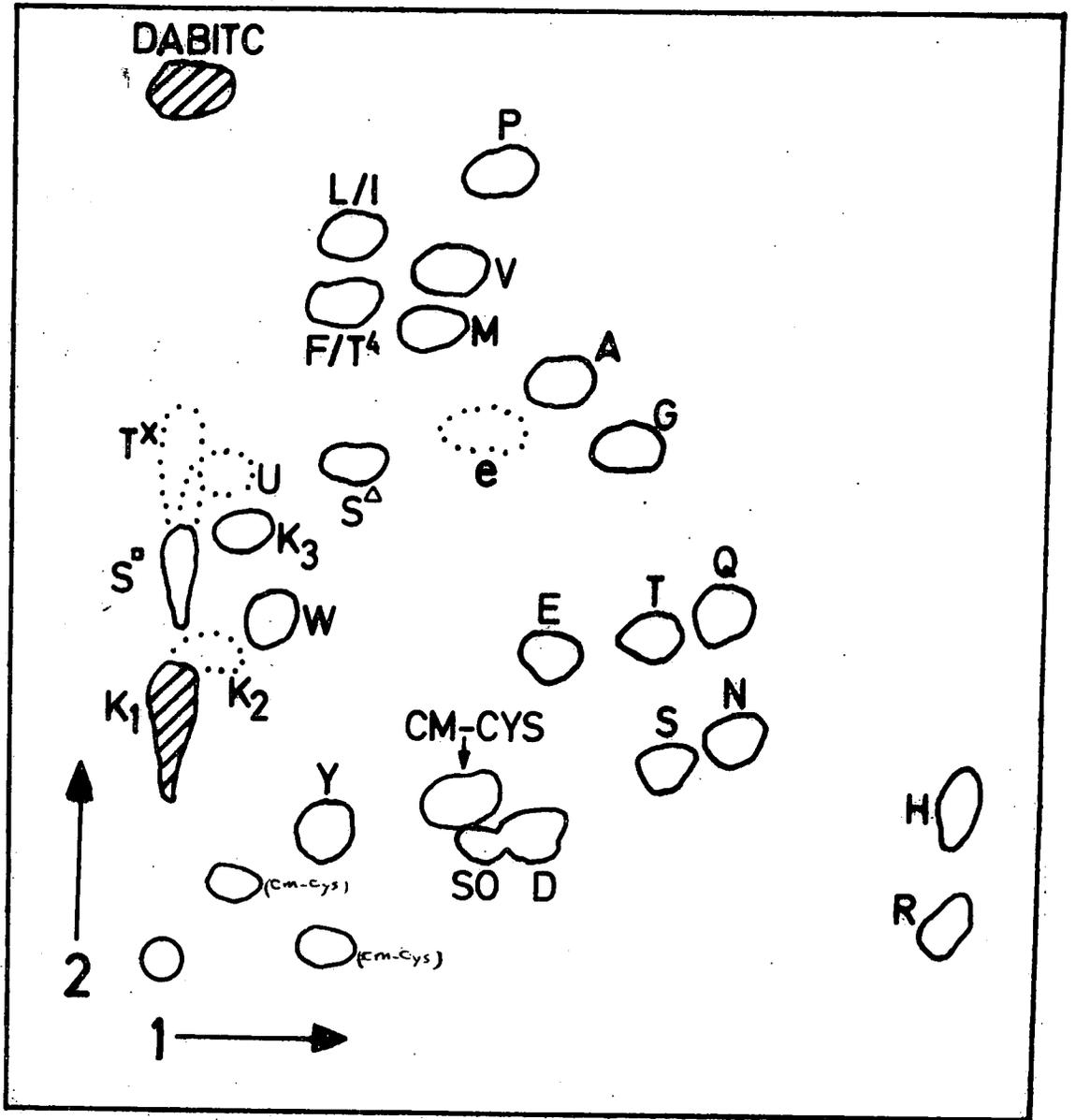
(T) indicates DABTH-Thr, (T^A) dehydro threonine

(T^X) is a blue spot of unknown nature

(K₁) is α -DABTH- ϵ -DABTC-lysine, (K₂) α -PTH- ϵ -DABTC-lysine,

, (K₃) α -DABTH- ϵ -PTC-lysine

Fig. 8



NaOH. The solution was neutralized with 100-150 μ l 0.1M HCl and made up to 1.5ml with 0.2M N-ethylmorpholine acetic acid buffer, pH 8.5.

Samples of the protein to be digested were dried in a Durham tube in vacuo over NaOH. 20 μ l of the carboxypeptidase A solution were added and the tube sealed with parafilm. The incubations took place for varying times, 15 secs to 1h., at 35°C, and the reaction was terminated by drying down in vacuo over NaOH. The liberated amino acids were determined as their dansyl derivatives after labelling, but without acid hydrolysis.

13. Nomenclature used to Describe Sequence Analysis Data

CNBr fragments and peptides derived from digestion of the whole protein are numbered on the basis of their order within the complete sequence, starting from the N-terminus of the protein and given a letter to indicate the enzyme used. Peptides resulting from sub-digestion of a larger fragment or peptide are numbered on the basis of their order within the parent fragment or peptide and given a letter subscript to the major fragment or peptide.

RESULTS AND DISCUSSION

I. EXTRACTION AND PURIFICATION OF α -AMYLASE INHIBITOR CIII

The overall scheme used for the isolation and purification of α -amylase inhibitor CIII is shown in figure 3.

The methods employed were similar to those commonly used in protein purification e.g. salt or organic solvent precipitation, gel-filtration and ion-exchange chromatography, and were a combination of the techniques previously used during other α -amylase inhibitor preparations (Saunders and Lang 1973 and O'Donnell and McGeeney 1976).

1Kg of wheat (Triticum aestivum) flour was suspended in 2L. of distilled water, and homogenized in a Waring blender for 2 minutes. Water has been found to be a satisfactory solvent in extracting these inhibitors and has been used previously as the extractant by Deponte et al. (1976), O'Donnell and McGeeney (1976) and Saunders and Lang (1973). Some other extractions have been carried out using low concentrations of NaOH and NaCl in aqueous solutions (Cantagalli et al. 1971; Saunders 1975; Redman 1975). In an attempt to maintain a low temperature during the homogenizing step, the wheat flour was suspended in cold (10°C) water before spinning in the homogenizer. The macerate was centrifuged at 2500g for 20 minutes at 10°C. The heavy pellets which formed were discarded and the supernatant was centrifuged again at 9000g for 20 minutes at 10°C. These centrifugation steps were found to be a more efficient and quicker method of removing the insoluble heavy matter than squeezing the homogenate through a muslin cloth.

Due to the large volume (1.6L) of the supernatant, concentration was achieved by rotary evaporation in vacuo at 55°C.

The volume was reduced by this means to about 300ml. The concentrated solution was then heated at 70°C for 10 minutes in a water bath. The proteins which were precipitated by this heat treatment were removed after cooling by centrifugation at 6000g for 20 minutes at 10°C.

The fact that α -amylase inhibitors have been found to be remarkably stable to extremes of heat (Marshall and Lauda 1975b; Buonocore et al. 1977 and Silano and Zahnley 1978) and denaturing conditions was utilized in the purification of CIII inhibitor. Heating the protein extract solution at 70°C for 10 minutes served to precipitate a majority of unwanted proteins, leaving the heat-stable proteins, including the inhibitors, in the solution. The heat treatment was also probably advantageous in destroying endogenous enzymes which could interfere with the subsequent purification or assay of the inhibitor.

A calculated volume of absolute ethanol was added slowly with stirring to the clear supernatant to bring the final concentration to 70% (v/v) alcohol. Care was taken to avoid ^alocalized high concentration of alcohol. The precipitate which formed was collected by centrifugation and discarded. The concentration of the supernatant was brought up to 90% (v/v) alcohol by adding slowly a calculated volume of absolute alcohol, and the solution was kept at 4°C for 8h. with occasional stirring. The resulting precipitate of protein which contained the amylase inhibitors was removed by centrifugation at 9000g for 20 minutes at 10°C. It has been reported that it is advisable to wash the precipitate several times with absolute alcohol and allow it to dry overnight at 30°C (O'Donnell and McGeeney 1976). Some other workers have preferred to use fractionation with ammonium sulphate instead of

ethanol, although there are reports of ammonium sulphate causing deamidation of some proteins (Flatmark 1966), but subsequent work done by Deponte et al. (1976); Redman (1975); Granum and Whitaker (1977) and Granum (1978) who used ammonium sulphate to fractionate α -amylase inhibitors from wheat, did not show any evidence of the deamidation of these inhibitors during their research. In this present work ethyl alcohol fractionation yielded satisfactory results.

The pellet was dissolved in a minimal volume of 50mM pyridine-acetate buffer pH 6.5 and the solution was dialysed extensively against the same buffer and then lyophilized.

Gel filtration and ion-exchange chromatography have played a major role in the purification of α -amylase inhibitors. In all of the purification schemes reported by various workers in different laboratories columns of Sephadex G-50 (Feillet and Nimmo 1970; Redman 1975 and O'Donnell and McGeeney 1976) or G-100 (Cantagalli et al. 1971; Marshall and Lauda 1975b and Deponte et al. 1976) have been used after the salt precipitation. In this work a column of Sephadex G-75 was found to produce a satisfactory resolution of the albumin extract.

A column (140cm x 6.8cm) of Sephadex G-75 was equilibrated with 50mM pyridine-acetate buffer pH 6.5. The lyophilized proteins were dissolved in 80ml of the same buffer and applied to the top of the column which was eluted with 50mM pyridine-acetate buffer pH 6.5. The column eluate was monitored at 280nm for the detection of protein and an assay was carried out on every second fraction to detect any inhibitory activity towards α -amylase. The fractions which showed inhibitory activity (indicated by bars) were pooled and lyophilized (Fig. 9). The first three albumin peaks eluted from

FIGURE 9

Chromatography of partially purified α -amylase inhibitor on a column (140 x 6.8cm) of Sephadex G-75 equilibrated in 50mM pyridine-acetate buffer pH 6.5. 1.1g protein in 80ml of the same buffer was applied. Fractions (14ml) were collected at a flow rate of 160ml/h. and assayed for inhibitory activity. -----absorbance at 280nm; ----- inhibitor activity. Fractions indicated by bars were pooled and lyophilized.

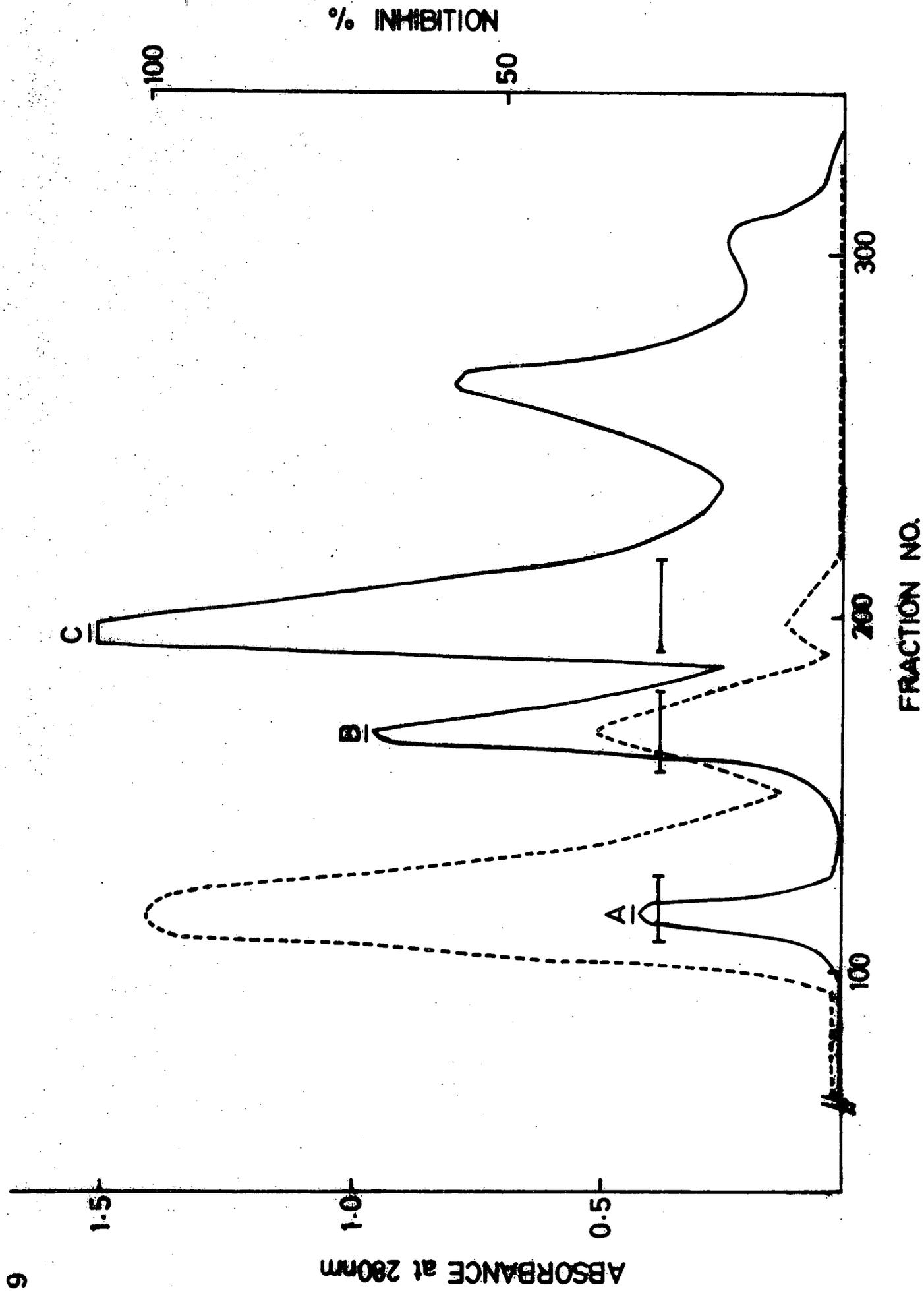


Fig. 9

the column showed an inhibitory activity against hog pancreatic α -amylase and were coded A, B and C. The proteins in peak C (the smallest molecular weight) appeared to be less active against hog pancreatic α -amylase than the proteins in peaks A and B. No other tests against any other source of α -amylase were carried out. It was assumed at this stage that peak C contained an α -amylase inhibitor as well as other contaminant proteins with more or less similar molecular weights (around 12,000 daltons).

Other workers (Cantagalli et al. 1971; Petrucci et al. 1974; Redman 1975 and Deponte et al. 1976) have reported similar results when submitting typical albumin preparations from hexaploid wheat flour to gel filtration which resolved them into three heterogeneous fractions with apparent molecular weights of 60,000, 24,000 and 12,000. The last of the three peaks has been further purified by Redman (1975); Cantagalli (1971) and Silano et al. (1973) by using either ion-exchange chromatography or preparative gel electrophoresis.

The α -amylase inhibitors purified by some other workers (Shainkin and Birk 1970; Saunders and Lang 1973; Saunders 1975 and Granum and Whitaker 1977) were not subjected to any gel filtration chromatography, instead the extracted albumins were directly loaded onto ion-exchange chromatography, while other workers (Marshall and Lauda 1975b, O'Donnell and McGeeney 1976 and Pick and Wöber 1978) applied this technique at a later stage of purification.

Lyophilized peak C was dissolved in 30ml of 50mM Tris-HCl buffer pH 8.4 and loaded onto a column (23cm x 2.5cm) of DE-52 cellulose equilibrated with the same buffer. The column was washed with about 4x the column volume of the buffer prior to

starting the salt gradient (0-0.1M NaCl). Five peaks of protein (I-V) were resolved by this method. Peaks I, II and III were eluted as the column was washed with the starting buffer while peaks IV and V were eluted by the salt gradient. On testing the inhibitory activity against hog pancreatic α -amylase peaks III, IV and V showed an inhibitory activity (Fig. 10). The fractions corresponding to central cuts of each active peak of figure 10 were pooled, dialyzed extensively against distilled water and freeze dried.

Ion-exchange chromatography is a powerful technique in the later stages of the purification of proteins, consequently most of the previous work on α -amylase inhibitors has employed either an ion-exchanger (Shainkin and Birk 1970; Feillet and Nimmo 1970; Marshall and Lauda 1975; Redman 1975; O'Donnell and McGeeney 1976 and Granum and Whitaker 1977) or a DEAE Sephadex ion-exchanger (Saunders and Lang 1973).

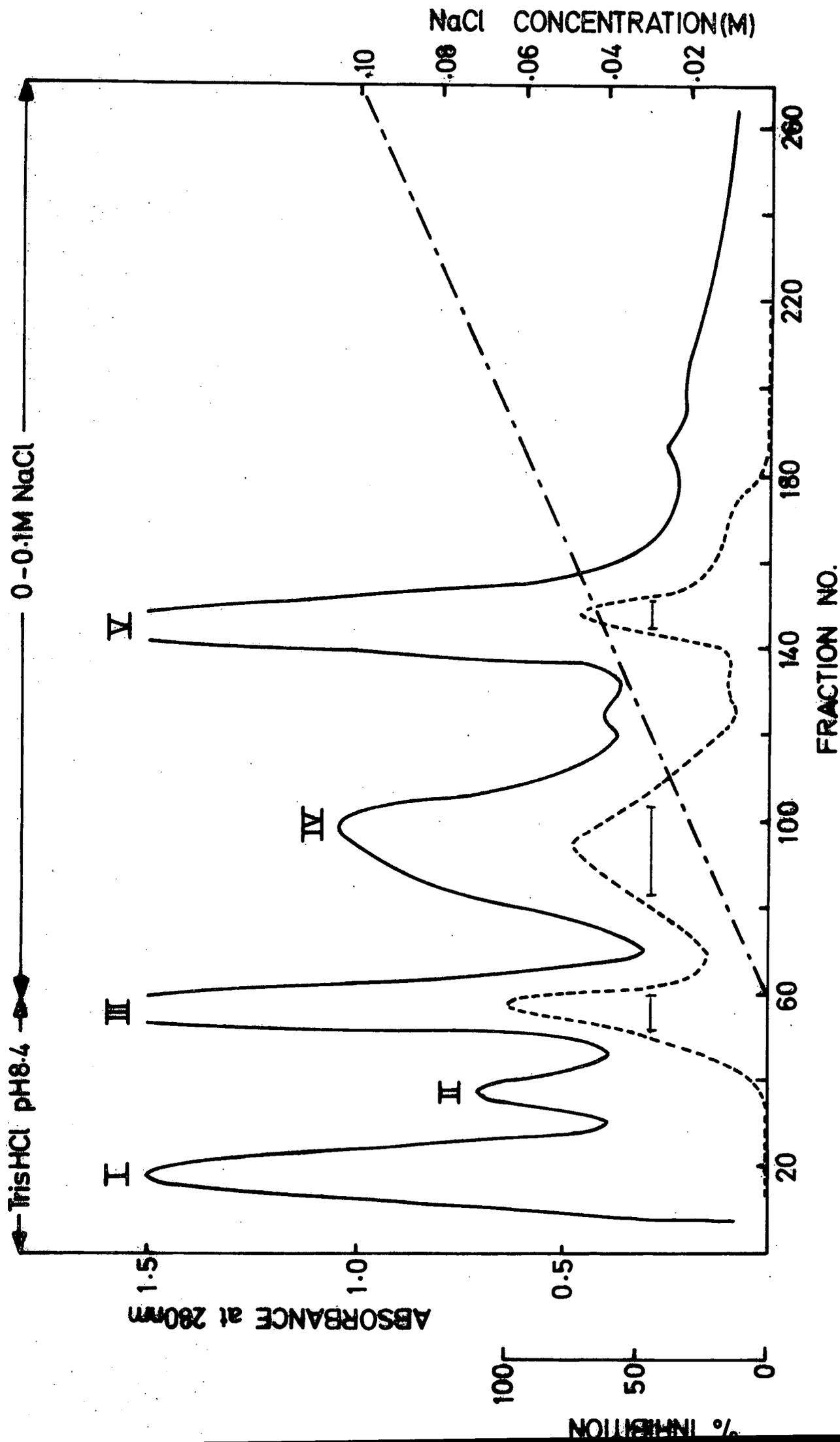
II. CRITERIA OF HOMOGENEITY

It was hoped that at this stage one of the three active peaks against hog pancreatic α -amylase might be pure enough to permit investigation of its structural composition. N-terminal analyses on samples of CIII, CIV and CV revealed serine in peak CIII as the only major N-terminal amino acid and only a trace of glycine. However peaks CIV and CV both showed serine and glycine as major N-terminal amino acids with traces of threonine and aspartic acid, and both clearly required further steps of purification. The N-terminal analysis reported by Redman (1975) and Petrucci et al. (1978) revealed serine which is identical to the

FIGURE 10

Chromatography of partially purified α -amylase inhibitor Peak C on a column (23 x 2.5cm) of DE 52 cellulose equilibrated with 50mM Tris-HCl buffer pH 8.4. A 30ml sample of Peak C in the same buffer was applied and the column was washed with 4x the column volume of this starting buffer before a gradient of 0 to 0.1M NaCl (1L of each) was applied. Fractions (6ml) were collected at a flow rate of 35ml/h., and assayed for inhibitory activity. ————— absorbance at 280nm; ----- inhibitory activity. Fractions corresponding to central cuts of each active peak were pooled, dialyzed against water and lyophilized.

Fig.10



CIII N-terminus.

Samples of the partially purified inhibitor (peak C) obtained by gel-filtration and the purified iso-inhibitors CIII, CIV and CV obtained after ion-exchange chromatography of peak C were examined by polyacrylamide rod gel electrophoresis at pH 8.3 as described by Ornstein (1964) and Davis (1964) (see Materials and Methods II 2). The results obtained are shown in figure 11. Peak CIII showed a single band with a mobility relative to bromophenol blue of 0.30.

As can be seen from figure 12 CIII always yielded a spherical shaped band on electrophoresis, even when run in low concentration and when other protein samples (pea Fraction I, small sub-unit) subjected to gel electrophoresis under the same experimental conditions as controls did not give such anomalous shaped bands (Fig. 12), however, the spherical nature of the band of CIII tended to disappear when the gels were kept for a week in the destaining solution (Fig. 13). A similar band shape has been reported for the α -amylase inhibitors purified from wheat flour by Granum and Whitaker (1977), suggesting that this unusual behaviour of CIII in electrophoresis at pH 8.3 could possibly be due to one of its characteristic bio-chemical properties.

Thus, the inhibitor CIII collected after the ion-exchange chromatography was considered to have purified to sufficient homogeneity as judged by electrophoresis at pH 8.3, using polyacrylamide gels and by N-terminal analysis using the dansyl method on samples of both the S-carboxymethylated and native protein. The near integral values obtained for the amino acid composition of CIII were very similar to those found by Redman (1976) and Silano *et al.* (1973) supporting the homogeneity of the

FIGURE 11

Polyacrylamide gel electrophoresis at pH 8.3 of partially purified α -amylase inhibitor (peak C) after chromatography on Sephadex G-75, and the iso-inhibitors (peak CIII, CIV and CV) resulting from chromatography of peak C on DE-52 cellulose. 40-70 μ g of protein was applied and the electrophoresis was performed for 70-100 min. at a current of 4mA/gel.

Fig.11

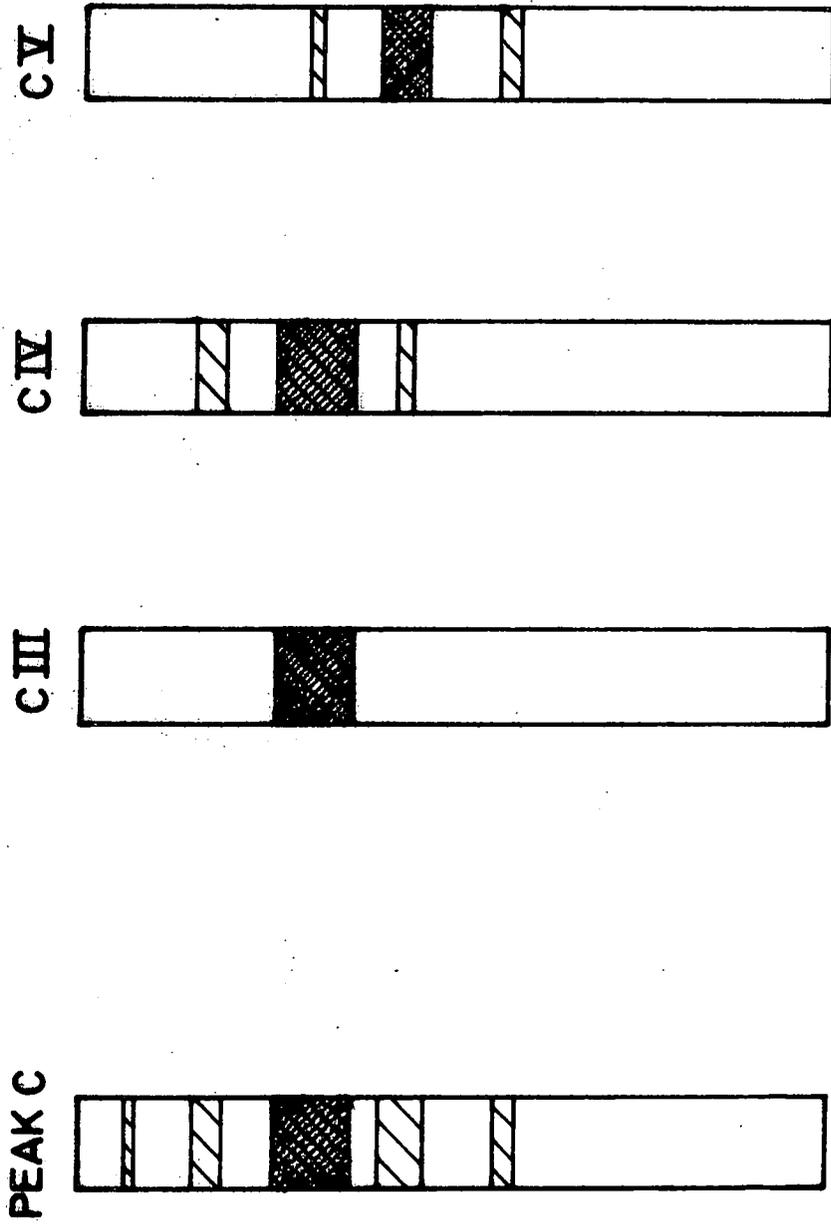


FIGURE 12

7% polyacrylamide gel electrophoresis at pH 8.3 of CIII α -amylase inhibitor from wheat (A) and the partially purified small subunit of Fraction I protein from peas (B). (Supplied as a gift from Mr. J. Gilroy).

10 μ l-20 μ l aliquots of the sample solutions which contained 2mg protein/ml dissolved in a 50% (w/v) sucrose solution were loaded on the top of (0.6 x 7cm) gels. The electrophoresis was performed for 60-90 min. with a current of 4mA/gel. Gels were stained in 0.25% Coomassie brilliant blue in methanol-acetic acid-water, 25:7:68 by volume and destained with the same solvent mixture.

FIGURE 13

Gels which have been described in Figure 12 and which have been kept immersed for seven days in several changes of the methanol-acetic acid-water (25:7:68 by volume) solvent mixture at room temperature.

Fig. 12

A

B



Fig. 13

A

B



purified inhibitor, and its identification as one of the 0.28 isoinhibitor family.

III. THE AMINO ACID SEQUENCE OF WHEAT (*Triticum aestivum*) α -AMYLASE INHIBITOR CIII

1. Amino Acid Composition

The amino acid composition of α -amylase inhibitor CIII is given in Table 3, together with the amino acid composition of the larger fragments resulting from cleavage of the protein with cyanogen bromide (X3, X4 and X5). Also shown for comparison are the values obtained by Redman (1975) for the wheat albumin (M) and its CNBr fragments. The result obtained for the amino acid composition of the whole protein was very similar to the amino acid composition reported by Redman (1975) except for the values of CM-cysteine, ^{lysine}aspartic acid, glycine and valine which were higher and the value of tyrosine which was lower. CNBr X3 fragment of inhibitor CIII was also similar in its amino acid composition to the CNBr-1 fragment of Redman (1975), while the X4 values showed a slight difference from the CNBr-3 of Redman (1975). Fragment X5 had similar amino acid composition to the CNBr-2 of Redman (1975) except for the values of aspartic acid, glycine and valine which were higher.

The results were in good agreement with those calculated from the complete sequence given in figure 16, although the values obtained for lysine and valine were both high, while the values for threonine and proline were both low. Consideration of the values for the CNBr fragments shows that in the CNBr X3 fragment the CM-cysteine value was low. In CNBr X4 fragment the aspartic value obtained indicated the presence of at least one aspartic

inhibitor
 Table (3) Amino acid composition of α -amylase CIII from Wheat (*Triticum aestivum*) seeds.

Whole Protein		C N B R F r a g m e n t s						
CIII	CIII (M)	X1 & X2 (Seq.)	X3 (Seq.)	X3 CNBr-1 (Seq.)	X4 (Seq.)	X4 CNBr-3 (Seq.)	X5 X5 & X6 CNBr-2 (Seq.)	
CM-CYS	9.74	10*	4.22	5	1.14	1	1.55	
ASP	10.24	10	4.97	5	1.19	0	3.13	
THR	2.45 ^a	3	0.07 ^a	0	0.01 ^a	0	1.29 ^a	
SER	8.75 ^a	8½*	2.84 ^a	3	0.29 ^a	½*	1.95 ^a	
HSE	-	-	1.07	1	1.00	1	-	
GLU	12.45	12	6.20	6	3.87	5	1.46	
PRO	9.09	9½*	1.26	1	1.06	1	4.87	
GLY	11.51	11½*	2.18	2	3.16	3	2.53	
ALA	10.72	10½*	2.30	2	1.56	1*	3.96	
VAL	14.83	14	4.00	4	4.37	4	4.31	
MET	3.03	3	-	0	-	0	-	
ILE	1.94 ^b	2	0.96 ^b	1	0.13 ^b	0	0.86 ^b	
LEU	8.97 ^b	9	3.59 ^b	4	2.48 ^b	3	1.19 ^b	
TYR	3.40	3½*	0.03	0	0.94	½*	1.26	
PHE	tr	0	tr	0	tr	0	tr	
LYS	6.62	5½*	0.96	1	2.30	2	2.13	
HIS	tr	0	tr	0	tr	0	tr	
ARG	7.23	7	1.30	2	2.58	3	1.17	
TRP	n.d.	4	n.d.	1	n.d.	0	n.d.	
TOTAL		123		38		25	36	

Figures in () are those reported for protein (M) by Redman (1975)

* indicates amino acids exhibiting micro-heterogeneity in sequence (Seq.) = number of amino acids found in sequence
 n.d. = not determined

a = calculated from 24 h. and 72 h. value assuming first order kinetics for destruction (Moore and Stein, 1963)
 b = calculated from 72 h. hydrolysis

acid residue in the fragment, but no such residue was detected during the sequencing of this fragment or any of its constituent peptides. It can also be seen that the values for glutamic acid and leucine are rather low.

Close examination of the amino acid composition reported for AmI₁ (Shainkin and Birk 1970) the wheat albumins (Redman 1975) and other members of the 0.28 family (Silano et al. 1973 and Granum and Whitaker 1977) provides more evidence that CIII α -amylase inhibitor is a member of the 0.28 family.

2. Digestion with Proteolytic Enzymes

The proteolytic enzymes used in this investigation were trypsin, α -chymotrypsin, thermolysin, Staphylococcus aureus V8 protease and carboxypeptidase A. The various sets of peptides resulting from each digest gave sufficient overlapping to logically establish most of the sequence. The peptides resulting from digestion with trypsin, thermolysin, Staphylococcus aureus V8 protease and α -chymotrypsin were subjected directly to molecular sieve chromatography on a (0.8cm x 190cm) column of Bio-gel P2. The first peak of 280nm absorbing material to elute from the P2 column (except for chymotrypsin) was collected, freeze-dried and applied onto a (1.0cm x 160cm) column of Sephadex G-50. Both columns were equilibrated with 50mM pyridine-acetate buffer pH5.6. High voltage paper electrophoresis at pH 6.5 and pH 1.9 as well as paper chromatography using the BAWP solvent mixture (see Materials and Methods) were subsequently employed to resolve those peptides which required further purification.

a. Trypsin Digest

A list of the peptides resulting from the tryptic digestion together with the details of the methods used for their purification

and the results obtained from the sequencing methods are given in Table 4.

<u>Peptide T1</u>	(1-14)	SER-GLY-PRO-TRP-SER-TRP-CYS-ASN-PRO- ALA-THR-GLY-TYR-LYS
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The electrophoretic mobility of this peptide at pH 6.5 showed that residue 8 (asparagine) was present in some cases as a deamidated form (aspartic acid). This peptide reacted positively with the Ehrlich reagent indicating the presence of tryptophan and this was confirmed by the pink colour which was noticed on adding trifluoroacetic acid (TFA) during the Edman degradation procedure. It was not possible to identify the amino acids present in position 4 and 6 by the dansylation procedure and it was thought that both of these positions might be ascribed to tryptophan. This was subsequently confirmed by other results (such as the results obtained during the sequencing of the CNBr X1 fragment using the DABITC method). After 13 steps of Edman degradation, the lysine in position 14 was identified by dansylation without hydrolysis as EPTC-LYS.

<u>Peptide T2</u>	(15-22)	VAL-SER-ALA-LEU-THR-GLY-CYS-ARG
<u>Peptide T3</u>	(23-26)	ALA-MET-VAL-LYS
<u>Peptide T4</u>	(27-40)	(LEU, GLX, CYS, VAL, GLY, SER, GLY, VAL, PRO GLX, ALA, VAL, LEU, ARG)

This large peptide was redigested with thermolysin for 4h. at 37°C and the resulting peptides (T4H1, T4H2 and T4H3) were separated by high voltage paper electrophoresis at pH 1.9 and sequenced by the dansyl-Edman method

<u>Peptide T4H1</u>	(27-29)	LEU-GLX-CYS
<u>Peptide T4H2</u>	(30-37)	VAL-GLY-SER-GLX-VAL-PRO-GLX-ALA
<u>Peptide T4H3</u>	(38-40)	VAL-LEU-ARG
<u>Peptide T5</u>	(41-55)	ASX-CYS-CYS-GLX-GLX-LEU-ALA-ASX

TABLE 4

Peptides resulting from trypsin digest of wheat (<u>Triticum aestivum</u>) α -amylase inhibitor CIII		Purification of Peptide		Dansyl-Edman Results	
Peptide No. and Position in Sequence	Peptides No. in Gel-Filtration ^a	Mobility of Peptides on High Voltage Paper Electrophoresis PH6.5 0, -0.60	PH1.9 -	BAWP ^b	
T1 (1-14)	P2 G-50 1 5	0.48	0.65	-	<u>SER-GLY-PRO-TRP-SER-TRP-CYS-ASN-ASP</u> <u>PRO-ALA-THR-GLY-TYR-LYS</u>
T2 (15-22)	3 -	0.48	0.65	-	<u>VAL-SER-ALA-LEU-THR-GLY-CYS-ARG</u>
T3 (23-26)	2 -	-	1.23	-	<u>ALA-MET-VAL-LYS</u>
T4 (27-40)	1 2	-0.57	-	-	LEU- digested with thermolysin for 4h. at 37°C
T4H1 (27-29)	-	-	0.43	-	<u>LEU-GLX-CYS</u>
T4H2 (30-37)	-	-	0.25	-	<u>VAL-GLY-SER-GLX-VAL-PRO-GLX-ALA</u>
T4H3 (38-40)	-	-	1.41	-	<u>VAL-LEU-ARG</u>
T5 (41-55)	1 2	-2.6	-	-	<u>ASX-CYS-CYS-GLX-LEU-ALA</u> <u>ASX (ILE, ASX, ASX, GLX, TRP, CYS, ARG)</u>
T6 (56-64)	1 3	-0.93	0.54	-	<u>CYS-GLY-ASP-LEU-SER-SER-MET-LEU-ARG</u>
T7 (65-73)	3 -	0	0.54	-	<u>SER-VAL-TYR-GLX-GLX-LEU-GLY-VAL-ARG</u>
T7a (64-67)	3 -	0	0.86	-	<u>ARG-SER-VAL-TYR</u>
T7b (68-73)	3 -	0	0.73	-	<u>GLX-GLX-LEU-GLY-VAL-ARG</u>

TABLE 4 Cont.

T8	(74-76)	3	-	0	1.17	-	<u>GLU-GLY-LYS</u>
T9	(77-84)	1	3	0	0.95	-	<u>GLU-VAL-LEU-PRO-GLY-CYS-ARG-LYS</u>
T10	(85-88)	2	-	0	0.92	-	<u>GLU-VAL-MET-LYS</u>
T11	(89-99)	1	2	-0.57	-	-	<u>LEU-</u>
T11H1	(89-91)	-	-	-	0.53	-	<u>LEU-THR-ALA</u>
T11H2	(92-99)	-	-	-	0.68	-	<u>ALA-SER-VAL-PRO-GLU-(VAL,CYS,LYS,)</u>
T12	(100-109)	2	-	-	0.66	-	<u>VAL-PRO-ILE-PRO-ASX-PRO-SER-GLY-ASX-ARG</u>
T13	(110-117)	1	5	-1.29	-	-	<u>ALA-GLY-VAL-CYS-TYR-(GLY,ASP,TRP,)</u>
T14	(118-123)	3	-	-1.56	-	-	<u>CYS-ALA-TYR-PRO-ASP-VAL.</u>

digested with thermolysin for
4 h. at 37°C

Footnote to Tables 4, 5

(→): Sequence determined by dansyl-Edman analysis.

(→): Sequence determined by DABITC method.

(a): Figures indicate the order of elution from the column.

(b): Paper chromatography using BAWP solvent (n-butanol-acetic acid-water-pyridine; 75:15:60:50 v/v)

(NF): Not found.

(ILE, ASX, ASX, GLX, TRP, CYS, ARG)

After seven cycles of Edman degradation, no further sequencing could be achieved. Therefore residues 47-55 were placed according to the semi-quantitative amino acid analysis done on a small sample of the peptide and from the overlapping with peptides H8, H9, V2, V3, C7, C8 and CNBr X3 fragment. This peptide showed a positive reaction with the Ehrlich reagent indicating the presence of tryptophan. The electrophoretic mobility of this peptide at pH 6.5 indicated the presence of several acidic residues, assuming CM-cysteine to have a -1 charge. It was impossible at this stage to locate them owing to the large number of possible amide/acid alternative forms.

Peptide T6 (56-64) CYS-GLY-ASP- \bar{L} EU-SER-SER-MET-LEU-ARG

The acidic mobility of this peptide during electrophoresis at pH 6.5 indicated that residue 58 was aspartic acid, assuming CM-cysteine to have a charge of -1 at pH 6.5.

Peptides T7, T7a and T7b (64-73) ARG-SER-VAL-TYR-GLX-GLX-LEU-GLY-VAL-ARG

Peptide T7 was sequenced completely by the dansyl-Edman procedure but in addition the trypsin used gave rise to anomalous cleavages of the Leucine (63) - arginine (64) bond and the tyrosine (67) - glutamic acid (68) bond forming peptides T7a and T7b. The specificity of these unexpected hydrolyses suggests that the trypsin used may have been contaminated with low levels of active chymotrypsin. In positions 65 and 67 only serine and tyrosine were observed in these peptides.

Peptide T8 (74-76) GLU-GLY-LYS

The electrophoretic mobility of this peptide at pH 6.5 indicated that residue 74 is glutamic acid.

Peptide T9 (77-84) GLU-VAL-LEU-PRO-GLY-CYS-ARG-LYS

The electrophoretic mobility of this peptide at pH 6.5 indicated that the residue in position 77 is glutamic acid. No cleavage of the arginine (83) - lysine (84) bond was detected.

Peptide T10 (85-88) GLU-VAL-MET-LYS

The electrophoretic mobility of this peptide at pH 6.5 indicated that the residue in position 85 is glutamic acid.

Peptide T11 (89-99) (LEU,THR,ALA,ALA,SER,VAL,PRO,GLU,
VAL,CYS,LYS)

This peptide was redigested with thermolysin for 4h. at 37°C and the resulting peptides (T11H1 and T11H2) were separated by high voltage paper electrophoresis at pH 1.9. The electrophoretic mobility of the T11 peptide at pH 6.5 indicated that the residue which was later found in position 96 was glutamic acid.

Peptide T11H1 (89-91) LEU-THR-ALA

Peptide T11H2 (92-99) ALA-SER-VAL-PRO-GLU- (VAL,CYS,LYS)

In peptide T11H2 Edman degradation was unsuccessful after four cycles and the residues in positions 97-99 were placed by examination of the semi-quantitative amino acid analysis and from the overlapping with peptide V9 and the CNBr X5 fragment.

Peptide T12 (100-109) VAL-PRO-ILE-PRO-ASX-PRO-SER-GLY-
ASX-ARG

The electrophoretic mobility of this peptide at pH 6.5 suggested that one of the residues in position 104 or 108 is aspartic acid and the other is asparagine.

Peptide T13 (110-117) ALA-GLY-VAL-CYS-TYR (GLY,ASP,TRP)

As the Edman degradation of this peptide was unsuccessful after four cycles, the residues in positions 115-117 were placed according to the semi-quantitative amino acid analysis and from the overlapping with peptides V9 and H20. The electrophoretic

mobility of this peptide was consistent with residue 116 which was found later to be aspartic acid.

Peptide T14 (118-123) CYS-ALA-TYR-PRO-ASP-VAL

This peptide was another which resulted from an anomalous chymotryptic-like cleavage by the trypsin at the tryptophan (117) - CM-cysteine (118) bond. In this digest residue 118 was found to be CM-cysteine. The electrophoretic mobility of this peptide at pH 6.5 indicated that residue 122 was aspartic acid.

The trypsin digestion of α -amylase inhibitor CIII yielded all but one of the expected number of peptides. The only expected peptide bond which failed to cleave was the bond between arginine (84) - lysine (85). In this work 16 tryptic peptides were isolated and sequenced which is one less than the number of peptides observed by Petrucci et al. (1978) in their "fingerprint" of the 0.28 protein.

b. Thermolysin Digest

20-30mg of S-carboxymethylated inhibitor CIII were digested with thermolysin in 0.2M N-ethylmorpholine-acetate buffer pH 8.5 for 4h. at 37°C. The resulting peptides were separated as described previously. The details of their purification and sequences are shown in Table 5.

Peptide H1 (1-14) SER-GLY-PRO (TRP, SER, TRP, CYS, ASX, PRO, ALA, THR, GLY, TYR, LYS)

This peptide showed a positive reaction with the Ehrlich reagent indicating the presence of tryptophan. After three successive Edman degradations the peptide failed to degrade any further. Residues 4-14 were placed by examination of the semi-quantitative amino acid analysis and the sequence data obtained from the sequences of the CNBr X2 fragment and the X2Cl, T1 and C2 peptides. Also residues 5-12 were placed from peptide H1b and residues 13 and 14 were placed from peptide H1c. The electrophoretic mobility at pH 6.5 indicated that the residue in position 8 was

TABLE 5

Peptides resulting from thermolysin digest of wheat (<i>Triticum aestivum</i>) α -amylase inhibitor CIII		Dansyl-Edman Results	
Peptide No. and Position in Sequence	Peptides No. in Gel-Filtration ^a	Purification of Peptide	
		Mobility of Peptides on High Voltage Paper Electrophoresis PH6.5 0	BAWP ^b
H1 (1-14)	P2 G-50 3 -	PH1.9 0.35	-
H1a (1-4)	5 -	-	-
H1b (5-12)	3 -	-0.77	0.66
H1c (12-14)	4 -	1.82	-
H2 (15-17)	3 -	0	0.56
H3 (18-23)	3 -	0	-
H3a (18-21)	3 -	-1.11	0.50
H3b (22-23)	4 -	1.89	-
H4 (24-26)	2 -	1.46	-
H4a (25-26)	3 -	1.85	-
H5 (27-29)	3 -	-1.51	-
H6 (30-37)	2 -	-0.70	-
H7 (38-45)	2 -	-1.53	0.25
H8 (46-48)	3 -	-1.31	-

SER-GLY-PRO-(TRP, SER, TRP, CYS, ASX, PRO, ALA, THR, GLY, TYR, LYS)

SER-GLY-PRO-(TRP)

SER-TRP-CYS-ASN-(PRO, ALA, THR, GLY)

TYR-LYS

VAL-SER-ALA

LEU-THR-GLY-CYS-ARG-ALA

LEU-THR-GLY-CYS

ARG-ALA

MET-VAL-LYS

VAL-LYS

LEU-GLN-CYS

VAL-GLY-SER-GLX-VAL-PRO-GLX-ALA

VAL-LEU-ARG-ASX-CYS-CYS-GLX-GLX

LEU-ALA-ASP

Table 5 cont.

H9	(49-58)	2	-	-1.80	0.44	-	ILE-ASN-ASN-GLU-TRP-CYS-ARG CYS-GLY-ASP
H10	(59-61)	3	-	0	0.46	0.54	LEU-SER-SER-MET
H11	(63-65)	4	-	1.52	1.25	-	LEU-ARG-SER
H12	(66-69)	4	-	-1.23	0.37	-	VAL-TYR-GLX-GLX
H13	(70-71)	4	-	0	0.60	0.63	LEU-GLY
H14	(72-77)	2	-	0	1.11	-	VAL-ARG-GLU-GLY-LYS-GLU
H15	(78-85)	2	-	0	0.93	-	VAL-LEU-PRO-GLY-CYS-ARG-LYS- GLU
H16	(86-88)	3	-	1.61	-	-	VAL-MET-LYS
H17	(89-91)	3	-	0	0.45	0.75	LEU-THR-ALA
H18	(92-99)	N.F.	N.F.	N.F.	N.F.	N.F.	(ALA, SER, VAL, PRO, GLX, VAL, CYS, PRO LYS) GLY
H19	(100-111)	1	1	0	0.48	-	VAL-PRO-ILE-PRO-ASN-PRO-SER-GLY- ASP-ARG-ALA-GLY
H19a	(108-111)	1	1	0	0.98	-	ASP-ARG-ALA-GLY
H20	(112-116)	3	-	-1.91	-	-	VAL-CYS-TYR-GLY-ASP
H20a	(112-113)	4	-	-1.96	-	-	VAL-CYS
H21	(117-118)	3	-	-1.83	-	-	TRP-CYS
H22	(119-123)	3	-	-1.11	0.32	-	ALA-TYR-PRO-ASP-VAL

See footnote to Table 4 for explanation of a, b and other symbols

asparagine.

Peptide H2 (15-17) VAL-SER-ALA

Peptide H3 (18-23) LEU-THR-GLY-CYS-ARG-ALA

Anomalous cleavage occurred in this peptide. Partial hydrolysis of CM-cysteine (21) - arginine (22) bond was noticed yielding both

Peptide H3a (18-21) LEU-THR-GLY-CYS

and

Peptide H3b (22-23) ARG-ALA

which have been sequenced.

Peptide H4 (24-26) MET-VAL-LYS

Peptide H4a (25-26) VAL-LYS

Peptide H5 (27-29) LEU-GLN-CYS

The electrophoretic mobility of this peptide indicated that the residue in position 28 is glutamine.

Peptide H6 (30-37) VAL-GLY-SER-GLX-VAL-PRO-GLX-ALA

The electrophoretic mobility of this peptide at pH 6.5 indicated that one of the two residues in positions 33 or 36 is glutamic acid while the other is glutamine.

Peptide H7 (38-45) VAL-LEU-ARG-ASX-CYS-CYS-GLX-GLX

The electrophoretic mobility of this peptide at pH 6.5 suggested that one of the ASX/GLX residues in it was in the acidic form.

Peptide H8 (46-48) LEU-ALA-ASP

This peptide was acidic on paper electrophoresis at pH 6.5 confirming that residue 48 is aspartic acid.

Peptide H9 (49-58) ILE-ASN-ASN-GLU-TRP-CYS-ARG-CYS
GLY-ASP

The DABITC method was found to be particularly useful for sequencing this peptide. It enabled the tryptophan residue in

position 53 to be detected easily and secondly it confirmed that the residues 50 and 51 were both asparagine while residue 52 was glutamic acid and 58 was aspartic acid.

Peptide H10 (59-62) LEU-SER-SER-MET

Peptide H11 (63-65) LEU-ARG-SER

Dansylation without hydrolysis after two Edman degradations yielded dansyl serine.

Peptide H12 (66-69) VAL-TYR-GLX-GLX

The electrophoretic mobility of this peptide at pH 6.5 indicated that one of the residues in positions 68 or 69 is glutamic acid while the other is glutamine.

Peptide H13 (70-71) LEU-GLY

Peptide H14 (72-77) VAL-ARG-GLU-GLY-LYS-GLU

The electrophoretic mobility of this peptide at pH 6.5 indicated that the residues in positions 74 and 77 were both glutamic acid.

Peptide H15 (78-85) VAL-LEU-PRO-GLY-CYS-ARG-LYS-GLU

The electrophoretic mobility of this peptide at pH 6.5 indicated that the residue in position 85 is glutamic acid.

Peptide H16 (86-88) VAL-MET-LYS

Peptide H17 (89-91) LEU-THR-ALA

Peptide H18 (92-99) (ALA, SER, VAL, PRO, GLX, VAL, CYS, LYS) GLY PRO

This peptide was not found. Possibly because it was only present in low yield due to the microheterogeneity in two residues.

Peptide H19 (100-111) VAL-PRO-ILE-PRO-ASN-PRO-SER-GLY-ASP-ARG-ALA-GLY

The electrophoretic mobility of this peptide at pH 6.5 indicated that one of the two residues in position 104 or 108 was aspartic acid and the other is asparagine, but the mobility

of H19a indicated that residue 108 was the acidic form.

Peptide H19a (108-111) ASP-ARG-ALA-GLY

Peptide H20 (112-116) VAL-CYS-TYR-GLY-ASP

The electrophoretic mobility of this peptide at pH 6.5 indicated that residue 116 is aspartic acid.

Peptide H20a (112-113) VAL-CYS

Peptide H21 (117-118) TRP-CYS

This peptide reacted positively with the Ehrlich reagent indicating tryptophan. Semi-quantitative amino acid analysis revealed CM-cysteine and strong traces of dansyl-tryptophan. Dansylation without hydrolysis after one Edman degradation yielded only dansyl-CM-cysteine. No trace of the possible TRP-ALA peptide indicated by other results (X6) was observed.

Peptide H22 (119-123) ALA-TYR-PRO-ASP-VAL

The electrophoretic mobility of this peptide at pH 6.5 indicated that the residue in position 122 is aspartic acid. No hydrolysis was noticed of the aspartic acid (122) - valine (123) bond.

The thermolysin digest was useful in confirming many of the sequences established from the tryptic peptides and it also provided information on some of the acid/amide placements. Observed enzyme specificities were consistent with those previously reported (Matsubara 1966) with the exception of the partial cleavage of the bond between tryptophan (4) - serine (5), cysteine (21) - arginine (22) and glycine (107) - aspartic acid (108).

c. Staphylococcus aureus V8 Protease Digest

20-30mg of S-carboxymethylated inhibitor CIII were digested with the S. aureus V8 enzyme in 50mM Na₂HPO₂ - NaH₂PO₄ buffer

pH 7.8 for 24h. at 37°C. The resulting peptides were purified by the methods described previously and are listed in Table 6. All of the peptides from this digest were sequenced using the DABITC method.

Peptide v1 (1-36) (SER, GLY, PRO, TRP, SER, TRP, CYS, ASX, PRO, ALA, THR, GLY, TYR, LYS, VAL, SER, ALA, LEU, THR, GLY, CYS, ARG, ALA, MET, VAL, LYS, LEU, GLX, CYS, VAL, GLY, SER, GLX, VAL, PRO, GLX)

This very large peptide was not obtained in a pure condition hence it was not sequenced.

Peptide v2 (37-52) ALA-VAL-LEU-ARG-ASP-CYS-CYS-GLN-GLN-LEU-ALA-ASP-ILE-ASN (ASX, GLU)

The DABITC method enabled direct identification of residues 41, 44, 45, 46, 50 as aspartic acid, glutamine, glutamine, aspartic acid and asparagine respectively, but the evidence after 14 cycles of DABITC degradation was less certain for residues 51 and 52 which were placed by examination of the semi-quantitative amino acid analysis and the overlapping with peptides H9 and C8. Furthermore the high specificity of the V8 enzyme (Houmard and Drapeau 1972) suggested that residue 52 was likely to be glutamic acid.

Peptide v3 (53-58) TRP-CYS-ARG-CYS-GLY-ASP

This peptide gave a purple colour with the Ehrlich reagent and the DABITC method gave a clear unambiguous identification of tryptophan as the N-terminal residue (53). The enzyme specificity indicated that the C-terminal amino acid was aspartic acid.

Peptide v4 (59-69) LEU-SER-SER-MET-LEU-ARG-SER-VAL-TYR-GLN (GLU)

During the sequencing of this peptide, serine was detected in position 65 and tyrosine in position 67 as further cases of

TABLE 6

Peptides resulting from protease V8 digest of wheat (Triticum aestivum) α -amylase inhibitor CIII

Peptide No. and Position in Sequence	Purification of Peptide		Mobility of Peptides on High Voltage Paper Electrophoresis	BAWP ^b	DABITC - Sequence Results
	Peptides No. in Gel-Filtration ^a	P2			
V1 (1-36)	1	G-50 1	PH6.5 N.F.	N.F.	(SER, GLY, PRO, TRP, SER, TRP, CYS, ASX, PRO, ALA, THR, GLY, TYR, LYS, VAL, SER, ALA, LEU, THR, GLY, CYS, ARG, ALA, MET, VAL, LYS, LEU, GLX, CYS, VAL, GLY, SER, GLX, VAL, PRO, GLX)
V2 (37-52)	1	1	-1.73	-	<u>ALA-VAL-LEU-ARG-ASP-CYS-CYS-GLN-GLX-LEU-ALA-ASP-ILE-ASN-ASX-GLU</u>
V2a (37-41)	2	-	0.88	-	<u>ALA-VAL-LEU-ARG-ASP</u>
V3 (53-58)	4	-	-	-	<u>TRP-CYS-ARG-CYS-GLY-ASP</u>
V4 (59-69)	2	-	0.54	-	<u>LEU-SER-SER-MET-LEU-ARG-SER-VAL-TYR-GLN-GLU</u>
V5 (70-74)	2	-	0.78	-	<u>LEU-GLY-VAL-ARG-GLU</u>
V6 (75-77)	3	-	1.18	-	<u>GLY-LYS-GLU</u>
V7 (78-85)	1	2	0.88	-	<u>VAL-LEU-PRO-GLY-CYS-ARG-LYS-GLU</u>
V8 (86-96)	1	1	0.51	-	<u>VAL-MET-LYS-LEU-CHR-ALA-ALA-SER-VAL-PRO-GLU</u>
V9 (97-123)	1	1	-1.2	0.45	<u>VAL-CYS-LYS-VAL-PRO-ILE-PRO-ASN-PRO-SER-GLY-ASP-ARG-ALA-GLY-VAL-CYS-TYR-GLY-ASP-TRP-CYS-(ALA, TYR, PRO, ASX, VAL,)</u>

See footnotes to Table 4

microheterogeneity which were confirmed by peptides H11 and H12.

<u>Peptide V5</u>	(70-74)	LEU-GLY-VAL-ARG-GLU
<u>Peptide V6</u>	(75-77)	GLY-LYS-GLU
<u>Peptide V7</u>	(78-85)	VAL-LEU-PRO-GLY-CYS-ARG-LYS-GLU
<u>Peptide V8</u>	(86-96)	VAL-MET-LYS-LEU-THR-ALA-ALA-SER- VAL-PRO (GLU)

After nine DABITC degradation cycles, residue 96 was placed by examination of the semi-quantitative amino acid analysis and the overlapping with peptide T11H2 and the CNBr X5 fragment. In addition the specificity of the V8 enzyme and the electrophoretic mobility at pH 6.5 indicated that glutamic acid was present in position 96.

<u>Peptide V9</u>	(97-123)	VAL-CYS-LYS-VAL-PRO-ILE-PRO-ASN- PRO-SER-GLY-ASP-ARG-ALA-GLY-VAL- CYS-TYR-GLY-ASP-TRP-CYS (ALA, TYR, PRO, ASX, VAL)
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CM-cysteine and lysine were found in positions 98 and 99 respectively as examples of microheterogeneity. This peptide showed a positive reaction with the Ehrlich reagent and tryptophan was identified in position 117. After 18 successive cycles of DABITC degradation which yielded unambiguous results, the residues in positions 119-123 were difficult to identify owing to problems of "hang-over" and were placed by examination of the semi-quantitative amino acid analysis and the overlapping peptides T14, H22, G14 and the CNBr X6 fragment.

d. Chymotrypsin Digest

Although 20-30 mg of α -amylase inhibitor CIII was digested with chymotrypsin, difficulties were experienced in separating the resulting peptides. Table 7 shows a list of those chymotryptic peptides which were obtained in a pure form together with their purification details and the results obtained from the sequencing

TABLE 7

Peptides resulting from chymotrypsin digest of wheat (*Triticum aestivum*) α -amylase inhibitor CIII

Peptide No. and Position in Sequence	Purification of Peptide		BAMP ^b	Dansyl-Edman Results
	Peptides No. in Gel-Filtration ^a	Mobility of Peptides on High Voltage Paper Electrophoresis PH6.5 PH1.9		
C1 (1-4)	P2 G-50 4 -	0	-	<u>SER-GLY-PRO</u> -(TRP)
C2 (5-13)	2 -	-0.71	-	<u>SER-TRP-GYS-ASN-PRO-ALA-THR-GLY-TYR</u>
C3 (14-18)	2 -	1.10	-	<u>LYS-VAL-SER-ALA-LEU</u>
C4 (19-24)	3 -	0	0.48	<u>THR-GLY-CYS-ARG-ALA-MET</u>
C5 (25-27)	4 -	1.66	-	<u>VAL-LYS-LEU</u>
C6 (28-39)	1 -	N.F.	N.F.	(GLX, CYS, VAL, GLY, SER, GLX, VAL, PRO, GLX, ALA, VAL, LEU)
C7 (40-46)	1 -	-1.42	-	<u>ARG-ASX-CYS-CYS-GLX-GLX-LEU</u>
C8 (47-53)	1 -	-1.42	-	<u>ALA-ASX-ILE-ASX-ASX-GLX-(TRP)</u>
C9 (54-63)	1 -	-1.21	-	<u>CYS-ARG-CYS-GLY-ASP-LEU-SER-SER-MET-LEU</u> ALA ALA
C10 (64-70)	N.F.	N.F.	N.F.	(ARG, SER, VAL, TYR, GLX, GLX, LEU)
C11 (71-87)	N.F.	N.F.	N.F.	(GLY, VAL, ARG, GLX, GLY, LYS, GLX, VAL, LEU, PRO, GLY, CYS, ARG, LYS, GLX, VAL, MET) <u>LYS-LEU</u>
C12 (88-89)	4 -	1.9	-	
C13 (90-113)	1 -	N.F.	N.F.	(THR, ALA, ALA, SER, VAL, PRO, GLX,

TABLE 7 cont.

C14	(114-117)	N.F.	-	N.F.	N.F.	N.F.	(GLY, ASX, TRP)	GLY PRO VAL, CYS, LYS, VAL, PRO, ILE, PRO, ASX, PRO, SER, GLY, ASX, ARG, ALA, GLY, VAL, CYS, TYR)	
C15	(118-123)	3	-	-1.4	0.35	-	$\overrightarrow{\text{CYS-ALA-TYR-PRO-ASP-VAL}}$		

See footnote to Table 4

methods.

Peptide C1 (1-4) SER-GLY-PRO (TRP)

This peptide gave a positive reaction with the Ehrlich reagent indicating the presence of tryptophan which was placed in the C-terminal position by the overlapping with the T1 and the CNBr fragment, and by the specificity of the enzyme used.

Peptide C2 (5-13) SER-(TRP)-CYS-ASN-PRO-ALA-THR-GLY-TYR

This peptide gave a positive reaction with the Ehrlich reaction indicating the presence of tryptophan which was placed in position 6 by homology using the Redman (1976) sequence, and the results of sequence analyses on other peptides (T1, H1b, X1). The electrophoretic mobility at pH 6.5 indicated asparagine in position 8.

Peptide C3 (14-18) LYS-VAL-SER-ALA-LEU

Peptide C4 (19-24) THR-GLY-CYS-ARG-ALA-MET

Peptide C5 (25-27) VAL-LYS-LEU

Peptide C6 (28-39) (GLX, CYS, VAL, GLY, SER, GLX, VAL, PRO, GLX, ALA, VAL, LEU)

This large peptide was not isolated probably due to the presence of glutamine (as determined later) in the N-terminal position. Cyclization of this glutamine (Smith et al. 1963) would prevent dansylation and/or reaction with ninhydrin and hence detection.

Peptide C7 (40-46) ARG-ASX-CYS-CYS-GLX-GLX-LEU

The electrophoretic mobility of this peptide at pH 6.5 indicated the presence of three acidic residues, assuming CM-cysteine to have a charge of -1.

Peptide C8 (47-53) ALA-ASX-ILE-ASX-ASX-GLX (TRP)

This peptide gave a positive reaction with the Ehrlich reagent indicating the presence of tryptophan. The electrophoretic mobility of the peptide at pH 6.5 suggested the presence of two residues in the acidic form.

Peptide C9 (54-63) CYS-ARG-CYS-GLY-ASP-LEU-SER-SER-MET-LEU

The electrophoretic mobility of this peptide at pH 6.5 showed that the residue in position 58 was aspartic acid. No hydrolysis was noticed of the peptide bond between methionine (62) and leucine (63).

Peptide C10 (64-70) (ARG, SER, VAL, TYR, ^{ALA}GLX, ^{ALA}GLX, LEU)

Peptide C11 (71-87) (GLY, VAL, ARG, GLX, GLY, LYS, GLX, VAL, LEU, PRO, GLY, CYS, ARG, LYS, GLX, VAL, MET)

These peptides were not isolated in a pure condition, possibly due to the failure of chymotrypsin to cleave the LEU (70)-GLY (71) bond.

Peptide C12 (88-89) LYS-LEU

Peptide C13 (90-114) (THR, ALA, ALA, SER, VAL, PRO, GLX, VAL, GLY, PRO, CYS, LYS, VAL, PRO, ILE, PRO, ASX, PRO, SER, GLY, ASX, ARG, ALA, GLY, VAL, CYS, TYR)

Peptide C14 (115-117) (GLY, ASX, TRP)

The peptides C13 and C14 were not detected.

Peptide C15 (118-123) CYS-ALA-TYR-PRO-ASP-VAL

The electrophoretic mobility of this peptide at pH 6.5 indicated that the residue in position 122 is aspartic acid.

The observed enzyme specificities were consistent with those normally expected for chymotrypsin (Smyth 1967) except that no evidence was observed for any hydrolysis of the LEU (70)-GLY (71) bond, the TYR (114)-GLY (115) bond or the ASN (54)-GLU (52) bond

e. Carboxypeptidase A Digest

Carboxypeptidase A was used to determine the C-terminal amino acid. Incubation of the native protein with the enzyme for varying times up to 24h. yielded only small amounts of valine as determined by dansylation of liberated amino acids. Prolonged digestion with carboxypeptidase A did not yield any other amino acids. This result suggested that the C-terminal residue was valine and that the attached amino acids were resistant to attack by the enzyme. Later sequencing data confirmed valine as the C-terminal amino acid with aspartic acid in the preceding position. This result agrees with the valine reported by Redman (1975) for the S and M isoinhibitor examples of the 0.28 albumin and Ewart (1972) for two different wheat albumins.

3. CNBr Cleavage

100mg of reduced and S-carboxymethylated α -amylase inhibitor CIII were dissolved in 2ml of 70% (v/v) formic acid and an 80 fold (w/w) excess of CNBr was added. The mixture was incubated in the dark for 24h. at room temperature. The solution was then diluted 10 times with distilled water and lyophilized.

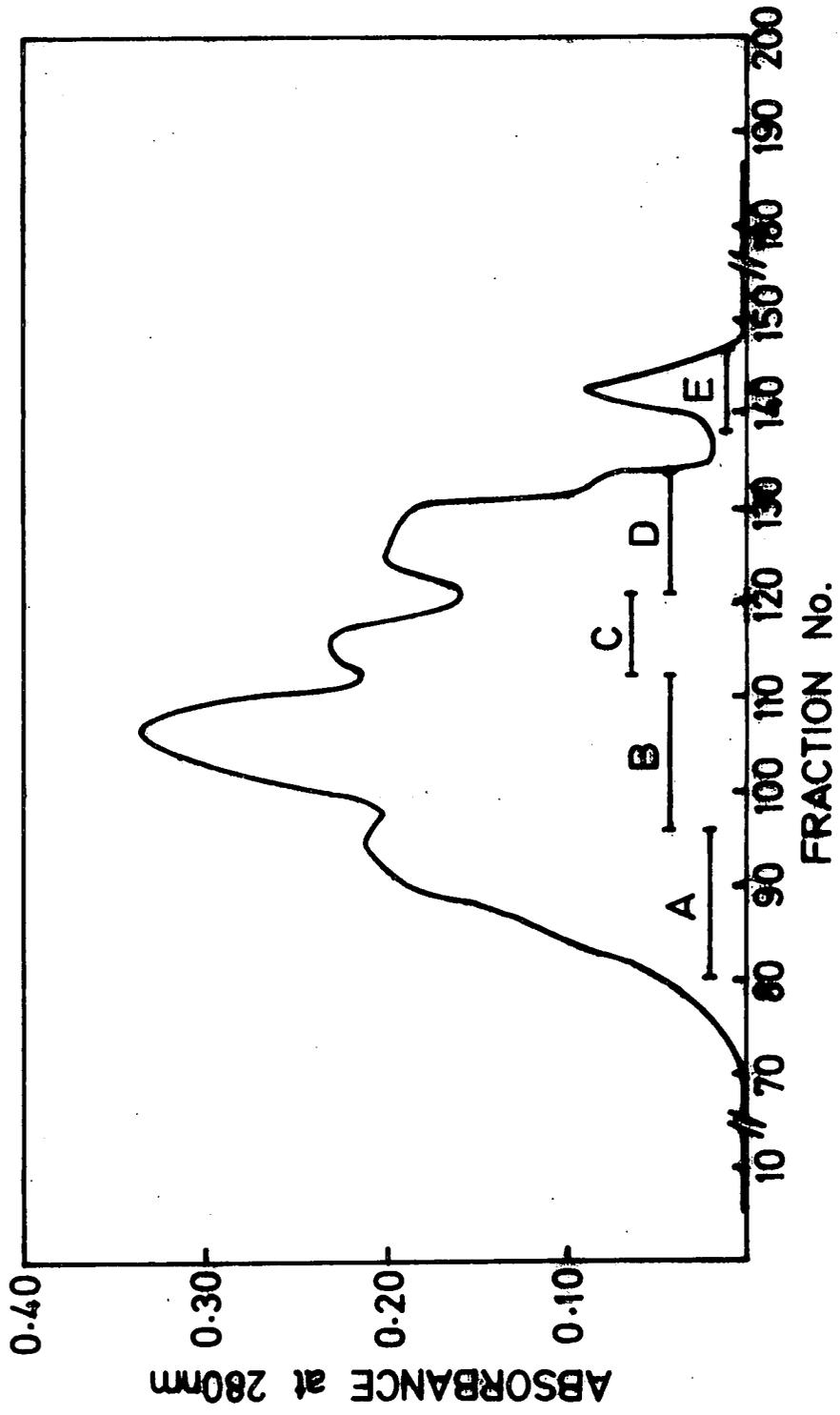
The cleaved fragments were separated on a column (200 x 3.2cm) of Sephadex G-75 equilibrated and eluted with 50mM pyridine-acetate buffer pH 5.4. The column eluate was monitored at 280nm for the presence of protein and peptide fragments (Fig. 14). Peaks A, B, C, D and E were collected and lyophilized.

The N-terminal analysis carried out on each of these peaks by the dansyl method revealed that valine, leucine and lysine (X3, X4 and X5) were present in peak A and B, CM-cysteine and a trace of lysine in peak C (X2 fragment), CM-cysteine and alanine

Figure 14

Separation of CNBr fragments (X1 - X6) on a (200cm x 3.2cm) column of Sephadex G-75 equilibrated and eluted with 50mM pyridine-acetate buffer pH 5.4. Fractions (10 ml) were collected at a flow rate of 55 ml/h. Those fractions, indicated by bars, representing peaks (A - E) were pooled and freeze-dried.

Fig. 14



in peak D (X6 fragment) and only serine in peak E (X1 fragment).

The CNBr fragments in peaks C (X2), D (X6) and E (X1) were sufficiently homogeneous for the determination of their sequences without further purification, but the fragments in peak A and B (X3, X4 and X5) were further separated by ion-exchange chromatography on CM 52 cellulose.

Lyophilized samples of peaks A and B were separately dissolved in 10ml of 5mM sodium acetate buffer pH 3.5 and loaded onto a column (30 x 1.6cm) of CM 52 cellulose equilibrated with the same buffer. The column was washed with about 400ml of starting buffer prior to the application of a salt gradient (from 0 - 0.17M NaCl). The eluate was monitored at 280nm. Figure 15a shows the separation of the fragments contained in the pooled peak A and figure 15b shows those from pooled samples of peak B.

N-terminal amino acid analysis by the dansylation method revealed valine (X3 fragment) in both peaks a, leucine (X4 fragment) in peak c lysine (X5 fragment) in all peaks b (Fig. 15a, b).

In both separations (Figures 15a and 15b) double peaks were found for the CNBr X3 fragment (peaks a) and the CNBr X5 fragment (peaks b). There are several possible explanations for this phenomena during ion-exchange chromatography. Such double peaks could be caused by the C-terminal residue of the CNBr-generated peptide existing either in the homoserine form or the homoserine lactone form (Gross 1967).

Figure 15a

Chromatography of partially purified CNBr fragments (X3, X4 and X5) on a column (30cm x 1.6cm) of CM-52 cellulose equilibrated in 5mM sodium acetate buffer pH 3.5. Peak A after chromatography of CNBr fragments on Sephadex G-75 was dissolved in 10 ml of the equilibrating buffer and applied to the column. Fractions (3 ml) were collected at a flow rate of 20ml/h. The column was washed with the original buffer prior to application of the salt gradient (from 0 - 0.17M NaCl). Short cuts of Peaks a (fragment X3), peaks b (fragment X4) and peaks c (fragments X5) were pooled, dialyzed against distilled water and lyophilized.

Fig.15a

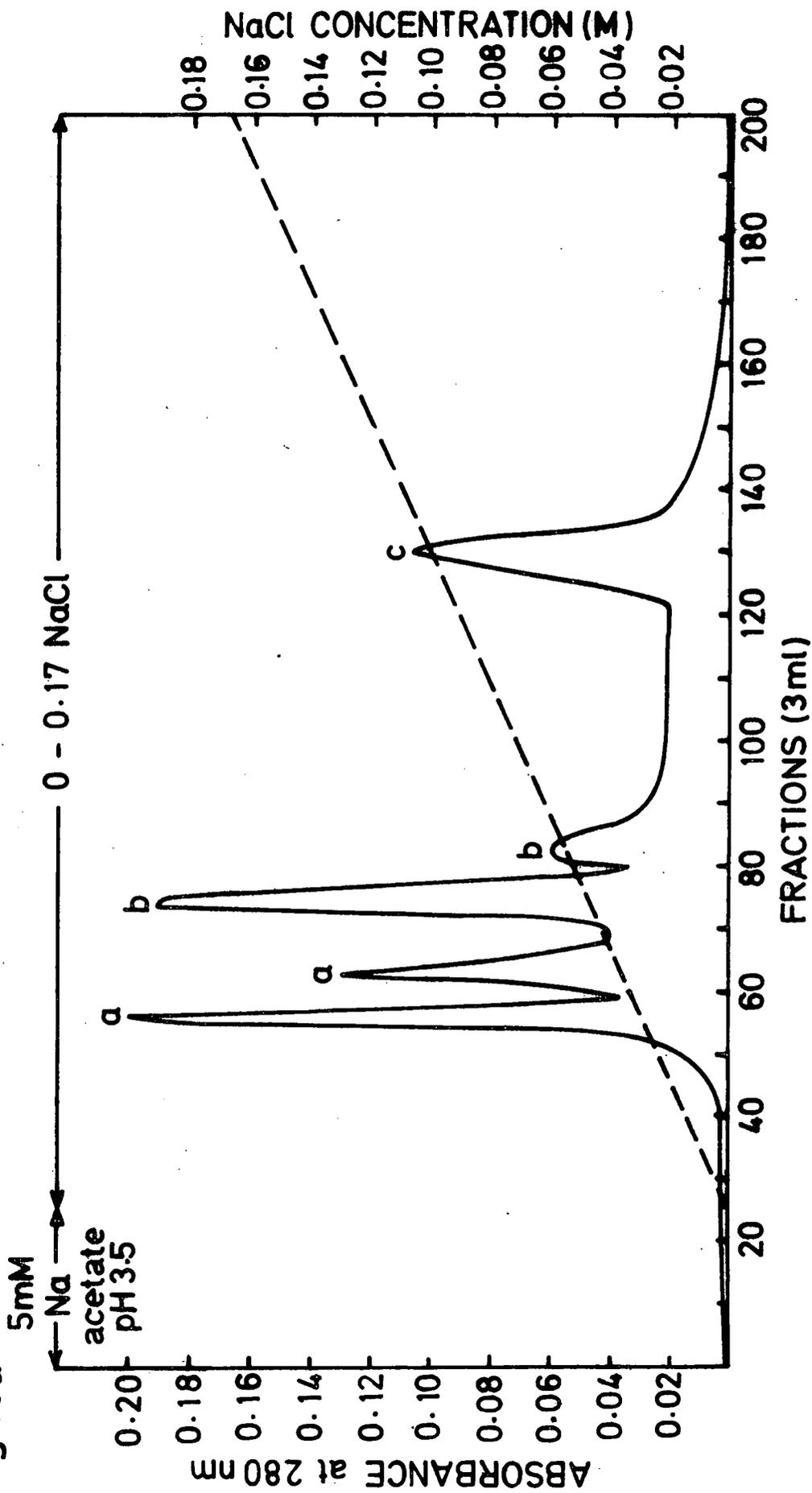


Figure 15 b

Chromatography of partially purified CNBr fragments (X3 X4 and X5) on a column (30cm x 1.6cm) of CM-52 cellulose equilibrated in 5mM sodium-acetate buffer pH 3.5. Peak B after chromatography of CNBr fragments on Sephadex G-75 was dissolved in 10 ml of the equilibrating buffer and applied to the column. Fractions (3 ml) were collected at a flow rate of 20 ml/h. The column was washed with the original buffer prior to application of the salt gradient (from 0 - 0.17M NaCl). Short cuts of peaks a (fragment X3), peaks b (fragment X4) and peak c (fragment X5) were pooled, dialyzed against distilled water and lyophilized.

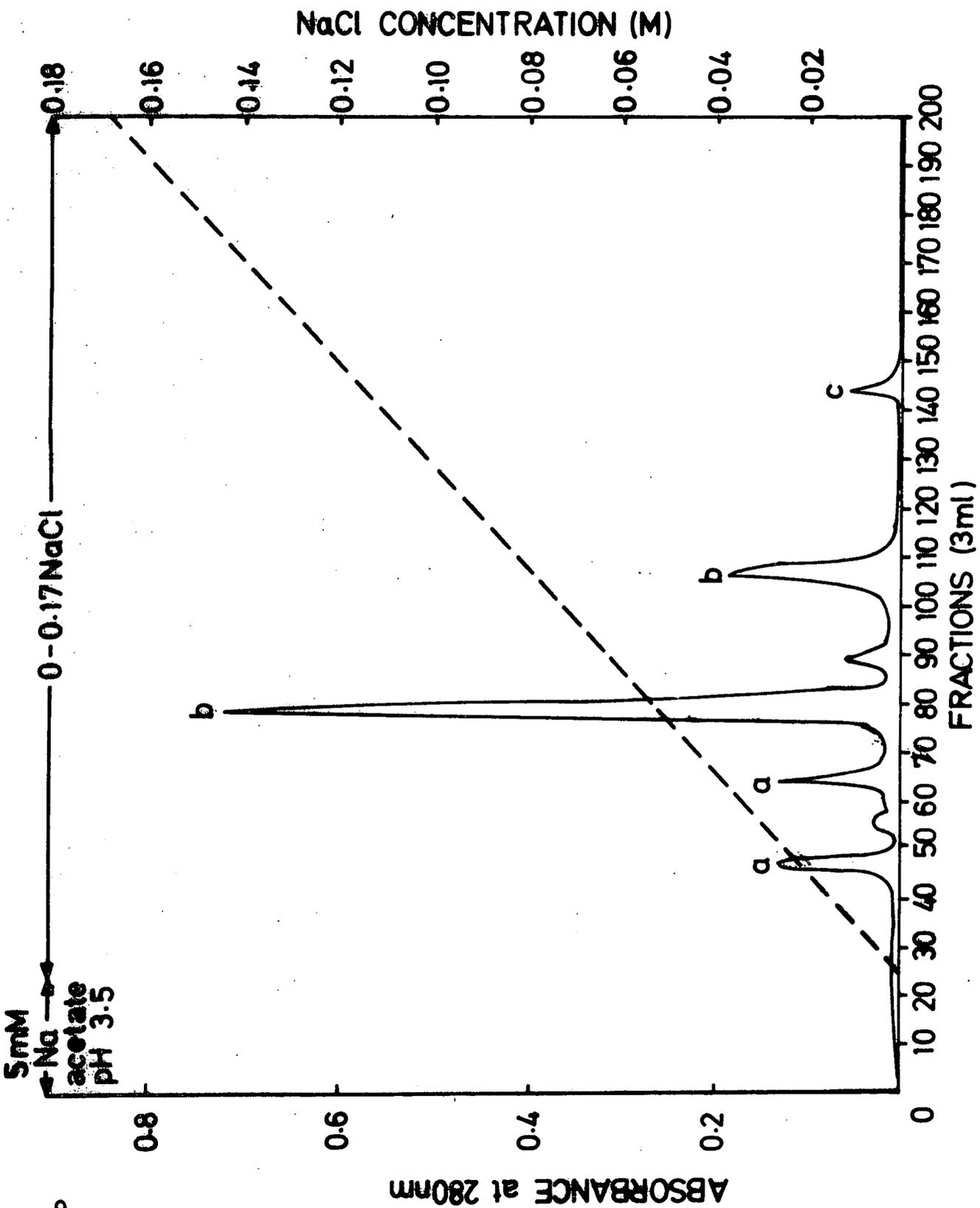


Fig.15 b

There was likely to have been an equilibrium mixture of these two forms as no specific treatment (alkali at room temperature, or heating with acid) was carried out to convert them all to the one form. This explanation cannot however apply to the CNBr X5 fragment (peaks b) as this peptide was generated from the C-terminal region of the protein and hence did not contain homoserine (or its lactone). Alternative explanations for the behaviour of the CNBr X5 fragment might be provided by the presence of the microheterogeneity involving charged amino acids which was subsequently discovered in residues 98 and 99 or an incomplete cleavage by the cyanogen bromide at the tryptophan (117) - cysteine (118) bond. It should be noted that the CNBr X4 fragment which only gave a single peak (peak c) did not exhibit microheterogeneity involving charged amino acids although it did contain homoserine (lactone).

A list of the CNBr fragments, together with the methods used for their purification and the DABITC sequence results are shown in Table 8.

Separation of the large CNBr fragments and the determination of major parts of their sequences by the DABITC method was of great assistance in confirming the complete amino acid sequence of the 0.28 α -amylase inhibitor CIII. The method used in separating these fragments was essentially as reported by Redman (1975) except that a linear salt gradient was used in this work instead of the convex salt gradient used by the other worker.

Treatment of isoinhibitor CIII with cyanogen bromide led to the expected cleavages at the three methionine residues and

TABLE 8

Fragments resulting from CNBr. cleavage of wheat (Triticum aestivum) α -amylase inhibitor CIII

Fragment No. and Position in sequence	Purification of Fragment		DABITC-Sequence Results
	Peak No. on G-75 Gel-filtration	Peak No. on CM 52 cellulose	
X1 (1-6)	E	-	<u>SER-GLY-PRO-TRP-SER-TRP</u>
X2 (7-24)	C	-	<u>CYS-ASN-PRO-ALA-THR-GLY-TYR</u> -(LYS, VAL,SER,ALA,LEU,THR,GLY,CYS,ARG, ALA,HSE)
X3 (25-62)	A & B	a	<u>VAL-LYS-LEU-GLN-CYS-VAL-GLY-SER-GLN-</u> <u>VAL-PRO-GLU-ALA-VAL-LEU-ARG-ASP</u> -(CYS, CYS,GLN,GLN,LEU,ALA,ASP,ILE,ASN,ASN, GLU,TRP,CYS,ARG,CYS,GLY,ASP,LEU,SER, SER,HSE)
X4 (63-87)	A & B	c	<u>LEU-ARG-ALA-VAL-ALA-GLN-GLU-LEU-GLY-</u> <u>VAL-ARG-GLU-GLY-LYS-GLU-VAL-LEU</u> -(PRO, GLY,CYS,ARG,LYS,GLU,VAL,HSE)
X5 (88-117)	A & B	b	<u>LYS-LEU-THR-ALA-ALA-SER-VAL-PRO-GLU-</u> <u>VAL-GLY-PRO-VAL-PRO-ILE-PRO-ASN-PRO-</u> <u>SER-GLY-ASP-ARG-ALA-GLY-VAL-CYS-TYR-</u> <u>GLY</u> -(ASP,TRP)
X6 (118-123)	D	-	CYS <u>ALA-ALA-TYR-PRO-ASP-VAL</u>

generated two CNBr fragments (X3 and X4) with the same terminal residues and very similar amino acid compositions to the CNBr-1 and CNBr-3 fragments reported by Redman (1975) for the 0.28 albumin M. In addition however the cyanogen bromide treatment used in this work produced anomalous cleavages of the tryptophan (6) - CM-cysteine (7) and tryptophan (117)-CM-cysteine (118) bonds leading to the formation of four additional fragments (X1, X2, X5 and X6) which clearly represent modified forms of the CNBr-4 and CNBr-2 fragments of Redman (1975). It is not known whether these anomalous cleavages were caused by the cyanogen bromide or were a result of the prolonged incubations in 70% formic acid. ~~In~~ *Ozols and Gerhard (1979) have reported similar anomalous cleavages when an excess of CNBr was employed* ^{alanine} ^{aspartic acid} ¹ this connection it is perhaps noteworthy that there was no evidence for any hydrolysis of the asparagine (8)- proline (9) by the 70% formic acid in contrast to its effects on the same peptide bond in other proteins (Piszkiwicz et al. 1970).

When the CNBr^{X1} fragment was redigested with chymotrypsin, the three peptides (X2C1 - X2C3) which resulted were separated by high voltage paper electrophoresis at pH 6.5 and sequenced by the dansyl-Edman method (Table 9).

The complete amino acid sequence of the wheat α -amylase isoinhibitor CIII is shown in Figure 16 together with the details of the CNBr fragments and the overlapping peptides from which it was deduced. This is the first report of the complete amino acid sequence for any of the wheat albumin proteins which inhibit α -amylase. The only region of the sequence which lacks support from good overlapping segments (3 or more residues) of peptides and fragments is the sequence of residues 116-120, but the sequence presented in Figure 16 is entirely compatible with the results of the amino acid analysis, and is in agreement with the tentative

TABLE 9

Peptides resulting from chymotrypsin digest of the CNBr X2 fragment

Peptide No. and position in sequence	Mobility of peptide on high voltage paper electrophoresis at pH 6.5	Dansyl-Edman result
X2C1 (7-13)	-0.89	<u>CYS</u> - <u>ASN</u> - <u>PRO</u> - <u>ALA</u> - <u>THR</u> - <u>GLY</u> - <u>TYR</u>
X2C2 (14-18)	1.15	<u>LYS</u> - <u>VAL</u> - <u>SER</u> - <u>ALA</u> - <u>LEU</u>
X2C3 (19-24)	0	<u>THR</u> - <u>GLY</u> - <u>CYS</u> - <u>ARG</u> - <u>ALA</u> - <u>HSE</u>

FIGURE 16

The amino acid sequence of the wheat (Triticum aestivum) protein α -amylase inhibitor CIII. T= trypsin peptides, C = chymotrypsin peptides, H = thermolysin peptides, V= peptides from digestion with S. aureus V8 protease, X = fragments resulting from cleavage with cyanogen bromide.

————> results from DABITC method applied to large fragments
<————> peptides sequenced by the DABITC method and / or the dansyl-Edman procedure; <----> peptides / residues which were not sequenced or yielded unsatisfactory results.

(/) DABITC sequence not determined

ordering of the CNBr fragments suggested by Redman (1975) and the identification of valine as the C-terminal residue (Redman 1975 and Ewart 1972). It is suggested that further work might be carried out in the future to strengthen this area of the sequence. One possible approach would be to isolate and purify the V9 peptide again and redigest it with the mouse submaxillary enzyme (Schenkein et al. 1977). The specificity of this enzyme is such that it should only hydrolyse the arginine (109)-alanine (110) peptide bond to give two peptides, the C-terminal of which could be sequenced by the DABITC method.

The highly sensitive DABITC micro-sequence method was particularly useful in the identification of the tryptophan residues in peptides X1, H1, H9, V3 and V9. Also this method was used in conjunction with the specificity of digestion by the S. aureus protease to resolve the identification of the acid/amide residues in those peptides (X3, X4, X5, H6, H7, H9, V2, V3, V9) where the placement could not be unambiguously assigned by the dansyl-Edman method and consideration of the electrophoretic mobilities.

The protein contains 123 amino acids which corresponds to an approximate molecular weight of 13,400. A more accurate molecular weight cannot be calculated owing to the degree of microheterogeneity exhibited by this protein. The value obtained is in agreement with the previous estimates of 12,500 made for the 0.28 albumin inhibitor by gel filtration (Petrucci et al. 1974 and Deponte et al. 1976) the values of 13,300 - 14,500 from sedimentation equilibrium studies (Silano et al. 1973 and Granum and Whitaker 1977) and the figures of 12,900 - 13,400 obtained from amino acid analyses (Shainkin and Birk 1970; Redman 1975;

Ewart 1972 and Feillet and Nimmo 1970).

The sequence of the first 24 amino acids in isoinhibitor CIII is identical with that reported by Redman (1976) for the N-terminal CNBr peptides from three inhibitors of the 0.28 family with the exception that residue 8 was usually present as asparagine although small amounts of the deamidated form were also observed.

This work also confirmed the previous suggestion based on the amino acid analysis of CNBr fragments (Redman 1975) that the glutamic acid/glutamine residues in the 0.28 inhibitor are located in the middle of the peptide chain. In fact all 12 of the glutamic acid/glutamine amino acids in the protein were found to be restricted to the central region between residues 28 and 96 (Fig. 16). The sequence of the protein was also notable for being completely lacking in phenylalanine and histidine residues. This is in agreement with most previous amino acid analyses carried out on this family of wheat albumins where both amino acids were absent (Shainkin and Birk 1970; Silano et al. 1973; Redman 1975; Ewart 1972 and Feillet and Nimmo 1970) or only present in very low amounts (Granum and Whitaker 1977).

Approximately equal amounts of serine and alanine were observed in position 65 in the various peptides and fragments covering this region. Other much lower levels of microheterogeneity were observed in residues 8 (aspartic acid as the minor form), 67 (alanine), 98 (glycine), 99 (proline) and 118 (alanine). The existence of such polymorphism is perhaps not surprising in view of the multiplicity of isoinhibitors which have been reported in the 0.28 family (Buonocore et al. 1977 and Silano et al. 1973).

The precise positions of the polymorphisms observed in the sequence of the CIII inhibitor in this work (a single example of microheterogeneity in the first 62 residues, and five examples in the last 61 residues) appear to confirm the general suggestions made by Redman (1975) as to the location of the variable regions in this family of isoinhibitors. Amino acid analyses carried out by the latter worker on the CNBr fragments derived from three isoinhibitors of the 0.28 and 0.39 groups indicated that there was likely to be little variation in the sequences of the first two fragments reading from the N-terminus (i.e. CNBr-4 and CNBr-1), but some considerable differences in the fragments (CNBr-3 and CNBr-2) in the C-terminal region.

It is important to mention that the amino acid sequence of the wheat α -amylase inhibitor CIII reported here showed no homology with the partial sequences of the wheat gliadin proteins reported by Kasarda et al. (1974), Bietz et al. (1977) and Autran et al. (1979). This is perhaps surprising in view of the suggestion based on the similarity of solubility data, pI values, amino acid compositions and molecular weights that the wheat albumin inhibitors bear a resemblance to the gliadins (Shainkin and Birk 1970).

Feillet and Nimmo (1970) were unable to detect any free sulphydryl groups in their ALB 13 B protein which appears to belong to the 0.28 family, suggesting that all of the cysteine residues must be combined as cystine. The absence of free thiol groups in the native 0.28 protein was also confirmed by Petrucci et al. (1978) who subsequently obtained nine radioactive spots in their tryptic peptide maps when the protein was reduced with dithiothreitol and carboxymethylated with iodo (^{14}C) acetic acid. A similar number

of tryptic peptides containing cysteine were found during the present work. These results and those of Redman (1975) indicate that the 0.28 iso inhibitor probably contains five disulphide bridges. It would be of interest to determine the location of the five intramolecular disulphide bridges in this 0.28 albumin as they appear to be essential for its activity, since reduction with β -mercaptoethanol (Buonocore et al. 1977) or dithiothreitol (Petrucci et al. 1978) completely destroys the inhibitory capacity.

Further sequence studies on other iso inhibitor forms of the 0.28 family might be revealing but of even greater interest would be the determination of the complete amino acid sequences for the two non-identical monomers which comprise the dimeric 0.19 inhibitor (Silano et al. 1973 and Petrucci et al. 1978). Only then will stronger evidence be available for the hypothesis that the multiple forms of these inhibitors originated from a common ancestral gene by a process of replication and mutation.

Other possible work to be carried out in the future on this protein is to determine the position of the single residue of reducing sugar which is thought to be present at the binding site for α -amylase (Petrucci et al. 1978 and Buonocore et al. 1980).

IV. THE AMINO ACID SEQUENCE OF SOYBEAN (Glycine max)
PROTEASE INHIBITOR PI IV

1. Amino Acid Composition

The amino acid composition of the soybean protease inhibitor PI IV is given in Table 10. The results obtained for serine and methionine were rather low when compared with their values calculated from the sequence. The other results were in good agreement with those calculated from the complete amino acid sequence shown in Figure 17. The amino acid composition also showed a slight difference to the values reported by Hwang et al. (1977) in which aspartic acid, threonine, and tyrosine were of higher values while alanine was obtained in lower values.

2. Digestion with Proteolytic Enzymes

The proteolytic enzymes used in this investigation were trypsin and chymotrypsin. The two sets of peptides resulting from these digests gave sufficient overlapping to logically establish most of the sequence.

TABLE 10

The amino acid composition of Soybean (Glycine max) Protease Inhibitor PI IV.

Amino Acid	Mean Values 24h. hydrolysis	Mean Values 72h. hydrolysis	Mean corrected Values	Sequence Values	Hwang † Values
Asp	12.00	14.60	13.30	13	12
Thr	2.91	2.81	2.96	3	2
Ser	9.58	8.97	9.62	12	12
Glu	6.30	5.80	6.01	6	6
Pro	5.10	5.30	5.20	5	5
Gly	1.683	1.03	1.05	1	1
Ala	0.40	0.33	0.36	0	1
Val	0.34	0.23	0.28	0	0
Met	1.99	2.38	2.18	3	2
Ile	0.95	1.00	1.00	1	1
Leu	2.65	3.55	3.55	3	3
Tyr	2.68	2.75	2.75	3	2
Phe	1.02	1.22	1.12	1	1
His	1.20	0.98	1.10	1	1
Lys	4.15	3.85	4.00	4	4
Arg	4.40	4.30	4.30	5	5
Cys*	-	-	-	14	12

*CM-Cys values could not be estimated as this amino acid was poorly separated from the aspartic acid peak.

† Hwang et al. (1977)

a. Trypsin Digest

A list of peptides resulting from the tryptic digestion together with the details of the methods used for their purification and the results obtained from the sequencing methods are given in Table 11.

Peptide T1 (1-24) (SER, ASX, GLX, SER, SER, SER, TYR, ASX, ASX, ASX, GLX, TYR, SER, LYS, PRO, CYS, CYS, ASX, LEU, CYS, MET, CYS, THR, ARG)

This peptide was treated with cyanogen bromide (CNBr) in the dark for 24h. The two expected fragments were separated by high voltage paper electrophoresis at pH 6.5.

Peptide T1X1 (1-21) SER-ASX-GLX-SER-SER-SER-TYR-ASX-ASX-ASX-GLX-TYR-SER-LYS- (PRO, CYS, CYS, ASX, LEU, CYS, HSE)

After 13 Edman degradations this peptide failed to degrade any further. Residues 15-21 were placed by examination of the semi-quantitative amino acid analysis and the overlapping with C2 peptide. The electrophoretic mobility indicated the presence of several acidic residues, when measured at pH 6.5.

Peptide T1X2 (21-24) CYS-THR-ARG

Peptide T2 (25-36) SER-MET-PRO-PRO-GLX-CYS-SER-CYS-GLX-ASX-ILE-ARG

The electrophoretic mobility of this peptide at pH 6.5 indicated the presence of several acidic residues which could not be placed at this stage.

Peptide T3 (37-45) LEU-ASX-SER-CYS-HIS-SER-ASX-CYS-LYS

The electrophoretic mobility of this peptide at pH 6.5 indicated that one of the residues in position 38 or 43 was aspartic acid, while the other was asparagine.

Peptide T4 (46-51) SER-CYS-MET-CYS-THR-ARG

TABLE 11

Peptides resulting from trypsin digest of soybean (Glycine max) Protease inhibitor PI IV

Peptide No. and Position in Sequence	Purification of Peptides			Dansyl-Edman Results
	Peptides No. in Gel-Filtration G-50	Mobility of Peptides on High Voltage Paper Electrophoresis	BAWP ^b	
T1 (1-24)	1	- PH6.5 PH1.9	-	<u>SER-</u> Treated with CNBr for 24h. at room temperature.
TIX1 (1-21)	-	-2.35	-	<u>SER-ASX-GLX-SER-SER-SER-TYR-ASX-ASX-ASX-GLX-TYR-SER-LYS-</u> (PRO, CYS, CYS, ASX, LEU, CYS, MET)
TIX2 (22-24)	-	0	-	<u>CYS-THR-ARG</u>
T2 (25-36)	2	-1.86	0.34	0.05 <u>SER-MET-PRO-PRO-GLX-CYS-SER-CYS-GLX-ASX-ILE-ARG</u>
T3 (37-45)	2	-0.73	-	- <u>LEU-ASX-SER-CYS-HIS-SER-ASX-CYS-LYS</u>
T4 (46-51)	2	-0.90	0.80	- <u>SER-CYS-MET-CYS-THR-ARG</u>
T5 (52-58)	2,3	0	0.73	0.11 <u>SER-GLN-PRO-GLY-GLN-CYS-ARG</u>
T6 (59-71)	2	-1.88	0.76	0.13 <u>CYS-LEU-ASX-THR-ASX-ASX-PHE-CYS-TYR-LYS-PRO-CYS-LYS</u>
T7 (72-75)	3	-1.10	1.02	- <u>SER-ARG-ASP-ASP</u>

Peptide T5 (52-58) SER-GLN-PRO-GLY-GLN-CYS-ARG

The electrophoretic mobility of this peptide at pH 6.5 indicated that the residues in positions 53 and 56 were both glutamine.

Peptide T6 (59-71) CYS-LEU-ASX-THR-ASX-ASX-PHE-CYS-
TYR-LYS-PRO-CYS-LYS

The electrophoretic mobility of this peptide at pH 6.5 indicated the presence of several acidic residues.

Peptide T7 (72-75) SER-ARG-ASP-ASP

The electrophoretic mobility of this peptide at pH 6.5 indicated the presence of two aspartic acid residues. After three Edman degradations dansylation without hydrolysis indicated dansyl aspartic acid.

The tryptic digest of the reduced and S-carboxymethylated trypsin inhibitor PI IV yielded all but one of the expected number of peptides. The only expected peptide bond which failed to cleave was the bond between arginine (73)-aspartic acid (74)

b. Chymotrypsin Digest

A list of the peptides resulting from the chymotrypsin digestion together with the methods used in their purification and the sequence data using the dansyl-Edman procedure are shown in Table 12.

Peptide C1 (1-7) SER-ASX-GLX-SER-SER-SER-TYR

This peptide was not isolated in a pure state.

Peptide C2 (8-12) ASP-ASP-ASP-GLU-TYR

The electrophoretic mobility of this peptide at pH 6.5 indicated that residues 8, 9 and 10 were aspartic acid while the residue in position 11 was glutamic acid.

TABLE 12

Peptides resulting from chymotrypsin digest of soybean (Glycine max) Protease inhibitor PI IV

Peptide No. and Position in Sequence	Purification of Peptide			Dansyl-Edman Results
	Peptides No. in Gel-Filtration ^a P4	Mobility of Peptides on High Voltage Paper Electrophoresis PH6.5 PH1.9	N.F.	
C1 (1-7)	N.F.	N.F.	N.F.	(SER, ASX, GLX, SER, SER, SER, TYR)
C2 (8-12)	5	-2.84	-	<u>ASP-ASP-ASP-GLU-TYR</u>
C3 (13-21)	3	-2.13	0.52	<u>SER-LYS-PRO-CYS-CYS-ASP-LEU-</u> <u>CYS-MET</u>
C4 22-24)	4	0	-	<u>CYS-THR-ARG</u>
C5 (25-38)	2	-0.85	0.47	<u>SER-MET-PRO-PRO-GLX-CYS-SER-</u> <u>CYS-GLX-ASX-ILE-ARG-LEU-ASN</u>
C6 39-48)	2	-1.25	0.82	<u>SER-CYS-HIS-SER-ASP-CYS-LYS-</u> <u>SER-CYS-MET</u>
C7 (49-60)	1	-0.65	-	<u>CYS-THR-ARG-SER-GLN-PRO-GLY</u> <u>GLN-CYS-ARG-CYS-LEU</u>
C8 (61-67)	5	-2.05	-	<u>ASX-THR-ASX-ASX-PHE-CYS-TYR</u>
C9 (68-75)	1	0	-	<u>LYS-PRO-CYS-LYS-SER-ARG-ASP-</u> <u>ASP</u>

Peptide C3 (13-21) SER-LYS-PRO-CYS-CYS-ASP-LEU-CYS-MET

The electrophoretic mobility of this peptide indicated that residue 18 was aspartic acid.

Peptide C4 (22-24) CYS-THR-ARG

This peptide resulted from an an anomalous cleavage with chymotrypsin.

Peptide C5 (25-38) SER-MET-PRO-PRO-GLX-CYS-SER-CYS-GLX-ASX-ILE-ARG-LEU-ASN

The electrophoretic mobility of this peptide indicated the presence of several acidic residues. The specificity of cleavage by chymotrypsin indicated that residue 38 was probably asparagine.

Peptide C6 (39-48) SER-CYS-HIS-SER-ASP-CYS-LYS-SER-CYS-MET

The electrophoretic mobility of this peptide at pH 6.5 indicated that residue 43 was aspartic acid.

Peptide C7 (49-60) CYS-THR-ARG-SER-GLN-PRO-GLY-GLN-CYS-ARG-CYS-LEU

The electrophoretic mobility of this peptide at pH 6.5 indicated that residues 53 and 56 were both glutamine.

Peptide C8 (61-67) ASX-THR-ASX-ASX-PHE-CYS-TYR

The electrophoretic mobility of this peptide at pH 6.5 indicated that one of the residues in the positions 61, 63 and 64 was asparagine, while the other two were aspartic acid.

Peptide C9 (68-75) LYS-PRO-CYS-LYS-SER-ARG-ASP-ASP

The electrophoretic mobility of this peptide at pH 6.5 indicated that residues 74 and 75 were aspartic acid.

It can be seen from the foregoing text that the residues in positions 8, 9, 10, 11, 18, 38, 43, 53, 56, 74 and 75 can be identified as the acid or amide form from the electrophoretic

mobilities of their peptides at pH 6.5. The acid/amide residues in positions 2, 3, 39, 33, 34, 61, 63 and 64 which could not be assigned by this means were tentatively identified by homology with the amino acid sequence of the DII inhibitor reported by Odani and Ikenaka (1976), CIII inhibitor reported by Odani and Ikenaka (1977) and Bowman-Birk inhibitor reported by Odani et al. (1971) (see figure 18).

The overlapping of chymotryptic and tryptic peptides as well as the homology with the DII protease inhibitor of Odani and Ikenaka (1976) gave the complete sequence of soybean (Glycine max) protease inhibitor PI IV (Fig. 17).

V. GENERAL DISCUSSION ON MANUAL SEQUENCING

The dansyl-Edman method for sequencing peptides is a very sensitive and reliable method. It has been used in a microform to determine the sequence of as little as 10 p mol of peptide (Bruton and Hartley 1970). When the dansyl derivatives are separated by polyamide thin layer chromatography (Woods and Wang 1967), it also has the advantage of giving an excellent resolution not only of all the normally occurring amino acid derivatives, especially leucine and isoleucine, but also of unusual amino acids such as E-N-trimethyllysine. A good separation of basic amino acids can also be achieved. The main difficulties of this and other 'end group' techniques, are those of identifying residues whose derivatives are labile during acid hydrolysis of the dansylated peptide. Thus, asparagine and glutamine are deamidated to the corresponding acids. If tryptophan has not been previously

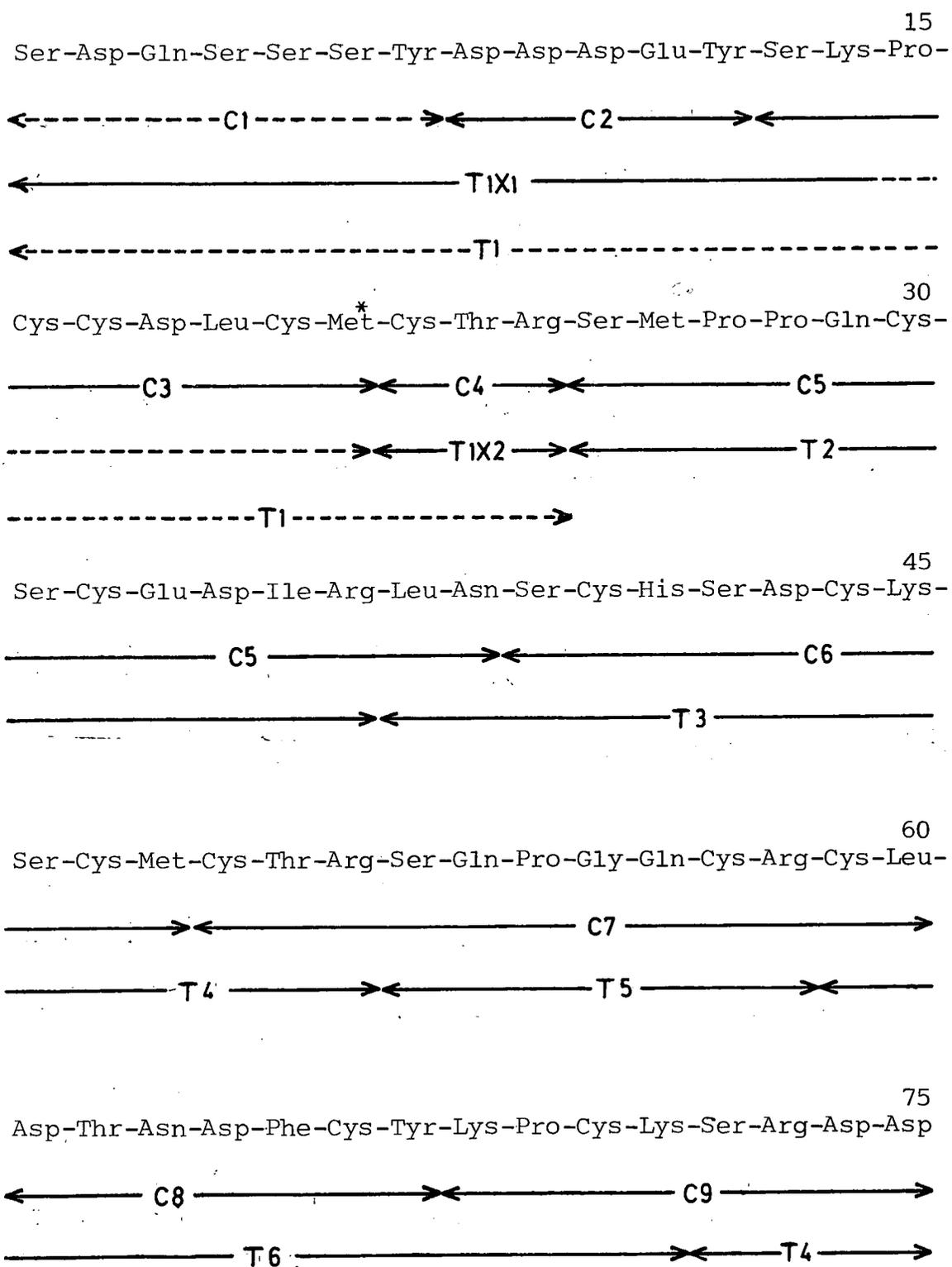
FIGURE 17

The complete amino acid sequence of soybean (Glycine max) protease inhibitor PI IV.

(x) represents the CNBr fragment. (\longleftrightarrow) indicates peptides sequenced by the manual dansyl-Edman analysis, ($\leftarrow\rightarrow$) indicates peptides not sequenced, (*) indicates homoserine was identified as the derivative from CNBr cleavage.

T and C indicates peptides obtained by tryptic and chymotryptic digestion of reduced and S- carboxymethylated protease inhibitor PI IV.

Fig. 17 The Complete Amino Acid Sequence of Soybean (Glycine
max) Protease Inhibitor PI IV



destroyed during the Edman degradation procedure its dansyl derivative is usually totally destroyed, although in some cases dansyl-tryptophan has been identified after 4-8h. hydrolysis as a spot on the polyamide sheet just below the dansyl bis-lysine. It has recently been suggested by Inglis et al. (1979) that it is advisable to carry out an initial oxidation of the tryptophan residues to kynurenine with sodium periodate as this facilitates the identification of the tryptophan when using the dansyl-Edman procedure.

After several steps of Edman degradation the internal lysine residues of peptides react their E-amino function with PITC to form E-PTC-lysine. The dansyl derivatives α -dansyl-E-PTC-lysine is rather unstable to acid hydrolysis and also tends to chromatograph in the region of dansyl-leucine and dansyl-phenylalanine, though with care these three amino acids can be resolved. Also bis-dansyl-histidine was degraded to α -N-dansyl-histidine which was sometimes difficult to distinguish from dansyl-arginine and E-dansyllysine. Dansyl-proline is degraded on prolonged acid hydrolysis so that when a proline residue was expected at the N-terminus of a 'dansylated' peptide, hydrolysis was only carried out for 6 h.

The dansyl method can only be qualitative when used in conjunction with Edman degradation, but dansylation of a peptide hydrolysate provided a semi-quantitative estimate of the amino acid composition of the peptide by an assessment of the relative intensities of the fluorescent spots under U.V. light following chromatography.

Dansylation without hydrolysis was capable of identifying free amino acids as impurities, the free C-terminal amino acid of

a fully degraded peptide and amino acids released from a peptide by digestion with carboxypeptidase A.

Theoretically, sequence determination using the Edman degradation method should be capable of elucidating the sequence of the complete peptide or protein. However various undesirable side reactions tend to limit the number of cycles that can be performed. One of the main problems encountered is due to the presence of oxygen during the coupling stage of the Edman degradation (Ilse and Edman 1963). In the manual degradation this is carried out as far as possible in an inert atmosphere of nitrogen, but it is impossible to exclude oxygen altogether.

One of the difficulties encountered in using the dansyl-Edman method was the identification of the amide residues present. The electrophoretic mobility of the peptide at pH 6.5 was often sufficient to assign most of the amide residues as described by Offord (1966). This is an additional advantage of this separation technique. However, certain amide residues remained unplaced. If sufficient peptide is available the mobilities of degraded peptides at pH 6.5 could be used to identify those residues. Otherwise, as in the case of the PI IV sequenced in this investigation, they had to be tentatively assigned by homology with the protease inhibitor DII of Odani and Ikenaka (1976) in which the amide residues have been experimentally determined, or by the new DABITC method of Chang et al. (1978) as in the case of the α -amylase inhibitor CIII.

This recently reported sequencing method (Chang et al. 1978) for the micro-sequence analysis of peptides and proteins is achieved using a coloured (4 N,N-dimethylaminoazobenzene 4'-isothiocyanate, DABITC-reagent in combination with phenylisothiocyanate

(PITC). This (DABITC-PITC) double coupling method was found to be a fast, simple and inexpensive technique. One degradation cycle takes about 140 min. Acid hydrolysis is not required in this method and it needs no special instrumentation or radioactive materials. Besides these advantages it also provided an excellent identification for all the amino acid derivatives on small (25 x 25mm) polyamide sheets except for leucine and isoleucine, which could be identified on plates or sheets of silica gel. Using this method tryptophan and amide/acid residues could be identified with no difficulties. During this investigation it was discovered that CM-cysteine could be identified by three coloured spots after exposing the polyamide micro-sheet to HCl vapour (see Fig. 8). This has not been reported by Chang *et al.* (1978). The sensitivity of this method and its relative freedom from the problems of 'hang-over' which occur with some amino acids in the dansyl method has meant that it could be used successfully for up to 15-18 cycles without any problems. The only problem encountered in using this technique was the very small volumes of solutions employed in each cycle, hence great care was necessary in removing the organic phases (heptane/ethyl acetate 2:1 (v/v)) and butyl acetate from the aqueous phase containing the peptide after centrifugation. Also DABITC is not very stable in pyridine, therefore stock solutions of DABITC were kept in acetone and suitable volumes of this solution were pipetted into clean test tubes, dried under vacuum, and redissolved in pyridine shortly before use.

BIBLIOGRAPHY

- Abe, M., Arai, S., Kato, H. & Fujimaki, M. (1980)
Agric. Biol. Chem. 44, 685
- Applebaum, S.W. (1964)
J. Insect Physiol. 10, 897
- Applebaum, S.W., Harpaz, I., Bondi, A. & Birk, Y. (1964)
Comp. Biochem. Physiol. 13, 107
- Applebaum, S.W. & Konijn, A.M. (1965)
J. Nutr. 85, 275
- Autran, J.C., Lew, J.L., Nimmo, C.C. & Kasarda, D.D. (1979)
Nature 282, 525
- Bailey, C & Ramshaw, J. (1973) Biochem. J. 135, 889
- Bedetti, C., Bozzini, A., Silano, V. & Vittozzi, L. (1974)
Biochim. Biophys. Acta. 362, 299
- Beitz, J.A., H^{ie}ubner, F.R., Sanderson, J.E. & Wall, J.S. (1977)
Cereal Chem. 54, 1070
- Beitz, J.A., Senkpiel, A. & Barth, A. (1978)
Biochem. Physiol. pflanz. 172, 413
- Bernfeld, P. (1955)
Methods Enzymol. 1, 149
- Bessho, H. & Kurosawa, S. (1967)
Eiyo To Shokuryo 20, 317

- Billett, E.E., Wallace, W. & Smith, H. (1978)
Biochim. Biophys. Acta. 524, 219
- Birk, Y. (1976)
Methods Enzymol. 45, 695
- Blömbäck, B., Blömbäck, M., Edman, P. & Hessel, B. (1966)
Biochim. Biophys. Acta. 115, 37
- Bock, W., Dongowski, G., Goebel, H. & Krause, M. (1975)
Nahrung 19, 411
- Bowman, D.E. (1945)
Science 102, 358
- Bruton, C.J. & Hartley, B.S. (1970)
J. Molec. Biol. 521, 165
- Buonocore, V., Petrucci, T., Silano, V. (1977)
Phytochemistry 16, 811
- Buonocore, V., Poerio, E., Gramenzi, F. & Silano, V. (1975)
J. Chromatogr. 114, 109
- Buonocore, V., Poerio, E., Silano, V. & Tomasi, M. (1976)
Biochem. J. 153, 621
- Buonocore, V., Gramenzi, F., Pace, W., Petrucci, T. & Poerio, E.
& Silano, V. (1980)
Biochem. J. 187, 637

Cantagalli, P., Di Giorgio, G., Morisi, G., Pocchiari, F.
& Silano, V. (1971)

J. Sci. Food Agric. 22, 256

Chang, J.Y. (1977)

Biochem. J. 163, 517

Chang, J.Y., Brauer, D. & Wittmann-Liebold, B. (1978)

FEBS Lett. 93, 205

Chang, J.Y., Greaser, E.H., Bentley, K.W. (1976)

Biochem. J. 153, 607

Chibnall, A.C. (1939)

'Protein Metabolism in Plants' Yale University Press,
New Haven

Chrzaszcz, T. & Janicki, J. (1933)

Biochem. Z. 260, 354

Chrzaszcz, T. & Janicki, J. (1934)

Biochem. J. 28, 296

Connor, J.K., Horwood, I.S., Burton, H.W. & Fuelling, D.E. (1969)

Aust. J. Exp. Agric. Anim. Husb. 9, 497

Crestfield, A.M., Moore, S. & Stein, W.H. (1963)

J. Biol. Chem. 238, 622

Davis, B.J. (1964)

Ann. N.Y. Acad. Sci. 121, 404

Dechary, J.M. (1970)

Econ. Bot. 24, 113

Deponte, R., Parlamenti, R., Petrucci, T., Silano, V. & Tomasi, M.
(1976)

Cereal Chem. 53, 805

Dulloo, R.M. (1980)

Ind. J. Exp. Biol. 18, 295

Easley, C.W. (1965)

Biochim. Biophys. Acta. 107, 386

Edman, P. (1956)

Acta. Chem. Scand. 10, 761

Elliot, B.B. & Leopold, A.C. (1953)

Physiol. Plantarum 6, 65

Elmore, D.T. & Toseland, P.A. (1956)

J. Chem. Soc. 1956, 188

Ewart, J.A.D. (1969)

J. Sci. Food Agric. 20, 730

Ewart, J.A.D. (1972)

J. Sci. Food Agric. 23, 701

Feillet, P. & Nimmo, C.C. (1970)

Cereal Chem. 47, 447

Fisher, M.L., Anderson, A.J. & Albersheim, P. (1973)

Plant Physiol. 51, 489

Flatmark, T. (1966)

Acta. Chem. Scand. 2, 1484

Fossum, K. & Whitaker, J.R. (1974)

J. Nutr. 104, 930

Fritz, H. & Tschesche, H. (1971)

'Proceedings of the First International Research Conference
on Proteinase Inhibitors' De Gruyter, Berlin

Fritz, H., Tschesche, H., Green, L. & Truscheit, E. (1974)

'Proteinase Inhibitors' Bayer-Symp. V Springer-Verlag,
Berlin & N.Y.

Frommer, W., Junge, B., Muller, L., Schmidt, D. & Truscheit, E.

(1979)

Planta Medica (or Journal of Medicinical Plant Research) 35, 195

Frommer, W., Puls, W., Schafer, D. & Schmidt, D. (1972)

German Offen. 2064092

Geddes, W.F. (1946)

in 'Advance in Enzymology' (ed. by F.F. Nord)

Vol. 6, p. 415 Interscience, N.Y.

Goebel, H. & Bock, W. (1978)

Die Nahrung 22, 809

- Gómez, J.C., Mosquera, M.I., Martin, J.C. & Diez, M.J. (1979)
Grasas y Aceites 30, 11
- Granum, O.E. (1978)
J. Food Biochem. 2, 103
- Granum, P.E. (1979)
Food Chem. 4, 173
- Granum, P.E. & Whitaker, J.R. (1977)
J. Food Biochem. 1, 385
- Gray, W.R. (1967)
Methods Enzymol. 11, 139
- Gray, W.R. & Hartley, B.S. (1963a)
Biochem. J. 89, 59p
- Gray, W.R. & Hartley, B.S. (1963b)
Biochem. J. 89, 379
- Greenwood, C.T. & McGregor, A.W. (1965a)
J. Inst. Brew. 71, 415
- Greenwood, C.T. & McGregor, A.W. (1965b)
Die Starke 17, 219
- Gross, E. (1967)
Methods Enzymol. 11, 238
- Heilmann, J., Barollier, J. & Watzka, E. (1957)
Hoppe-Seyler's Z. Physiol. Chem. 309, 219
- Hemberg, T. (1967)
Acta. Chem. Scand. 21, 1665
- Hemberg, T. (1975)
Physiol. Plantarum 31, 11
- Hemberg, T. & Larsson, I. (1961)
Physiol. Plantarum 14, 861
- Hernandez, A. & Jaffé, W.G. (1968)
Acta. Cient. Venezolana 19, 183

Hopkins, R.H. & Bird, R. (1954)

Biochem. J. 56, 86

Houmard, J. & Drapeau, B.R. (1972)

Proc. Natl. Acad. Sci. USA 69, 356

Hwang, D.L., Lin, D.K., Yang, W.K. & Foard, D.E. (1977)

Biochim. Biophys. Acta. 495, 369

Ilse, D. & Edman, D.E. (1963)

Aust. J. Chem. 16, 411

Inglis, A.S., Rivett, D.E. & McMahon, D.T. (1979)

FEBS Lett. 104, 115

Jaffé, W.G. & Lette, C.L. (1968)

J. Nutr. 94, 203

Jaffé, W.G., Moreno, R. & Wallis, U. (1973)

Nutr. Rep. Int. 7, 169

Jolly, S.O. & Tolbert, N.E. (1978)

Plant Physiol. 62, 197

Kasarda, D., ^{Da Roza} , D. & Ohms, J. (1974)

Biochim. Biophys. Acta. 351, 290

Kassel, B. (1970)

Methods Enzymol. 19, 839

- Kim, B.M., Shimoda, T. & Tunatsu, M. (1977)
J. Fac. Agric. Kyushu Univ. 21, 1
- Kneen, E. & Sandstedt, R.M. (1943)
J. Am. Chem. Soc. 65, 1247
- Kneen, E. & Sandstedt, R.M. (1946)
Arch. Biochem. 9, 235
- Kruger, J.E. (1972)
Cereal Chem. 49, 391
- Kunitz, M. (1945)
Science 101, 668
- Kunitz, M. (1946)
J. Gen. Physiol. 29, 149
- Lang, J.A. & Chang-Hum, L.E., Reyess, P.S. & Briggs, M. (1974)
Fed. Proc. 33, 718
- Laskowski, M. & Sealock, R.W. (1971)
in 'The Enzymes' (ed. by P.D. Boyer, 3rd ed.) 3, 375 Acad. Press
N.Y.
- Lau, A., Ako, H. & Werner-Washburne, M. (1980)
Biochem. Biophys. Res. Commun. 92, 1243
- Liener, I.E. (1974)
J. Agric. Fd. Chem. 22, 17

Liener, I.E. & Kakade, M.L. (1969)

in 'Toxic Constituents in Plant Foodstuffs' (ed. by I.E. Liener)
pp. 8 Acad. Press N.Y.

Macri, A., Valfrè, F., Parlamenti, R. & Silano, V. (1977)

Poultry Sci. 56, 434

Majunder, A.L. & Biswas, B.B. (1973)

Phytochemistry 12, 321

Malhotra, O.P. & Rani, I. (1978)

Ind. J. Biochem. Biophys. 15, 229

Marshall, J.J. (1975)

Am. Chem. Soc. Symp. Series 16, p. 244

Marshall, J.J. (1977)

Carbohydrate Research 57, C27

Marshall, J.J. & Lauda, C.M. (1975a)

Die Starke 27, 274

Marshall, J.J. & Lauda, C.M. (1975b)

J. Biol. Chem. 250, 8030

Matsubara, H. (1966)

Biochem. Biophys. Res. Commun. 24, 427

Matsushita, K. & Uritani, I. (1976)

J. Biochem. 79, 633

- Mattoo, A.K. & Modi, V.V. (1970)
Enzymologia 39, 237
- Maxson, E.D. & Rooney, L.W. (1972)
Cer. Sci. Today 17, 260
- Maxson, E.D. & Rooney, L.W. Lewis, R.W., Clark, L.E. & Johnson, J.W.
(1973)
Nutr. Rep. Int. 8, 145
- Means, G., Ryan, C.A. & Feeney, R.E. (1974)
Acc. Chem. Res. 7, 315
- Militzer, W., Ikeda, C & Kneen, E. (1946a)
Arch. Biochem. 9, 309
- Militzer, W., Ikeda, C. & Kneen, E. (1946b)
Arch. Biochem. 9, 321
- Miller, B.S. & Kneen, E. (1947)
Arch. Biochem. 15, 251
- Moore, S. & Stein, W.H. (1963)
Methods Enzymol. 6, 819
- Mosolov, U.V., Longinova, M.D., Malova, E.L. & Benken, I.I. (1979)
Planta. 144, 265
- Murao, S. & Miyata, S. (1978)
Agric. Biol. Chem. 44, 219

Murao, S. & Ohyama, K. (1975)

Agric. Biol. Chem. 39, 2271

Murao, S., Ohyama, K. & Ogura, S. (1977)

Agric. Biol. Chem. 41, (6), 919

Nelson, N. (1944)

J. Biol. Chem. 153, 375

Niwa, T., Inouye, S., Tsuruoka, T., Koaze, T. & Niida, T. (1970)

Agric. Biol. Chem. 34, 966

Odani, S. & Ikenaka, T. (1976)

J. Biochem. 80, 641

Odani, S. & Ikenaka, T. (1977)

J. Biochem. 82, 1523

Odani, S., Koide, T. & Ikenaka, T. (1971)

Proc. Japan Acad. 47, 621

O'Donnell, M. & McGeeney, K.F. (1976)

Biochim. Biophys. Acta. 422, 152

Offord, R.E. (1966)

Nature 211, 591

Ornstein, L. (1964)

Ann. N.Y. Acad. Sci. 121, 321

Ozols, J. & Gerhard, C. (1977) J. Biol. Chem. 252, 5986

- Pace, W., Parlamenti, R., Urrab, A., Silano, V. & Vittozzi, L. (1978)
Cereal Chem. 55, 244
- Peeters, H. (1975)
in 'Protides of the Biological Fluid' Pergamon, Oxford p. 27
- Petrucci, T., Rab, A., Tomasi, M. & Silano, V. (1976)
Biochim. Biophys. Acta. 420, 288
- Petrucci, T., Sannia, G., Parlamenti, R. & Silano, V. (1978)
Biochem. J. 173, 229
- Petrucci, T., Tomasi, M., Cantagalli, P. & Silano, V. (1974)
Phytochemistry 13, 2487
- Pick, K.H. & Wóber, G. (1978)
Hoppe-Seylers Z. Physiol. Chem. 359, 1371
- Piszkiewicz, D., Landon, M. & Smith, E.L. (1970)
Biochem. Biophys. Res. Commun. 40, 1173
- Puls, W. & Keup, N. (1973)
Diabetologia 9, 97
- Pusztai, A. (1967)
Nutr. Abstr. Rev. 15, 59
- Ramshaw, J.A., Thompson, E.W. & Boulter, D. (1970)
Biochem. J. 119, 535

- Rao, M.N., Shurpolekar, K.S. & Sundaravalli, O.E. (1967)
Ind. J. Biochem. 4, 185
- Rao, M.N., Shurpolekar, K.S. & Sundaravalli, O.E. (1970)
Ind. J. Biochem. 7, 241
- Read, J.W. & Hass, L.W. (1938)
Cereal Chem. 15, 59
- Redman, D.G. (1975)
Biochem. J. 149, 725
- Redman, D.G. (1976)
Biochem. J. 155, 193
- Reich, E., Refkin, D.B. & Shaw, E. (1975)
in 'Proteases and Biological Control' p. 367 Cold Spring Harbour
Lab., Cold Spr. N.Y.
- Richardson, M. (1977)
Phytochemistry 16, 159
- Royer, A. (1975)
in 'Les Proteines des Graines' (J. Miegé, ed) p. 159 Geneva
- Ryan, C.A. (1973)
Ann. Rev. Plant Physiol. 24, 173
- Ryan, C.A. (1979)
in 'Herbivores: Their Interaction with Metabolites' (ed. by
G.A. Rosenthal and D.H. Janzen) p. 599 Acad. Press

Sandstedt, R.M. & Beckord, O.C. (1946)

Cereal Chem. 23, 548

Saunders, R.M. (1975)

Cereal Food World 70, 282

Saunders, R.M. & Lang, J.A. (1973)

Phytochemistry 12, 1237

Savaiano, D.A., Powers, J.R., Castello, M.J., Whitaker, J.R.
& Clifford, A.J. (1977)

Nutr. Rep. Int. 15, 443

Schenkin, I., Levy, M., Franklin, E.C. & Frangione, B. (1977)

Arch. Biochem. Biophys. 182, 64

Shainkin, R. & Birk, Y. (1966)

Israel, J. Chem. 3, 96

Shainkin, R. & Birk, Y. (1970)

Biochim. Biophys. Acta. 221, 502

Silano, V., Pocchiari, F. & Kassarda, D. (1973)

Biochim. Biophys. Acta. 317, 139

Silano, V., Poerio, E. & Buonocore, V. (1977)

Molecular and Cellular Biochem. 18, 87

Silano, V., Furia, M., Gianfreda, A., Macri, A., Palescandolo, R., Rab, A., Scardi, V., Stella, E. & Valfré, F. (1975)

Biochim. Biophys. Acta. 391, 170

Silano, V. & Zahnley, J. (1978)

Biochim. Biophys. Acta. 533, 181

Smith, I. (1958)

Chromatographic and Electrophoretic Techniques 1, p. 7
London: Heinmann

Smith, D.G., Stein, W.H. & Moore, S. (1963)

J. Biol. Chem. 238, 227

Smyth, D.G. (1967)

in 'Methods in Enzymology' (Colowick, S.P. and Kaplan, N.O., eds.), 11, p. 214 Acad. Press N.Y.

Sodini, G., Silano, V., De Agazio, M., Pocchiari, F., Tentori, I. & Vivaldi, G. (1970)

Phytochemistry 9, 1167

Sorenson, J.C. & Scandalios, J.G. (1976)

Plant Physiol. 57, 351

Stankovic, S.C. & Markovic, N.D. (1963)

Glasnik Hem. Drustva 25-26 519 (Chem. Abstr. 59, 3084d)

Steers, E., Craven, G.R. & Anfinsen C.B. (1965)

J. Biol. Chem. 240, 2478

- Strumeyer, D.H. (1972)
Nutr. Rep. Int. 5, 45
- Strumeyer, D.H. & Fisher, B.R. (1973)
Fed. Proc. 32, 624
- Strumeyer, D.H. & Malin, M.J. (1969)
Biochim. Biophys. Acta. 184, 634
- Strumeyer, D.H., & Romano, A.T. (1966)
in 'Technicon International Symposium in Automation' in Anal.
Chem. p. 469 Technicon N.Y.
- Surendranathan, K.K. & Nair, P.M. (1978)
Plant Sci. Lett. 12, 169
- Takemoto, D.J., Kresie, R & Vaughn, D. (1980)
Biochem. Biophys. Res. Commun. 94, 332
- Thompson, E.W. Laylock, M.W. Ramshaw, J.A. & Boulter, D. (1970)
Biochem. J. 117, 183
- Tschesche, H. (1974)
Angew. Chem. Int. Ed. 13, 10
- Ueda, S. & Koba, Y. (1973)
Agric. Biol. Chem. 37, 2025
- Umezawa, H. (1976)
in 'Methods in Enzymology' (Lorand ed.) 45, 678 Acad Press N.Y.

Vanlelyveld, L.J. & Bester, A.I. (1978)

Phytopathol. Z 93, 69

Vesely, D.L., Graves, W.R., Lo., T.M., Fletcher, M.A. & Levey, G.S.
(1977)

Biochem. Biophys. Res. Commun. 77, 1295

Vittozzi, L. & Silano, V. (1976)

Theoretical and Applied Genetics 48, 279

Vittozzi, L., Morisi, G. & Silano, V. (1976)

J. Sci., Food Agric. 27, 449

Vogel, R., Trautschold, I. & Werle, E. (1968)

'Natural Proteinase Inhibitors' Acad. Press N.Y.

Warchalewski, J.R. (1975)

13th Ann. Meeting of the Polish Biochemical Soc., Torun,
Poland, Abstr. p. 124

Warchalewski, J.R. (1976)

Bull. Acad. Polon. Sci. Ser. Sci. Biol. 24, 559

Warchalewski, J.R. (1977)

Bull. Acad. Polon. Sci. Ser. Sci. Biol. 25, 725

Weinland, E. (1903)

Z. Biol. 44, 1

Werle, E. & Zickgraf-Rudel, G. (1972)

Z. Klin. Chem. Klin. Biochem. 10, 139

Whitaker, J.R. & Feeney, R.E. (1973)

in 'Toxicants Occuring Naturally in Food' (Ed. by Ryan C.A.)
p. 276 Nat. Acad. Sci. Washington D.C.

Wierzbowski, J.G. & Skupin, A. (1965)

'Final Technical Rep., F G-Po-109-60' Project No. UR-E 21-(10)-1,
Poznan p. 36

Wilding, P. (1963)

Clin. Chim. Acta. 8, 918

Woods, K.R. & Wang, K.T. (1967)

Biochim. Biophys. Acta. 133, 369

Yetter, M.A., Saunders, R.M. & Boles, H.P. (1979)

Cereal, Chem. 56, 243



