Studies on a-amylase inhibitors from seeds of sorghum bicolor

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STUDIES ON α-AMYLASE INHIBITORS FROM SEEDS OF *Sorghum bicolor*

A thesis presented by

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ABSTRACT for Degree of Ph.D 1991 **CARLOS BLOCH JUNIOR**

Studies on α-Amylase Inhibitors from Seeds of *Sorghum bicolor*

Six inhibitors (SIα1, SIα2, SIα3, SIα4, SIα5 and SIα6) of α-amylase from mammalian, insect, bacterial and fungal sources were purified from seeds of *Sorghum bicolor* (L) Moench by saline extraction, precipitation with ammonium sulphate, affinity chromatography on Red Sepharose, preparative and analytical reverse phase HPLC on Vydac C_{18} columns. The complete primary structures of five of these inhibitors (SIα1-5) were determined by automatic degradation of the intact, reduced and S-alkylated proteins and by manual DABITC/PITC microsequencing of peptides obtained from enzyme digests.

The first three inhibitors consist of 47 (SIα1) and 48 (SIα2, SIα3) amino acids with respective molecular weights of 5,396, 5,310, and 5384. These basic proteins (pI predictions above 8) were found to be highly homologous between themselves and with the recently isolated γ-hordothionin, γ_1-and γ_2-purothionins (Colilla et al., 1990; Mendez et al., 1990) and are, therefore, considered to be thionin-like inhibitors. Four disulphide bonds were identified and their positions determined in the sequence of SIα1. It has been reported that the α-amylase inhibitory activity of thionins is due to competition for calcium ions which is the most important co-factor for this enzyme activity (Matsuura et al., 1984; Buisson et al., 1987). Calcium binding motifs have been located in the sequences of SIα1, SIα2 and SIα3 and their structural significance has been investigated by molecular modelling.

SIα4 and SIα5 which consist of 118 (MW 12,485) and 116 (MW 12,761) amino acids respectively are also basic polypeptides (pI predictions above 8). These proteins were found to be 35% homologous between themselves and showed significant homology (range 21-42%) with the members of the cereal superfamily. Hydrophobicity plots and secondary structure prediction results also revealed common features between these proteins and those of the cereal superfamily.

Only a preliminary N-terminal sequence was obtained for SIα6 which was found to inhibit human salivary α-amylase and locust gut α-amylase.

This thesis is entirely the result of my work. It has not been submitted for a degree in this or any other University, and it is not being submitted for any other degree.

To my best friend my wife,
and to our parents

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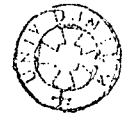
ABBREVIATIONS

BSA	bovine serum albumin
C-terminal	carboxy terminal
DABITC	4-N,N-dimethylaminoazobenze-4'-isothiocyanate
DTT	dithiothreitol
g	grammes or force of gravity
HPLC	high performance liquid chromatography
L	liters
(L/I)	leucine or isoleucine residue
mA	milliampere
NMR	nuclear magnetic resonance
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
PITC	phenylisothiocyanate
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N',tetramethylene diamine
TFA	trifluoracetic acid
Tris	2-amino 2-hydroxymethyl propane-1,3-diol
U.V.	ultraviolet

Other abbreviations used in this thesis are as recommended in "Instructions to Authors", *Biochem. J.* (1975) **131**: 1-20.

INTRODUCTION



1.1 General Introduction

It is known that many plants, animals and microorganisms are able to produce several substances which act as natural inhibitors for a variety of enzymes such as: trypsin, chymotrypsin, subtilisin, papain, bromelain, α-amylases, transferases and many others. These inhibitors include a wide range of molecules such as drugs, polyanions, low-molecular weight compounds and macromolecules.

Studies at the beginning of the 1930's (Chrazaszcz and Janicki, 1933), confirmed later by Kneen and Sandstedt (1943, 1946), demonstrated the existence of plant protein inhibitors of α-amylase (1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) in extracts of various cereals. Simultaneously, Kunitz (1945) isolated and purified a heat-labile protein from soybean which inhibited trypsin.

A consequence of the work of Kunitz, Kneen and Sandstedt was that attention towards plant protein inhibitors of enzymes increased dramatically. The potential therapeutic, biochemical, nutritional and agricultural values of these molecules have stimulated the interest of many research groups and led to a substantial number of publications on this subject resulting in a great deal more work and many publications on these inhibitors (for general reviews see: Ryan, 1973, 1979,1981; Ryan and Walker-Simons, 1981; Birk, 1976; Richardson, 1977, 1981a, 1981b, 1991; Liener and Kakade, 1980; Laskowski and Kato, 1980; Xavier-Filho and Campos, 1984, 1987; Xavier-Filho and Ventura, 1988; Whitaker, 1981, 1983; Gatehouse, 1984; Warchalewski, 1983; Silano, 1986; Buonocore and Silano, 1986; Rackis *et al.*, 1986; Weder, 1986; Garcia-Olmedo *et al.*, 1987). Due to their molecular similarities and other common features, these inhibitors are currently grouped in at least ten different families (Table 1).

α-Amylase inhibitors may be found in a variety of species, including microorganisms, and the fruits, tubers and seeds of many plants. Amongst the richest sources of these inhibitors are the grains of cereals. In fact, α-amylase inhibitors have

been purified from seeds of wheat (*Triticum*), barley (*Hordeum*), ryes (*Secale*), corn (*Zea mays*), coix (*Coix lacryma-jobi*), oats (*Avena sativa*) and various millets (*Panicium*, *Pennisetum*, *Eleusine*, etc). Up to now the majority of the α-amylase inhibitors from plant origin have been placed in four well defined families, and among them the Cereal superfamily (Table 1) is the one where most examples of α-amylase inhibitors are found (Fig.1). This Cereal superfamily includes a wide range of other inhibitory and non-inhibitory proteins (Shewry et al., 1984) whose structural relationship, previously unsuspected, have only recently been fully demonstrated. Studies on the structure of numerous enzyme inhibitors found in cereals and many other sources show that this family is distributed beyond the plant kingdom (Odani et al , 1983b; Shewry and Tatham, 1990).

Apart from the activity of the inhibitors against their target α-amylases it has been suggested (Richardson, 1991) that these proteins may have, in addition, other important roles in the seed. Several recent observations (Richardson et al., 1987; Singh et al., 1987, 1989; Osborn et al., 1988; Ary et al., 1989; Moreno and Chrispeels, 1989) have encouraged the idea that these inhibitors and proteins showing structural identities with them might be part of a general defence mechanism against tissue damage, environmental stress, invasions by pathogens, and/or consumption by predators, as well as probably acting as seed storage proteins (Richardson, 1991).

The present thesis deals with work done on the purification, characterization and complete amino acid sequences of five proteins from seeds of *Sorghum bicolor* which have inhibitory activity against the α-amylases from human saliva and the guts of adult cockroaches and *Spodoptera litoralis* and locust larvae.

TABLE 1: Enzyme inhibitor families

Family	Mr (kDa)	Enzymes inhibited	Distribution
1.Bowman-Birk	8-9	Trypsin* Chymotrypsin Elastase	Leguminosae Gramineae
2.Kunitz	21-22	Trypsin Chymotrypsin Subtilisin Kallikrein Amylase	Leguminosae Gramineae Araceae Alsmataceae
3.Potato I	8-9	Chymotrypsin Trypsin Subtilisin	Solanaceae Gramineae Leguminosae Polygonaceae Cucurbitaceae *Hirudo medicinalis*
4.Potato II	6	Trypsin Chymotrypsin	Solanaceae
5.Cucurbit	3	Trypsin Hageman factor	Cucurbitaceae
6.Cereal Superfamily	12-13	Amylase** Trypsin Hageman factor	Gramineae (CM, 2S storage proteins in Cruciferae Euphorbiaceae Lecythidaceae Leguminosae)
7.Ragi AI2/ barley, rice PAPI	9	Amylase**	Gramineae
8.Thaumatin/ PR like	22-23	Amylase** Trypsin	Gramineae Solanaceae
9.Carboxy-peptidase	4	Carboxy-peptidase	Solanaceae
10.Cystatin-like	12	Cysteine-proteinases	Gramineae animals

*Double-headed; ** Bifunctional (Based on Richardson, 1991).

Fig.1: Cereal superfamily, sequence homologies.

(1) Barley trypsin inhibitor (Odani et al., 1983a); (2) bifunctional trypsin/α-amylase inhibitor from ragi *Eleusine coracana* (Campos & Richardson, 1983); (3) maize trypsin inhibitor (Mahoney et al., 1984); (4) rye trypsin inhibitor (Lyons et al., 1987); (5) amino acid sequence deduced from barley cDNA clone pUP-13 (Paz-Ares et al., 1986); (6) wheat α-amylase inhibitor 0.19 (Maeda et al., 1983a); (7) wheat α-amylase inhibitor 0.28 (Kashlan & Richardson, 1981); (8) wheat α-amylase inhibitor 0.53 (Maeda et al., 1985a); (putative α-amylase inhibitor; sequence deduced from barley cDNA clone pUP-44 (Lazaro et al., 1988a); (10) rye α-amylase inhibitor (Lyons et al., 1987); (11), (12), (13) and (14) the chloroform-methanol soluble (CM) proteins from barley (Barber et al., 1986a); (15), (16), (17) and (18) CM proteins from wheat (Barber et al., (1986b). Note that the sequences (10) to (18) are all incomplete. The arrow indicates the location of the reactive sites in the trypsin inhibitors. (Taken from Richardson, 1991)

1.2 Distribution of α-Amylase Inhibitors

Plant inhibitors of exogenous and endogenous α-amylases have been described in a number of species. Most of the proteins or glycoproteins which show α-amylase inhibitory activity have been detected in cereals and legumes. In many instances, more than one inhibitor has been identified within the same plant tissue.

1.2.1-CEREALS

Cereals have been a major source of proteinaceous α-amylase inhibitors from plants. Although the available information for some of them is not as complete as that for those of wheat and barley, steady progress has been made towards a better understanding of them.

Wheat *(Triticum)*

In wheat, the protein inhibitors have been extracted from seeds of a number of *Triticum* and genetically related *Aegilops* species (Bedetti et al., 1974; Vittozzi and Silano, 1976; Warchalewski, 1976; Konarev, 1982). Studies by Kneen and Sandstedt (1943, 1946), and Militzer et al. (1946a, 1946b) established the protein nature of α-amylase inhibitors from wheat flour. It was observed by Kneen and Sandstedt (1943) that most of the inhibitors were located in the endosperm. This observation has been confirmed by microscopic studies (Sandstedt and Beckord, 1946) and by the assay of the inhibitor content in different milling fractions (Saunders, 1975). The linear relationship observed between the inhibitor and starch contents of different wheat milling fractions indicates that the inhibitors are closely associated with starch and are probably endospermic in nature. The production of wheat inhibitors during kernel development was followed qualitatively by Sandstedt and Beckord (1946) and

quantitatively by Pace et al. (1978). It was found that the synthesis of inhibitors started about 8 days after fertilization and rapidly increased with maturation up to a maximum reached at the full maturity. Pace et al. (1978), also observed that the content of inhibitors rapidly decreased during germination and that no detectable amount of it was found in roots and coleoptiles from germinated seeds.

It is now well established that wheat contains not one but a mixture of protein inhibitors of α-amylase. Shainkin and Birk (1970) isolated two protein inhibitors (Called AmI_1, and AmI_2) from an aqueous extract of wheat kernel by ammonium sulphate fractionation and ion exchange chromatography. AmI_1 and AmI_2 were electrophoretically homogeneous and differed in their specificities towards α-amylases from different origins, and in their molecular weights and amino acid compositions.

In addition to AmI_1, and AmI_2, four other inhibitors (Sodini et al., 1970; Saunders and Lang, 1973; Silano et al., 1973; O'Donnel and McGeeney, 1976) were purified from similar crude wheat inhibitor preparations. It was found later that three of these inhibitors showed similar inhibition patterns, almost identical amino acid compositions and molecular weights all close to 25,000 (Buonocore et al., 1977).

The very close similarities observed among these three inhibitors were the first indications that the wheat α-amylase fractions contained several related forms of protein capable of inhibiting mammalian, insect and other α-amylases. This possibility was confirmed by Deponte et al. (1976) when three albumin fractions with apparent molecular weights of 60,000, 24,000 and 12,000 were obtained after gel filtration chromatography of a wheat protein extract, preciptated with ammonium sulphate.

The work of Cantagalli et al.(1971) and Silano et al.(1973), showed that the albumin fraction of 12,000 molecular weight (Deponte et al., 1976) consisted of five closely related components with gel electrophoretic mobilities around 0.28. It was then suggested that these albumins compose a wheat α-amylase inhibitor family: the 0.28 family (Cantagalli et al., 1971). This suggestion was strongly supported by Redman's

work (1975, 1976) who first showed a close homology between the partially sequenced N-terminals and peptides of some components of this family. Finally, this hypothesis was confirmed when the complete primary structures of three of these inhibitors were determined (Kashlan and Richardson, 1981; Maeda et al., 1985a).

Polyacrylamide gel electrophoresis of the albumin fraction of 24,000 molecular weight showed a highly heterogenous fraction with at least ten components, all of which were active in inhibiting human salivary and *Tenebrio molitor* L. α-amylases. Further studies on this fraction showed reversible dissociation of the protein in the presence of guanidine hydrochloride or SDS into two subunits with molecular weights close to 13,000 (Deponte et al., 1976).

Due to these features, Deponte et al. (1976) concluded that most of the components of the albumin fraction with molecular weights of 24,000 constitute a second family of related isoinhibitors. This family was designated the 0.19 wheat α-amylase inhibitor family because of the gel electrophoretic mobility of its main component. Later Maeda et al. (1983a) determed the complete sequence of a 0.19 wheat inhibitor active against human salivary, avian and insect α-amylases. Purified inhibitors from the 12- and 24-kDa families have differing activities against mammalian and amylases but are particularly effective (*in vitro*) against amylases from granivorous insects such as *Sitophilus*, *Tenebrio*, *Tribolium*, and *Oryzaephilus* (Silano et al.,1975). Among these latter species, *Sitophilus* weevils have highly active midgut amylases (Baker and Woo, 1985; Baker, 1986). Baker (1987b, 1988) also identified and purified an α-amylase inhibitor from wheat (*Triticum aestivum*) called 0.31 which complexed with one of the rice weevil amylase isoenzymes in an apparent 2:1 molar ratio.

A third family was proposed, when preliminary investigations on the albumin fraction of 60,000 molecular weight (Petrucci et al., 1974), showed that as much as 80% of its protein component underwent reversible dissociation into subunits of 12,000

molecular weight (Deponte et al., 1976) suggesting a tetrameric organization of these subunits.

However, much more needs to be done to establish a clear pattern of these molecules and to clarify whether the α-amylase inhibitory activity observed belongs to the dissociable components or to the oligomeric protein itself.

Studies on different species of wheat also revealed a similar distribution of inhibitors within their particular families, i.e. *Triticum urartu* was found to have one inhibitor fraction with 24,000 molecular weight which inhibited both mammalian and insect amylases. In tetraploid *Triticum* species (*turgidum* and *timopheevi*) α-amylase inhibitory patterns were equal in complexity to those found in the diploid *Aegilops* and *Triticum* species (in Buonocore et al., 1977).

Wheat germ was found to contain at least one bifunctional proteinase K/insect α-amylase inhibitor called PKI-3 (Jany and Lederer, 1985). This inhibitor was shown to have a molecular weight of 21,000, to be active against insect and wheat amylases, and an amino acid composition similar to that of the barley subtilisin inhibitor (Hejgaard et al., 1983; Svendsen et al., 1986). The bifunctional proteinase K/α-amylase inhibitor from wheat germ was successfully crystallized alone and in its complex with proteinase K by Pal et al. (1986). The three-dimensional structure of PKI-3 (Zemke et al., 1991) was solved by molecular replacement of the x-ray data collected to 2.3Å resolution on the basis of the atomic coordinate of the homologous *Erythrina caffra* DE-3 inhibitor (Onesti et al., 1990). The structure of PKI-3 (Fig.2) is stabilized by two disulphide bridges and has a central β-barrel with distorted β-structure. The binding site for proteinase K was assumed to be located on the surface of the protein (Gly66-Ala67). However, the binding site for α-amylase could not be determined with the structural information available.

In comparison with the albumin α-amylase inhibitors, less work has been done on the non-albumin inhibitors from wheat. The first to describe the existence of such molecules were Strumeyer and Fisher (1973). They extracted from commercial wheat

gliadin a protein inhibitor active against several amylases including pig pancreatic amylase. This inhibitor was characterized as a 50,000 molecular weight protein. It showed two major bands on disc-electrophoresis corresponding in electrophoretic mobility to those of the α-gliadins and its amino acid composition showed an extremely high content of glutamine (30%) and proline, but a very low level of lysine (1%). The inhibitory behavior of this preparation was identical to that of some albumin inhibitors prevously discussed.

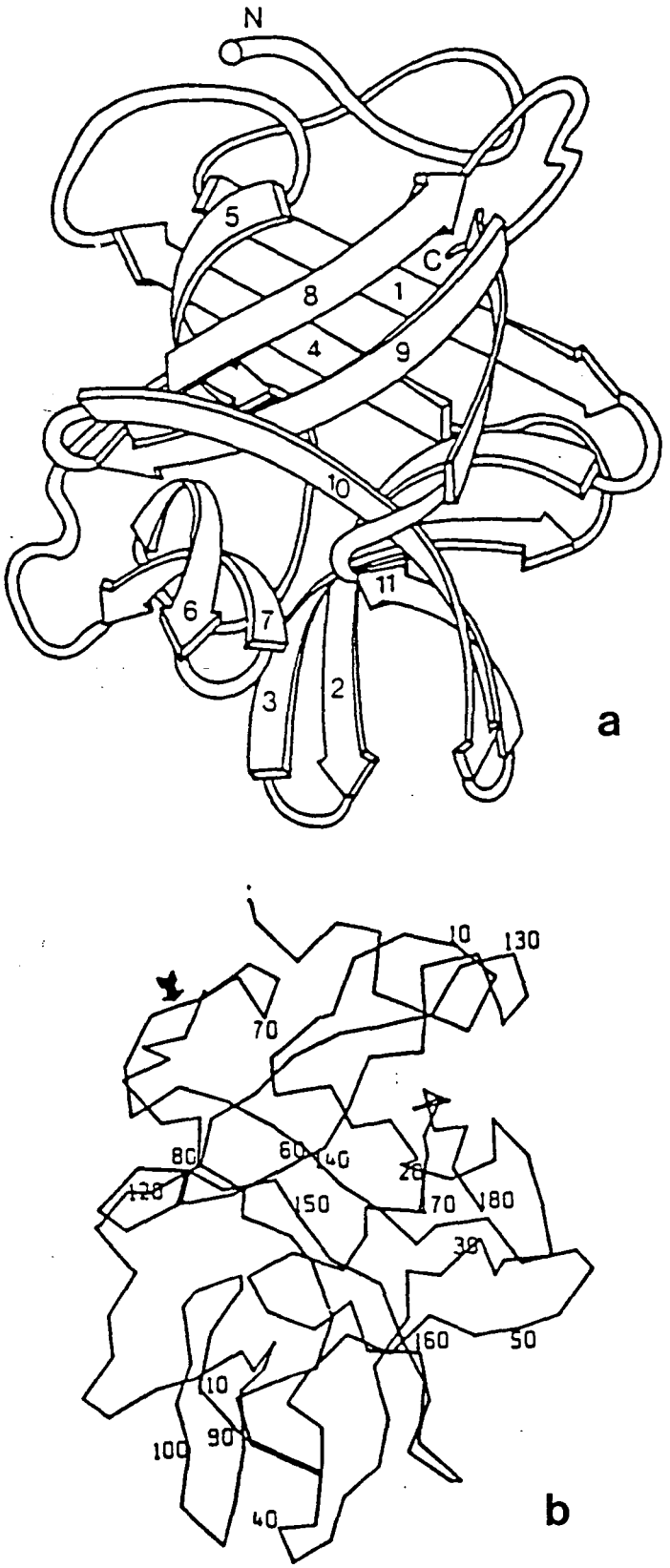


Fig 2: PKI3 three-dimensional structure representations.

(a) cartoon resentation; the β-strands are related by a three-fold pseudo-symmetry. Strands 1, 4, 5, 8, 9, and 12 form antiparallel β-barrel; (b) stereo plot of the Cα-backbone of PKI3; the binding site for proteinase K is assumed to be located in a loop; it is marked by an arrow. Disulphide bridges are indicated by thick lines. (Taken from Zemke et al., 1991)

In a short communication, Garcia-Olmedo and Carbonero (1970) described the purification of two components present in the chloroform-methanol (2:1, v/v) protein extract from endosperm of *T.aestivum* L. This new class of proteins, known as CM1 and CM2, were later demonstrated to be part of a highly heterogenous family called the CM proteins (Rodrigues-Loperena et al., 1975; Barber et al., 1986a). N-terminal sequences of the CM1, CM2, CM3, CM16 and CM17 proteins (Shewry et al., 1984; Barber et al., 1986a) showed considerable homology with members of the trypsin α-amylase inhibitor family of wheat (Cereal Superfamily). It has been recently demonstrated (Guitierrez et al., 1990) that some of the CM proteins which have been thought to be inactive against α-amylases are indeed inhibitors of various amylases.

Inhibitors of endogenous α-amylases were first observed by Warchalewski (1976) who studied multiple forms of inhibitors of native α-amylase from malted wheat. A total of eight inhibitors were purified and some of these were also active against *B.subtilis* and *A.oryzae* enzymes. Electrophoretic studies (Peruanskii and Gabsattarova, 1980) of the α-amylase inhibitor fraction of two varieties of wheat indicated that the component inhibitors had definite isoforms and that these isoforms expressed clear specificities towards individual α-amylase isoenzymes. The inhibitors were highly active against α-amylases from the same source but less active against amylases from other types of wheat, indicating a highly specific interaction between the α-amylase and its natural protein inhibitor.

In 1982, Konarev confirmed the presence in wheat kernels of multiple albumin inhibitors which were active against the endogenous wheat α-amylase as well as exogenous (animal) α-amylases. Konarev (1982) showed that the albumin inhibitors which were active against exogenous α-amylases could be differentiated from those active on endogenous amylases by their electrophorectic mobilities, pIs, and molecular weights. It was demonstrated that the molecular weight of the inhibitors active on

wheat amylase were 19,500 in comparison to the 12,800 - 15,000 molecular weight reported for those inhibitors which were active against animal α-amylases.

In 1984, Mundy et al. purified from *T.aestivum* a bifunctional endogenous α-amylase and subtilisin inhibitor, called WASI. This single polypeptide inhibitor had a molecular weight of 20,500, a pI of 7.2 and an amino acid composition very similar to the soybean trypsin inhibitors, members of the Kunitz trypsin inhibitor family. Later it was confirmed by Maeda (1986) that the 180 amino acid single chain inhibitor exhibited a high sequence homology to the Kunitz-type trypsin inhibitors in soybean (31%). More recently, Warchalewski (1987) has purified another bifunctional endogenous α-amylase and trypsin inhibitor from winter wheat (*Triticum aestivum* cv. Beta). This dimeric inhibitor coded A/T-WI has 24,000 molecular weight (12,000 for each subunit) and was considered to be "double headed" due to its activity towards both native α-amylase and porcine pancreatic trypsin.

Barley *(Hordeum)*

The first studies on an α-amylase inhibitor from barley were carried out independently by Weselake et al. (1983a, 1983b) and Mundy et al. (1983). Both investigations led to the purification of a bifunctional inhibitor, called BASI, which was active against the endogenous barley malt α-amylase and the microbial subtilisin. BASI was initially isolated from barley kernels as a complex of barley α-amylase isoenzyme II and a heat labile proteinaceous factor, previously reported as barley α-amylase isoenzyme III (Weselake et al.,1983b).

This proteinaceous factor was found to be a 19,865 molecular weight inhibitor with a pI between 7.2 - 7.3. Its physicochemical properties and amino acid sequence were very similar to WASI and other proteins in the soybean Kunitz trypsin inhibitor family (in Garcia-Olmedo et al., 1987, Richardson 1991). The inhibitor produced

maximum inhibition of 40% at a 1:1 molar ratio against barley α-amylase II but failed to inhibit α-amylase I.

The existence of α-amylase inhibitors corresponding to the three size families (60, 24 and 12 kDa) found in wheat has been demonstrated in barley by Sanchez-Monge et al. (1986b), who showed that some of the CM proteins isolated from barley endosperm (Barber et al., 1986a) were subunits (CMa, CMb and CMd) of the tetrametric insect α-amylase inhibitor.

The N-terminal sequences of five proteins (CMa-e) (Odani et al., 1983; Shewry et al., 1984; Lazaro et al., 1985; Barber et al., 1986a, Halford et al., 1988) showed high homology to those of the monomeric and dimeric inhibitors from wheat but only one of them, CMa, has been found to be active against α-amylase on its own (Barber et al., 1986a, 1986b; Sanchez-Monge et al., 1986b). Dissociation-reassociation experiments indicated that all binary mixtures of these monomers were active, including those between inactive subunits, and that the mixture of the three subunits had the highest specific activity (Sanchez-Monge et al., 1986b). The tetrameric form was active against the α-amylase from *T.molitor* but showed no effect against salivary α-amylase. Another tetrameric inhibitor with similar subunits has been reported in the wild barley *Hordeum chilense* by Sanchez-Monge et al. (1987).

A 10,000 molecular weight barley seed protein (Svensson et al., 1986) homologous with an α-amylase inhibitor from Indian finger millet (Campos and Richardson, 1984) was cloned and its primary structure was obtained by both cDNA (Mundy and Rogers, 1986) and amino acid sequence (Svensson et al., 1986). This polypeptide of 91 amino acid residues is approximately 50% homologous with the α-amylase inhibitor I-2 from ragi seeds and has distant homology with two Bowman-Birk type protease inhibitors. However, the barley protein did not inhibit any of the tested enzymes (Svensson et al., 1986). This protein was subsequently found to have strong sequence homology with phospholipid transfer proteins (Bernard and Sommerville, 1989) and indeed was shown

to be capable of transferring phosphatidyl choline in liposomes to potato mitochondria (Breu et al., 1989).

NMR and UV spectroscopies, revealed that the protein possesses tertiary structure and interacts with porcine pancreatic α-amylase. For these reasons this protein was called a probable amylase/protease inhibitor (PAPI) and belongs to the Ragi I-2 family of inhibitors (Table 1 and Fig.4).

Maize *(Zea mays)*

Three α-amylase inhibitors with PAGE mobilities of 0.33, 0.45 and 0.60 have been obtained from kernels of *Zea mays* H-28 hybrid (Blanco-Labra and Iturbe-Chinas, 1981). When the inhibitor mixture was submitted to PAGE in the presence of dissociating agents, only one protein band, ranging from 28,900 to 30,400 was observed. This inhibitor mixture was active not only against several insects and *B.subtilis* α-amylases, but also against the endogenous α-amylase from 5-day-germinated H-28 maize seeds.

A 22,000 molecular weight α-amylase inhibitor purified and sequenced by Richardson et al. (1987), showed a bifunctional inhibitory activity against mammalian trypsin and insect α-amylases. A surprising sequence homology (Fig. 3) of 52% identity with the intensely sweet protein thaumatin II from the fruits of *Thaumatococcus daniellii* and 57% identity with a so called pathogenesis related (PR) protein induced in tobacco plants by tobacco mosaic virus infection (Cornellisen et al., 1986; Edens et al., 1982) has suggested that these proteins of as yet unknown function might have an inhibitory effect on hydrolytic enzymes. The 22 KDa inhibitor from maize was also found to be homologous with drought- and NaCl- induced proteins in tomato and tobacco (Fig. 3). A 12,000 Molecular weight trypsin inhibitor from maize (Mahoney et al., 1984) was later shown to be also an inhibitor of insect α-amylases (Valdes-Rodriguez and Richardson, 1991, unpublished results).

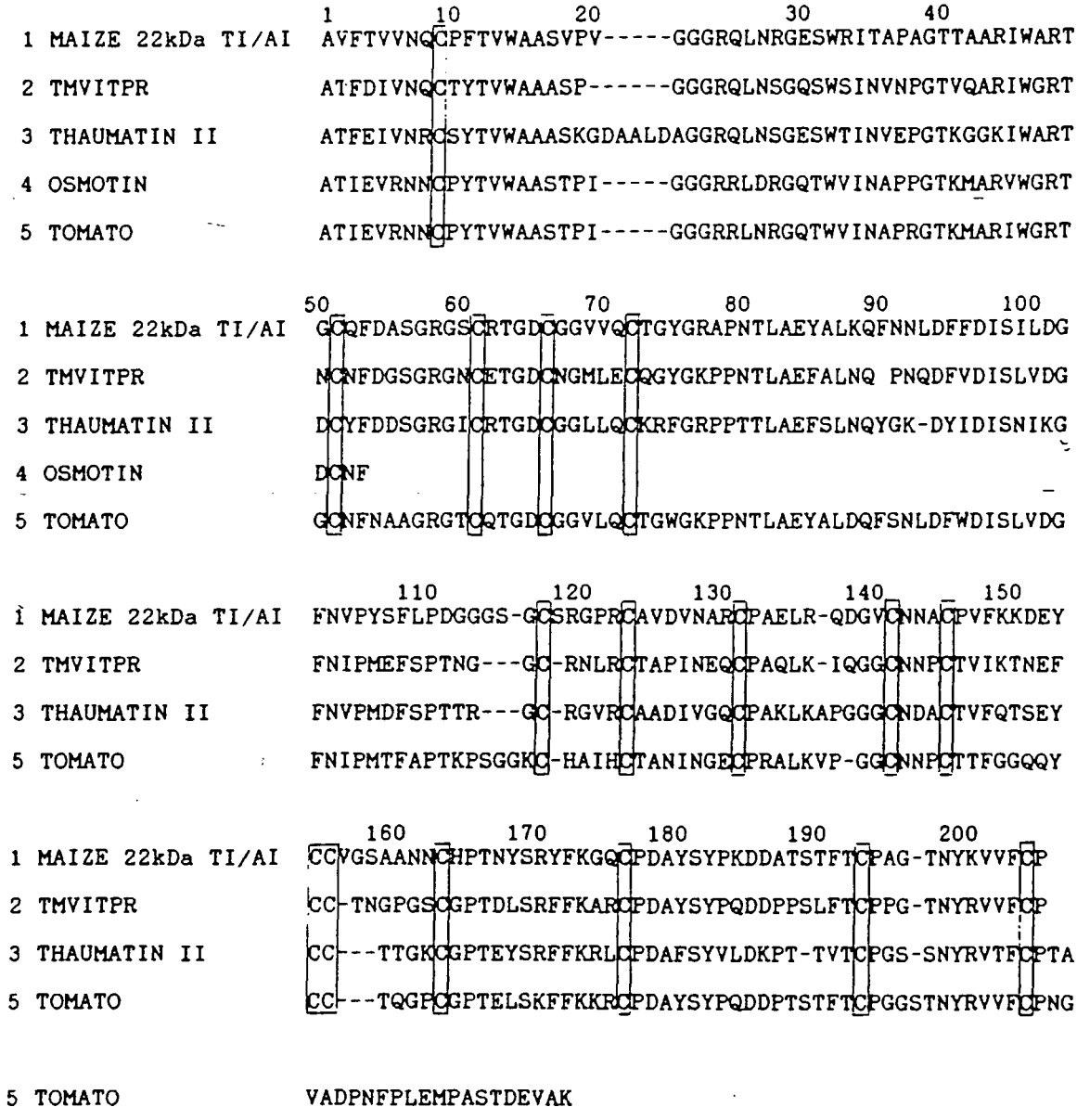


Fig 3: The homology of the maize 22kDa inhibitor/thaumatin family.

(1) The 22kDa bifunctional trypsin/α-amylase inhibitor from maize (Richardson et al., 1987); (2) pathogenesis-related (PR) protein induced in tobacco by infection with tobacco mosaic virus (Cornellisen et al., 1986); (3) thaumatin, the intensily sweet protein from berries of *Thaumatococcus danielli* (Edens et al., 1982); (4) N-terminal sequence of osmotin protein induced in cultured tobacco cells by osmotic stress (Singh et al., 1987); (5) protein NP24 induced in roots of tomato by exogenous NaCl; sequence deduced from cDNA clone pNP24 (King et al., 1988). (Taken from Richardson, 1991)

Ragi (*Eleusine coracana*)

A bifunctional α-amylase/trypsin inhibitor was purified and characterized from kernels of ragi (Indian finger millet) by Shivaraj and Pattabiraman (1980). The inhibitor was found to contain 122 amino acid residues with a molecular weight of about 14,000 and a pI above 10 (Campos and Richardson, 1983). This inhibitor was able to inactivate α-amylase and trypsin independently as well as to form a ternary complex with the two enzymes. The α-amylases of porcine pancreas, human pancreas and human saliva were inhibited in 5:5:1 ratio. The amino acid sequence of this bifunctional inhibitor clearly indicates that it belonged to the cereal superfamily. A probable isoform of this inhibitor was later isolated by Manjunath et al. (1983) who reported a 12,000 molecular weight bifunctional α-amylase/trypsin inhibitor called FMTI.

Another α-amylase inhibitor (Ragi I-2) was isolated and characterized from seeds of *Eleusine* by Shivaraj and Pattabiraman (1980, 1981). This inhibitor was found to be a basic protein with an apparent molecular weight determined by gel filtration of about 8,000. The amino acid sequence determination of the Ragi I-2 protein (Campos and Richardson, 1984) revealed a completely different structure from that of the bifunctional inhibitor from the same seed. This evidence and reports of similar proteins in other cereals indicate that the Ragi I-2 inhibitor probably belongs to a new and separate family (Richardson, 1991). (See Fig. 4)

Rice (*Oryza sativa*)

A probable amylase/protease inhibitor (PAPI) from rice seeds (*Oryza sativa* L.) was purified and sequenced by Yu et al. (1988). The 9,000 molecular weight basic protein shows high homology with the Ragi I-2 α-amylase inhibitor (Campos and Richardson, 1984) and with the so-called probable amylase/protease inhibitor from

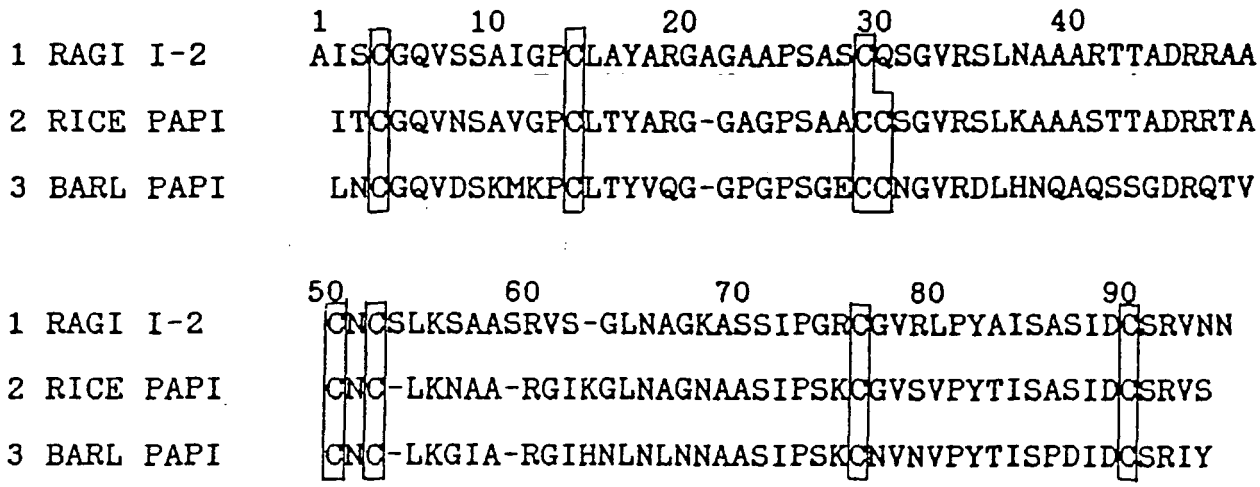


Fig. 4: The homology of the ragi I-2 inhibitor family.

(1) The α-amylase inhibitor I-2 from ragi *Eleusine coracana* (Campos and Richardson, 1984); (2) putative α-amylase proteinase inhibitor (PAPI) from rice (Yu et al., 1988); (3) putative α-amylase/proteinase inhibitor (PAPI) from barley (Mundy and Rogers, 1986; Svenson et al., 1986). (Taken from Richardson, 1991)

barley (Svensson et al., 1986), but like the barley protein the rice PAPI molecule also did not show inhibitory activity against any of the proteases and amylases tested.

Due to the high homology of the amino acid sequences of the three proteins (ragi I-2, barley PAPI and rice PAPI) they have been classified as a probable new family of inhibitors: the ragi I-2 inhibitor family (Fig. 4).

Rye (*Secale cereale*)

An α-amylase inhibitor from rye flour closely related to the 0.19 inhibitor family from wheat was purified and characterized by Granum (1981). The inhibitor had an approximate molecular weight of 28,000 by polyacrylamide gel electrophoresis and a pI of 5.8. Under denaturating conditions the molecular weight was determined as 14,000, indicating that the native molecule was composed of two subunits identical in size. The inhibitor was active towards *B. subtilis* and *A. oryzae* α-amylases.

The partial amino acid sequence of an α-amylase inhibitor from rye (Lyons et al., 1987) with similar characteristics to the protein isolated by Granum (1981) revealed a close relation to the barley α-amylase inhibitor CMa and to the wheat CM1 and CM2 proteins, and indicated that it belonged to the cereal superfamily.

Strumeyer (1972), isolated and purified amylase inhibitors from rye flour. Similarly to wheat, the gliadin-like inhibitors from rye showed potent activity against human salivary and porcine pancreatic amylases.

Oat (*Avena sativa*)

Elliot and Leopold (1953) studied a water-soluble, nondialyzable, heat-stable α-amylase inhibitor from oat (*Avena sativa*) seeds which was also active against β-amylase. This protein was found to inhibit plant germination but was inactive on catalase, peroxidase, ascorbic acid oxidase, and polyphenol oxidase systems. This

inhibitor was stable for 20 min. at 100°C but was partially inactivated after incubation with papain. The inhibition towards α- and β-amylase was found to be prevented by sulphydryl compounds like glutathione and 1,2-dithiopropanol.

Coix (*Coix lachryma-jobi*)

An inhibitor of locust gut α-amylase was purified from seeds of Job's Tears by Ary et al. (1989) using ammonium sulphate precipitation, affinity chromatography on Red Sepharose and reverse-phase HPLC. The inhibitor consists of two major isomers, each of which were reported to be a dimer of closely similar or identical subunits of about 26,400 molecular weight, and associated by interchain disulphide bonds. These isomers also had similar amino acid compositions. Assay of the major isomer with amylases from five different sources (human saliva, porcine pancreas, *B. subtilis*, *A. oryzae* and barley malt) showed no inhibitory activity.

The partial amino acid sequence of the major isoform revealed a high degree of homology with prevously reported sequences of endochitinase enzymes from several species (tobacco, potato, barley and bean) and endochitinase activity was demonstrated by following the release of radioctivity from tritium-labelled chitin.

This unique combination of enzymatic and inhibitor functions suggests a possible role of the protein as a concerted protection mechanism of the seed against insect feeding and fungal infection. However, up to now there is no report of any other endochitinase/α-amylase inhibitor. It implies that much more needs to be done to clarify the real significance of these novel findings.

Because of its unique characteristics, the Job's Tears bifunctional inhibitor cannot be assigned to an existing family of the inhibitors (Richardson, 1991).

Sorghum (*Sorghum bicolor*)

Miller and Kneen (1947) were the first to describe α-amylase inhibitory activity in Leoti sorghum. However, the nature of the inhibitor was later characterized by Strumeyer and Malin (1969) as condensed tannins.

In 1981, proteinaceous inhibitors of human salivary α-amylase were found in ten examined varieties of sorghum (Chandrasekher et al., 1981). The inhibitor fraction obtained showed no activity against bovine and porcine pancreatic α-amylase and appeared to be relatively thermolabile when compared with the inhibitors from other tested cereals.

Kutty and Pattabiraman (1986a, 1986b, 1986c, 1987) have demonstrated an effective method of purifying α-amylase inhibitors from sorghum by affinity chromatography using Red Sepharose CL-6B. Later, using the same technique, Kutty (1987), purified a 21,000 molecular weight inhibitor from *Sorghum bicolor* seeds which behaved as a 10,000 molecular weight protein during polycrylamide gel electrophoresis in the presence of denaturating agents. This inhibitor was reported to be active against both human pancreatic and salivary amylases in a linear range ratio of 1:1.25, respectively. It also showed weak activity against porcine pancreatic amylase but no inhibition of either *B. subtilis* or *A. oryzae* α-amylase was observed.

Two types of complex formation between the sorghum inhibitor and the human pancreatic amylase was reported (Kutty, 1987). The first type which appears to be formed due to loose interactions, dissociates under the amylase assay conditions, whereas the second type seems to be due to strong non-covalent interactions between the enzyme and inhibitor. The *Ki* value reported (calculated based on a molecular weight of 21,000) was 2.02×10^{-8}M in a non-competitive mode of inhibition.

Other Cereals

Chandrasekher et al. (1981) investigated the presence of α-amylase inhibitors in eight different species of millets including many varieties for each species. The inhibitor preparations were tested against four enzymes: human salivary and human, bovine and porcine pancreatic amylases. However, only setaria (*Setaria italica*), ragi (*Eleusine coracana*) and sorghum (*Sorghum bicolor*) were reported to be active against at least one of the enzymes.

A dimeric α-amylase inhibitor from *Setaria italica* grains was purified using affinity chromatography by Nagaraj and Pattabiraman (1985). The 24,000 molecular inhibitor produced two dissimilar polypeptides when run on SDS-PAGE. The setaria inhibitor was active on human salivary and pancreatic amylases as well as porcine pancreatic amylase, but had no action on *B. subtilis* and *A. oryzae* amylases.

Probably a similar α-amylase inhibitor from setaria grains was isolated by Tashiro and Maki (1986). Gel filtration chromatography performed with a crude inhibitor preparation revealed a 19,000 molecular weight inhibitor against porcine pancreatic and human salivary amylases, but it did not inhibit *B. subtilis* α-amylase.

Nagaraj and Pattabiraman (1985a) reported the purification of a 14000 molecular weight α-amylase inhibitor from proso grains (*Panicium miliaceum*) which was active against human salivary and pancreatic amylases.

α-Amylase inhibitors have also been detected in pearl millet (*Pennisetum typhodeum*) (Chandrasekher and Pattabiraman, 1983) and sanwa millet (*Echinocloa fruneutacea*) (Kutty, 1987). Sousa and Richardson (unpublished) have obtained N-terminal sequences of pearl millet α-amylase inhibitors homologous to the barley CM proteins (CMc and CMd). These 14,000 molecular weight inhibitors were previously isolated by Chandrasekher and Pattabiraman (1983) in a dimeric form. The

inhibitor fraction was found to be most effective on human salivary and pancreatic amylases but did not exhibit any action against *B. subtilis* and *A. oryzae*.

A number of varieties of *P. scorbiculatum* and *Echinocloa colona*, have been screened for α-amylase inhibitory activity (Chandrasekher et al., 1981), but so far no inhibitor has been found.

1.2.2 - LEGUMES

Bowman (1945) and Hernandez and Jaffe (1968) were the first to identify inhibitors of pancreatic amylase in aqueous extracts of beans. Later, Jaffe et al. (1973) described an α-amylase inhibitor in 79 legume cultivars. The most significant inhibitory activity was found in kidney beans, the other cultivars displayed moderate or very low inhibitory activity.

Beans *(Phaseolus)*

Several authors have purified α-amylase inhibitors from beans. These inhibitors differ from those purified from wheat, particularly in their higher carbohydrate contents and molecular weights.

An α-amylase inhibitor called phaseolamin was purified from white kidney bean (*Phaseolus vulgaris*) by Marshall and Lauda (1975a, 1975b). The inhibitor was found to be specific for animal α-amylases, having no activity towards the corresponding plant, bacterial, and fungal enzymes. The molecular weight of this inhibitor was assumed to be about 49,000 and it was also characterized as a glycoprotein containing between 9 and 10% of carbohydrate.

Powers and Whitaker (1977) purified an inhibitor from red kidney beans very similar to phaseolamin in its physicochemical properties and pattern of inhibition of α-amylases from different sources. Later, the structural features of this glycoprotein α-amylase inhibitor from red kidney bean were examined (Wilcox and Whitaker, 1984). It was found that the inhibitor contained 13.0% carbohydrate of which there were 25 mannose, 2 xylose, 1 fucose and 17 N-acetylglucosamine residues per mol. The glyco chains apparently play no role in the inhibition mechanisms of α-amylase, as when these intact chains alone were tested against porcine pancreatic amylase they revealed no activity even in excess molar ratio. Moreover, the glyco chains of the inhibitor have a structure which is very different from those of the known natural substrates of inhibitors of α-amylase (Wilcox and Whitaker, 1984).

Another glycoprotein containing approximately 15% carbohydrate with 43,000 molecular weight from *Phaseolus vulgaris* was found to have inhibitory activity against porcine pancreatic amylase (Pick and Wober, 1978). This inhibitor appears to consist of three or four identical subunits with a molecular weight of either 15,500 or 11,000 respectively.

An amylase inhibitor from black beans was characterized (Lajolo and Finardi Filho, 1985) as having an apparent molecular weight of 53,000, isoelectric point of 4.35, and a sedimentation coefficient of 4.45. It contains mannose, xylose, galactose (5.4%) and glucosamine (3%). The inhibitor is active against mammalian α-amylase and amyloglucosidase from *Rhizopus genus* and *Aspergillus niger*.

In cranberry bean, Kotaru et al. (1987a, 1987b) purified a 47,000 molecular weight glycoprotein inhibitor coded CBAI. The inhibitor contained 14% of carbohydrate and was effective towards several mammalian pancreatic α-amylases. CBAI exhibited a maximal inhibition at pH 5.5 against porcine pancreatic amylase in the 1:1 ratio.

Insect α-amylase inhibitors from kidney bean have been studied by Ishimoto and Kitamura (1988, 1989). A 17,000 molecular weight protein was found to affect larval

growth and α-amylase activity in three examined Coleoptera species, *Callosobruchus chinensis*, *C. maculatus* and *Zabrotes subfasciatus*.

A lectin-like α-amylase inhibitor was purified and sequenced by Moreno and Chrispells (1989). The 246 amino acid (cDNA deduced sequence) inhibitor was active against insect and mammalian α-amylase but not towards plant α-amylases. The lack of homology of this inhibitor with other families of enzyme inhibitors suggests that this may be the first member of a new family of plant inhibitors of α-amylase.

Chickpea (*Cicer arietinum* L.)

Singh et al. (1982) investigated amylase inhibitory activity against pancreatic and salivary amylases in protein extracts from two cultivars of chickpea (Desi and Kabuli). The extract showed higher inhibitory activity towards pancreatic amylase than salivary amylase. It was also observed that in the Desi cultivar the inhibitor activity was slightly higher than in the Kabuli cultivar.

Peanut (*Arachis hypogea*)

A 25,000 molecular weight α-amylase inhibitor has been purified from aqueous extract of peanut meal (Irshad and Sharma, 1981). This inhibitor was found to be active against human salivary and porcine pancreatic amylases, but did not show any inhibition of *B. subtilis* or wheat and barley α-amylases.

1.2.3 - TUBERS

Yam (*Dioscorea alata*)

A glycoprotein exhibiting α-amylase inhibitor activity was purified from tubers of *Dioscorea alata* by Krishna Sharma and Pattabiraman (1982b). The 70,000 molecular weight inhibitor containing 64% carbohydrate was found to be active against human salivary, human pancreatic, and porcine pancreatic amylases. The inhibitor had no action on *B. subtilis* or *A. oryzae* amylases.

Colocasia (*C. antiquorum*)

Two inhibitors (I-1 and I-2) active against mammalian α-amylase were purified from *Colocasia antiquorum* tubers (Krishna Sharma and Pattabiraman, 1980). The two basic proteins, I-1 and I-2 showed molecular weights of 14,300 and 12,500, respectively. I-1 was also found to be a glycoprotein containing 5.4% carbohydrate.

Tubers and Bulbs

Tubers and bulbs such as potato (*Solanum tuberosum*), sweet potato (*Ipomea batatas*), tapioca (*Manihot utilissima*), coleus (*Coleus paviflorus*), arrow root (*Maranta arundinacea*), alocasia (*Alocasia macrorhiza*), amorphophallus (*Amorphophallus companulatus*), yam (*Dioscorea esculenta*), ginger (*Zinigiber officinale*), onion (*Allium cepa*) and garlic (*Allium sativum*) were examined by Shivaraj et al. (1979) for proteinaceous α-amylase inhibitor, but so far, no inhibitory activity has been found in them.

1.2.4 - OTHER PLANT SOURCES

α-Amylase inhibitors have been found in mango fruit (*Mangifera indica*) and bananas (*Musa paradisiaca*) by Matoo and Modi (1970). Heat-labile α-amylase inhibitors from mango and banana were both reported to have a competitive type of inhibition towards their respective α-amylases.

Carob fruit (*Ceratonia siliqua*) and acorn (*Quercus pedunculata*) have been shown to contain α-amylase inhibitors (Corcoran, 1966; Stankovic and Markovic, 1960). In acorn β-amylase inhibitors were also detected.

1.2.5 - MICROORGANISMS

A number of microorganisms are known to produce compounds active against α-amylases. The *Actinomycetales* order, in particular, has proved to be a rich source of several pseudo-oligosaccharides or glycopeptides which can be regarded as substrate-like inhibitors (Ohyama and Murao, 1977; Murao and Ohyama, 1979).

However, protein or glycoprotein α-amylase inhibitors have been mostly reported in *the Streptomyces* genus and in *Cladosporium herbarum* (Murao et al., 1980a, 1980b; Goto et al., 1983; Ashauer et al., 1981, 1983; Vertesy and Tripier, 1985; Saito, 1982). Among those inhibitors, only the inhibitors from *S. tendae* 4158/Tendamistat-HOE 467 (Ashauer et al. 1981, 1983; Vertesy et al., 1984; Pflugrath et al., 1986; Kline et al., 1988; Braun et al., 1989; Billeter et al., 1989) and *S. griseosporeus* YM-25/ HAIM I-II (Murao et al., 1980a, 1980b, 1981; Goto et al., 1983, 1985; Murai et al., 1985; Yoshida et al., 1990) have been fully characterized and their three-dimensional structures determined. Their restricted specificity towards mammalian α-amylases suggests a common inhibitory pattern for these molecules.

S.tendae HOE 467 (Tendamistat)

Tendamistat is probably the best studied microbial α-amylase inhibitor. This polypeptide of 74 amino acid residues (Ashauer et al., 1981), binds porcine pancreatic α-amylase irreversibly to form a stoichiometric 1:1 complex. A characteristic of Tendamistat is its tight-binding, pH-independent kinetics of inhibition and the specific action on mammalian α-amylases. Studies on the mode of action revealed that the α-amylase inhibitory activity was linked to the intact disulphide bridges of the inhibitor (Vertesy et al., 1984).

The crystal and NMR structure determinations (Plugrath et al., 1986; Billeter et al., 1989) confirmed the existence of an exposed triplet Trp 18-Arg 19-Tyr 20 which may play an important role in the inhibitory action (Goto et al., 1985) (Figs.5 and 6).

S.griseosporeus YM-25 (HAIM)

The HAIM I and II inhibitors (Murao et al. 1980a, 1980b, 1981; Goto et al., 1983) are very similar to Tendamistat. They consist of 78 and 77 amino acid residues respectively (Fig. 5), including two disulphide bridges which are conserved relative to those of the Tendamistat (Murai et al., 1985; Yoshida et al., 1990).

HAIM II strongly inhibited α-amylases from vertabrates, and weakly inhibited those from Mollusca, Annelida, and Arthropoda. This inhibitor was reported to form a stoichiometric 1:1 complex with porcine pancreatic α-amylase after preincubation for 3 min at 37°C (Goto et al., 1985).

The three-dimensional structure of HAIM I (Yoshida et al., 1990) revealed that the HAIM molecule consists of two β-sheets, as is the case in the homologous Tendamistat, but one of its β-strands is much shorter than that of the *S.tendae* inhibitor. The exposed triplet Trp18-Arg-19-Tyr-20 is also present in HAIM, this segment forms a β-turn in both inhibitors and it is thought to be the binding site for the

α-amylase (Goto et al., 1985; Vertesy and Tripier, 1985). Neither inhibitor was found to be a glycoprotein.

S.corchorusii (PAIM)

Mammalian α-amylase inhibitors have been found in at least two more strains of *Streptomyces*. Two small inhibitors, PAIM I and PAIM II were isolated from cell culture filtrate of *S.corchorusii* by Murao et al. (1983). The molecular weights for PAIM I and II were found to be 4,300 and 4,800 respectively. Both molecules inhibited the pancreatic α-amylases of pig, dog, cow and horse pancreas, but not that of humans.

S.aurofaciens (AI-3688)

Vertesy and Tripier (1985) isolated and sequenced an α-amylase inhibitor, AI-3688, from *S.aurofaciens*. This 3,936 molecular weight inhibitor consists of 36 amino acid residues (Fig.5) with only two intramolecular cysteines forming a disulphide bridge. The inhibitor sequence showed more than 55% of homology when compared to the Tendamistat.

C.herbarum F-828

A 18,000 molecular weight glycoprotein α-amylase inhibitor from *Cladosporium herbarum* F-828 was isolated by Saito (1982). This much bigger inhibitor was found to contain about 10% carbohydrate and its amino acid analysis showed high contents of glycine, asparagine, glutamic acid, serine, alanine and threonine residues. The inhibitor was specific to mammalian α-amylases exhibiting a non-competitive mode of inhibition towards human salivary α-amylase.

```
1   DTTVSEPAPSCVTLYQSWRYSQADNGCAETVTVKVVYEDDTEGLCYAVAPGQITTVGDGY
2   DAGNRIAAPACVHFTADWRYTFVTNDCSIDYSVTVAYGDGTDVPCRSANPGDILTFP-GY
3   -----IAAPACVHFTADWRYTFVTNDCSIDYSVTVAYGDGTDVPCRSANPGDILTFP-GY
4   ATG--SPAPDCVESFQSWRYTDVRNGCSDAVTVVVQY-E--------------------
          .**.**    ..***. . *.*. . .* * * .

1  IGSHGHARYLARCL-----------
2  -GTRGNEV-LGAVLCATDGSA----
3  -GTRGNEV-LGAVLCATDGSALPVD
4  -------------------------
```

Fig.5: Sequence homologies of α-amylase inhibitors from *Streptomyces*

(1) Tendamistat (Ashauer et al., 1981);(2) HAIM (Yoshida et al., 1990);(3) HAIM II (Murai et al., 1985); (4) AI-3688 (Vertesy and Tripier, 1985).(*) Match across all sequences; (·) Conservative substitutions.

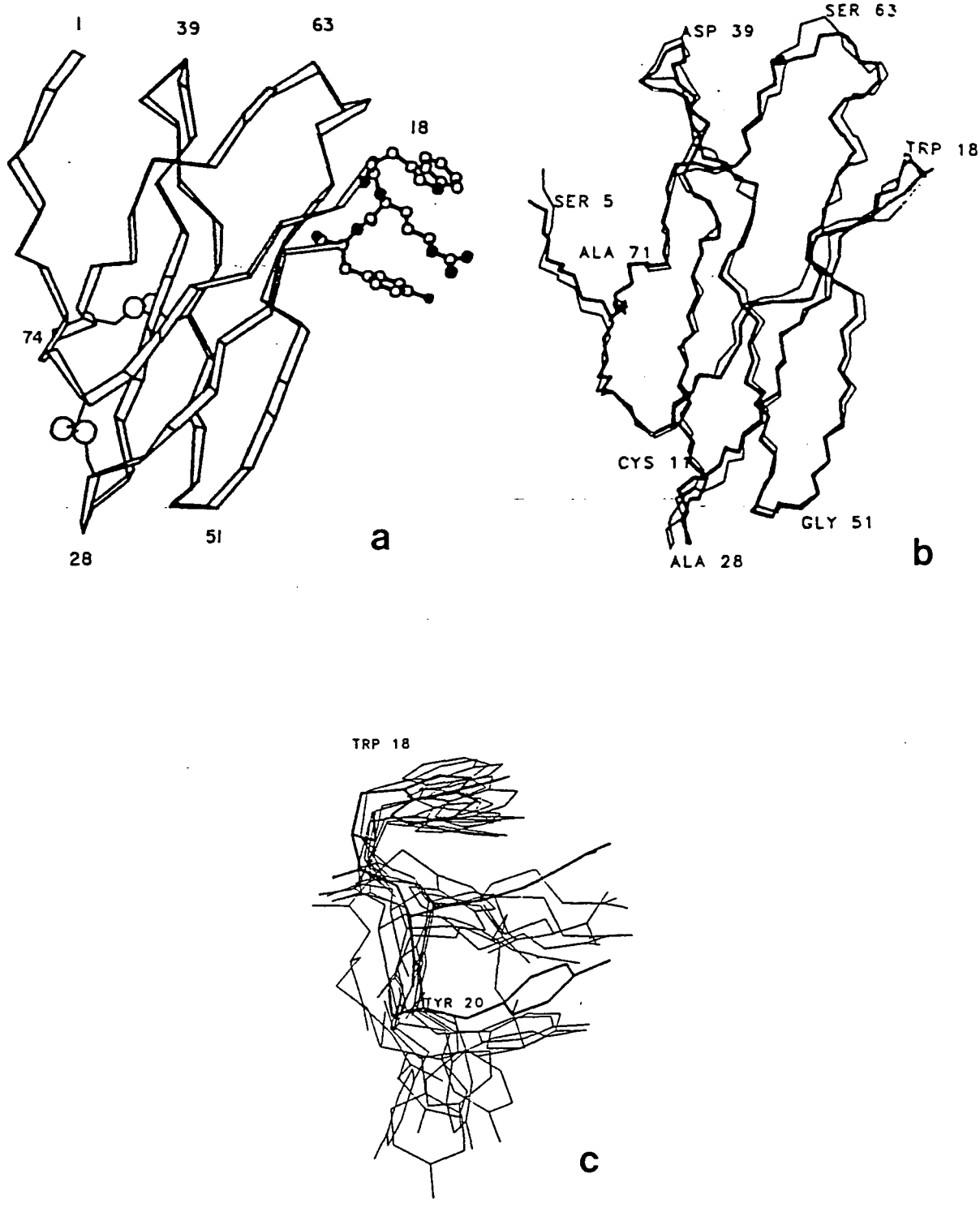


Fig.6: Diagrams of the three-dimensional structure of Tendamistat.

(a) Stereo ribbon of the Cα chain (from the crystal data) with the two disulphide bridges indicated by large open circles and the triplet Trp 18-Arg 19-Tyr 20 shown in "ball and stick" form where open circles are carbon atoms (taken from Pflugrath et al., 1986); (b) NMR structure from the 5 to 73 residues and (c) NMR stereo view of the fragment Trp 18-Arg 19-Tyr 20 (taken from Billeter et al., 1989).

1.3 Detection

The assay for α-amylase inhibitors in biological extracts is usually performed by adding the test extract to a buffered solution of the chosen α-amylase and then determining amylase activity after an appropriate period of preincubation. In this type of assay four steps are required: 1. Preincubation (enzyme + inhibitor); 2. Incubation (enzyme, inhibitor + substrate); 3. Enzyme inactivation; and 4. Assay of substrate hydrolysis. The practical procedure for this type of assay will be presented in the following chapter 'Materials and Methods'.

Preincubation

Investigations on protein inhibitors from plant tissues (Buonocore et al., 1976a; Warchalevski, 1983; Whitaker, 1983) have shown that inhibition of α-amylase activity is a time-dependent reaction and that both the α-amylase and inhibitor must be preincubated together before the substrate is added if maximal inhibition is to be observed. In most cases preincubation times have not exceeded more than 30 min. (Silano, 1986).

Incubation

In the incubation step the substrate is added to the already preincubated solution at a constant temperature for a specific time of hydrolysis.

Inactivation

In this step the enzyme is usually exposed to chemical inactivation (e.g. acidification) to stop the hydrolysis reactions.

Assay of Substrate Hydrolysis

Depending on the substrate, the hydrolysis product of the enzyme reaction (in the presence of the inhibitor) can be determined by using one of the following three techniques: 1. Saccharogenic (Samogyi, 1938; Nelson, 1944; Bernfeld, 1955); 2. Iodometric (Bird and Hopkins, 1954; Smith and Roe, 1957); or 3. Chromogenic (Ceska et al., 1969; Tuzhilin et al., 1982; Soyama and Ono, 1983; Parviainen et al., 1984).

The saccharogenic technique is probable the most frequently used for the screening of plant extracts. It is based on the measurement of the reducing sugar formation with 3,5-dinitrosalicylic acid (Bernfeld, 1955) or an alkaline copper reagent (Pick and Wober, 1978a, 1978b) using soluble starch as the substrate.

In the iodometric (also called amyloclastic) technique, the remaining starch levels can be estimated by iodine staining. However, iodine-staining methods can not be used for a wide range of starch concentrations and also have the disadvantage of possibly being affected by inactive proteins, if these are present (Wildring, 1963).

In the chromogenic technique the enzyme activity can be measured continuously by the formation of a colored product which is released during hydrolysis of the substrate. Usually, these substrates are chemically modified starch or smaller oligosaccharides such as: Procion yellow starch (Nagaraj and Pattabiraman, 1986), carboxymethylated starch, maltotetraose (Parviainen et al., 1984) or p-nitrophenyl-α-D-maltoheptaoside (Okabe et al., 1984).

Fossum and Whitaker (1974) described a qualitative method for detecting α-amylase inhibitors in biological materials by impregnating certain areas of a starch-agar gel plate with the test substance and then exposing it to an α-amylase solution. Iodine solution is used to stain the remaining starch present in the areas which contain α-amylase inhibitors. Alternatively, Suehiro et al. (1981) described the use of polycrylamide gel electrophoresis for a similar procedure.

Polyacrylamide-amylopectin gel electrophoresis has also been used to detect α-amylase inhibitors (Finardi Filho and Lajolo, 1982). Gels containing bands of inhibitors are immersed in solutions of α-amylase, and then stained with iodine-potassium iodide solution. The residual undigested bands of amylopectin indicate the location of the inhibitors.

Baker (1987) showed that bands of α-amylase inhibitors on polyacrylamide gel slabs can be detected by blotting onto solidified agar-amylase layers. After the polymerization another layer of agarose containing starch is added on top of the previous polymerized layers. Subsequently, a suitable period of incubation is observed, and then a solution of iodine is poured over the upper layer to stain the bands corresponding to unhydrolysed starch wherever the α-amylase inhibitors are located.

Immunological methods have also been developed for assaying α-amylase inhibitors, their electrophoretic comparison and determination of cellular localization (Lauriere et al., 1985; Sadowski et al., 1986 and Lecommandeur et al., 1987 in Richardson, 1991).

1.4 Enzyme-Inhibitor Interactions

Several investigations have shown that in spite of the strength with which plant proteinaceous α-amylase inhibitors bind to their specific enzymes, the majority of the enzyme-inhibitor complexes are reversible. Maltose (or other small competitive inhibitors), extreme pH values, variations of buffer polarity and changes of ionic strength are the factors most commonly responsible for affecting the stability of the binary EI complexes (Kneen and Sandstedt, 1943, 1946; Buonocore et al., 1975; Marshall and Lauda, 1975; Petrucci et al., 1976; Granum, 1978; Pick and Wober, 1979; O'Connor and Mc Geeney, 1981b; Shivaraj and Pattabiraman, 1981; Krishna Sharma and Pattabiraman, 1982a; Shivaraj et al., 1982; Weselake et al., 1983a; 1983b; Wilcox and Whitaker, 1984).

In contrast, proteinaceous α-amylase inhibitors from microorganisms seem to act in a different manner, as far as the enzyme-inhibitor complex stability and rate of formation is concerned (Vertesy et al., 1984; Goto et al., 1985). Tendamistat (HOE 467) showed pH-independent inhibition kinetics with a 1:1 complex formation. This complex could not be separated into its individual components by sodium dodecyl sulphate PAGE or molecular sieve chromatography (Vertesy et al., 1984).

However, inhibitors from both taxonomic kingdoms often require preincubation with the target enzyme before the addition of any substrate, if maximum inhibition is to be achieved (except for the In3 and In4 from wheat. O'Connor and McGeeney, 1981a). Preincubation times for studies on enzyme-inhibitor interactions involving plant α-amylase inhibitors may vary between 5 to 120 min. (Warchalewski, 1976, 1978; Weselake et al., 1983b) and 6 to 12h., sometimes (Frels and Rupnow, 1985).

Preincubation times no longer than 5 min. have been reported for the *Streptomyces* α-amylase inhibitors to achieve maximun inhibition (Vertesy et al., 1984; Goto et al., 1985; Vertesy and D. Tripier, 1985). However, the glycoprotein α-amylase

inhibitor from *C.herbarum* required 20 min. preincubation at room temperature to elicit maximal inhibitory response against human salivary α-amylase (Saito, 1982).

Enzyme-Inhibitor Complex Formation

The fact that preincubation is required for the maximum formation of the inactivated complex (*EI*) and its reversible nature, suggest that slow rate multi-order reactions might be involved in plant α-amylase inhibitor kinetics.

Another important aspect of the formation of the enzyme-inhibitor complex concerns the concentration of the two molecular forms (*E* and *I*). In the experimental conditions used for testing a number of α-amylase inhibitors, the inhibitor and the target enzyme concentrations are not much different. This implies that classical kinetic treatments (where inhibitor concentration is generally assumed to be much higher than the enzyme concentration) are not suitable to explain this particular case.

In fact, Wilcox and Whitaker (1984) showed that the complex formation between red kidney bean inhibitor and porcine pancreatic amylase was achieved in two steps. Moreover, when the rate constants, obtained from the rate of loss of enzyme activity in the presence of excess of inhibitor (10-fold higher) were plotted as a function of inhibitor concentration, they did not fall on a straight line as it is required for a simple one-step reversible process (where $[I] >> [E]$). Therefore, *Ki* values and inhibition mode descriptions based only on classical methods (Lineweaver-Burk and Dixon plots) seem to provide meaningless results as far as the mechanistic aspect of the α-amylase inhibition by a plant proteinaceous inhibitor is regarded. Non-steady state kinetics must be used.

Bieth (1974) described a treatment to determine *Ki* values graphically in cases where the inhibitor concentration is reduced by its combination with the enzyme (Eq.1).

$$[I^\circ] = \frac{1-a}{a} Ki + [E^\circ](1-a) \qquad (1)$$

i.e.

[Total inhibitor]=[Free inhibitor]+[Bound inhibitor] (1')

Where *a* is the fraction of total enzyme which is not bound to the inhibitor, *Ki* is the dissociation constant of the complex *EI*, [*I*°] is the total inhibitor concentration and [*E*°] is the enzyme concentration.

This demonstrates clearly that the binding behaviour of the presented enzyme-inhibitor system depends upon [*E*°]/*Ki* and not only upon *Ki* as is often assumed. Bieth also considered the competing effect of the substrate on the binding behaviour of the enzyme-inhibitor systems, expressing it on the following equations (Eqs.2 and 3).

$$[I^\circ] = \frac{1-a}{a} Ki \left(1 + \frac{[S^\circ]}{Km} \right) + [E^\circ](1-a) \qquad (2)$$

Where [*S*°] is the initial substrate concentration and K_m is the Michaelis constant. The equation 2 indicates that the binding behaviour will now depend upon the ratio [*E*°]/*Ki*(*app*)

where

$$Ki(app) = Ki \left(1 + \frac{[S^\circ]}{Km} \right) \qquad (3)$$

The amylase-inhibitor kinetics in this case were studied by applying to the amylase-(protein) inhibitor system the approach suitable for mutual depletion systems (Edsall and Wyman, 1958; Tanford, 1961; Webb, 1963) characterized by strong binding of the inhibitor to the amylase and by partial dissociation of the enzyme-inhibitor complexes after addition of the substrate (Bieth, 1974).

Buonocore et al. (1980, 1984), applied this kinetic treatment to a study of the interactions of wheat α-amylase inhibitors with insect and avian α-amylases. Powers and Whitaker (1977), and Mundy et al. (1983) have used a similar approach to investigate both the porcine pancreatic amylase-red kidney bean inhibitor systems and the barley amylase II-BASI systems, respectively.

However, this treatment does not consider the time-dependence of the interaction between enzyme and inhibitor, and does not provide much information about the mechanism by which the inhibition occurs.

Tight-binding Inhibition

Tight-binding inhibition has been used to describe those systems in which the rate of establishment of equilibrium between enzyme, inhibitor and enzyme-inhibitor complex is considered fast (similar to the classical steady-state (*EI*) complex formation). In the case where the initial concentration of the inhibitor is comparable to the initial concentration of the enzyme, the derivation of rate equations must allow for the reduction in the inhibitor concentration that occurs on formation of enzyme-inhibitor complex. Therefore, the first steady-state assumption of the classical Michaelis-Menten treatment is no longer valid, because the concentration of *EI* is not negligibly small compared with the total concentration of inhibitor (I_t) and the free concentration of inhibitor is not equal to its added concentration. The resulting equation in the case where [*E*] and [*I*] are comparable (Williams and Morrison, 1979) is a quadratic function with squared and linear terms in velocity and predicts that plots of velocity against total enzyme concentrations (E_t) at different total inhibitor concentration (I_t) will be curvilinear (Morrison, 1969). It also predicts that a double-reciprocal plot of velocity against substrate concentration in the presence of the inhibitor will have curved and linear portions. In this situation misinterpretation between competitive and non-competitive inhibition might occur if one is not aware that, with tight-binding inhibition,

the slopes of the lines of a double-reciprocal plot do not vary as a linear function of I_t (Morrison, 1969).

Morrison (1982) explains that as the strength of an interaction between an enzyme and a tight-binding inhibitor increases, a stage is reached when the equilibrium of the reaction cannot be established rapidly.

For the reaction $E \underset{K_2}{\overset{K_1 I_t}{\rightleftarrows}} EI$

the apparente first rate constant ($K_1 I_t$) will be small when only a very low concentration of I_t is required to demonstrate the inhibition. The slow establishment of the full inhibition of enzymes has been long known and frequently circumvented by preincubating the enzyme with the inhibitor before the addition of substrate.

Slow-binding and Slow, Tight-binding Inhibition

Slow-binding inhibition occurs when the concentration of inhibitor is ten or more times greater than that of the enzyme and the rate of establishment of the equilibrium between *E*, *I*, and *EI* is slow. William and Morrison (1979) showed that when the equilibrium for the interaction of an inhibitor with any form of enzyme is not established rapidly, a transient or pre-steady-state phase will occur in a plot of product formation as a function of time. The shape of the progress curve would vary according to whether or not the inhibitor was preincubated with the enzyme (Fig.7).

When a reaction, in the presence of a slow-binding inhibitor, is started by the addition of enzyme the relatively rapid initial velocity decreases to a slower steady-state rate (Fig.7). If the enzyme is preincubated with inhibitor and the reaction started with substrate, there will be a slow release of inhibition and ultimately a steady-state

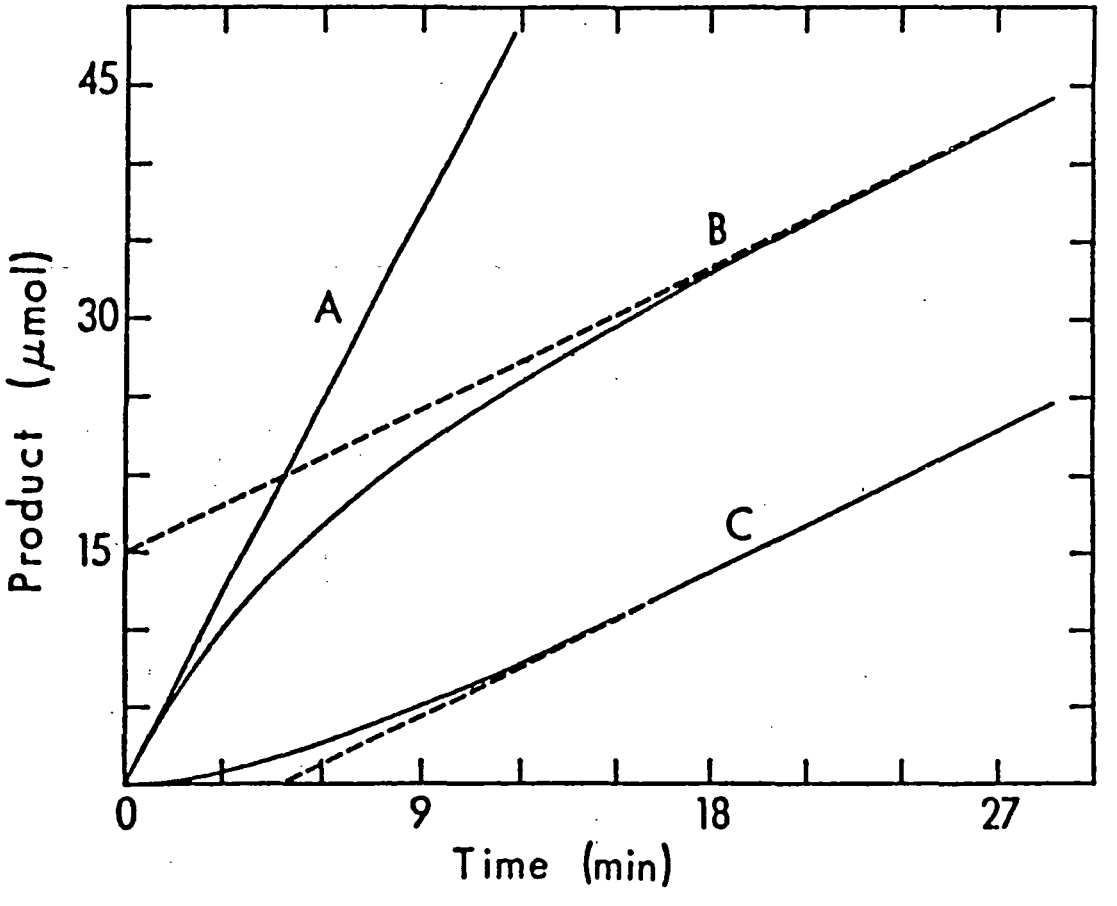


Fig.7: Theoretical plots of progress curves for an enzyme-catalyzed reaction in the absence (curve A) and in the presence (curves B and C) of a slow-binding inhibitor. B is the expected curve when reaction is started by the addition of enzyme; C is the expected curve when reaction is started by the addition of substrate after preincubation of the enzyme with the inhibitor. The dashed lines represent the true steady-state rate (v_s) in the presence of the inhibitor. (Taken from William and Morrison, 1979)

rate will be reached. The two steady state rates will be identical provided that there is no significant enzyme inactivation, substrate depletion or product inhibition. It should be noted that slow-binding inhibition is in effect transient-state kinetics on a steady-state time scale (Morrison, 1982), i.e. in minutes rather than in the millisecond range.

Williams and Morrison (1979) also explained that a time-dependent interaction between enzyme (*E*) and inhibitor (*I*) could involve the rapid formation of an *EI* complex, which then undergoes a slow isomerization to an inactive EI^* complex. Under such circumstances the overall dissociation constant (Ki^*) for the EI^* complex is likely to be low, as it would be a function of the *Ki* for the *EI* complex as well as of the forward and reverse isomerization constants associated with the interconversion of *EI* and EI^*. Alternatively, the slow establishment of the equilibrium between *E*, *I*, and *EI* could be due simply to the low value of the pseudo-first-order rate constant for the dissociation of *EI*. There is, of course, the possibility that the *EI* complex thus formed could undergo a further slow interconversion to EI^*. For reaction of this type, the dissociation constants must be low, but they need not to be so low that the inhibitors would exhibit the kinetic characteristics of tight-binding inhibitors.

However, if slow-binding inhibition occurs at inhibitor concentrations which are comparable to that of the enzyme, this type of inhibition is called *slow, tight-binding*. The strong interaction of an inhibitor with an enzyme can lead not only to a delay in the establishment of the steady-state velocity, but also to the need to allow for depletion of inhibitor concentration. If the steady-state is reached without any significant change in substrate concentration, then the steady-state velocity data can be analysed in the same way as when the equilibria involving the tight-binding inhibitor are rapidly established.

Mechanisms that describe reversible slow-binding and slow, tight-binding inhibition for a number of systems have been proposed by several authors (Cha, 1976,

1980; Williams e Morrison, 1979; Williams et al., 1979; Uehara et al., 1980; Zhou et al., 1989; Longstaff and Gaffney, 1991).

Wilcox and Whitaker (1984) were the first to propose a kinetic model to explain the inactivation of porcine pancreatic α-amylase by the red kidney bean α-amylase inhibitor. The inhibition was described as non-competitive, slow, tight-binding, occuring in two steps. Three selected mechanisms were investigated to see which one would best fit the obtained kinetic data. It turned out to be a complex model, which assumes that [*E*], [*ES*], [*EI*], and [*ESI*] are at steady state, as well as the interconversion of EI^* and ESI^* (Fig.8). A step involving interconversion of the enzyme-inhibitor complex was included ($EI \rightleftarrows EI^*$, where EI^* is the inactive α-amylase-inhibitor complex and is very slowly reversible) to explain the ability of the complex to bind maltose and starch. The model also assumes that the amount of substrate converted to product during the measurements is insignificant in comparison to total substrate present and also that $K_m \equiv K_S$(Fig.8).

The final equations described in terms of [*ES*] had to be integrated numericaly and fit to ten progress curves of [*P*] vs time.

The measured *Ki* for red kidney bean α-amylase of $3x10^{-11}$M (Powers and Whitaker, 1977) is a combination of two steps (Fig.8). The first step ($E + I \rightleftarrows EI$ and $ES + I \rightleftarrows ESI$, where *ESI* is able to form product) has a calculated *Keq* of $3.09x10^{-5}$M at pH 6.9 and 30 °C. The second step is slower ($EI \rightleftarrows EI^*$) has a rate constant (K_1) of 3.05 $min.^{-1}$.

Conversely, Tendamistat (HOE 467) seems to inactivate porcine pancreas α-amylase in a tight-binding manner (Vertesy et al., 1984). However, no mechanism of microbial α-amylase inhibition has been proposed up to now. The *Ki* of $2x10^{-10}$M for tendamistat and the porcine α-amylase was obtained indirectly using a reversible competitive inhibitior with known *Ki* towards the porcine enzyme.

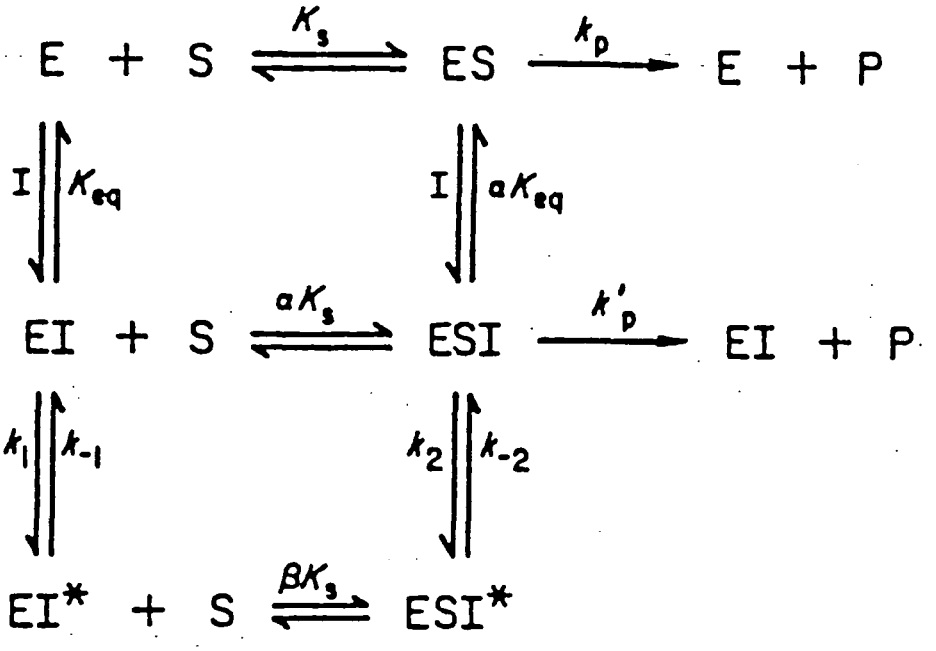


Fig.8: Model "C" that better explains the inactivation of porcine pancreatic α-amylase by the red kidney bean α-amylase inhibitor (Wilcox and Whitaker, 1984)

α-Amylase Inhibitor "Active Site"

Silano et al.(1977, 1980) proposed a hypothetical model to explain the interaction between insect, avian, or mammalian α-amylase and the monomeric (0.28) and dimeric (0.19) inhibitors from wheat. This model provides an explanation based on the stoichiometrical behaviour of the two inhibitors (*E*:*I* = 1:1 for 0.19 and 1:2 for 0.28). According to Silano (1986) the higher affinity shown by the 0.19 family and the stoichiometric data for the 0.28 family, strongly suggest that each 0.19 inhibitor subunit and 0.28 molecule has one binding site for the amylases and that the amylases have two binding sites for inhibitors. It was speculated (Buonocore et al., 1980), that a sugar moiety in the inhibitors was involved in the enzyme inhibitor binding process and that the complexes were further stabilized by secondary protein-protein binding forces. Studies of the difference spectra studies of amylase-wheat inhibitor complexes indicated that structurally related tryptophanyl side chains are involved in the binding of the inhibitors to the enzymes (Buonocore et al., 1980; 1984).

Studying the interaction of barley α-amylase II with an endogenous barley α-amylase inhibitor, Halayko et al. (1986), observed that a tryptophan residue in the enzyme (which is essential for productive enzyme-substrate binding) was affected when the inhibitor bound the enzyme. It was also observed that a red-shift difference occured in the spectrum of α-amylase II, which was induced by the binding of the inhibitor. This indicated that a more hydrophobic region was being formed around the essential tryptophan residue. However, evidence for the involvement of a structural sugar moiety in the interaction between the two molecules have not been obtained yet. In fact, Garcia-Olmedo et al. (1991, unpublished) have shown that a wheat α-amylase inhibitor synthesized by E. coli (not glycosylated) was active.

The available crystal and NMR three-dimensional structures of *Streptomyces* α-amylase inhibitors (Pflugrath et al., 1986; Braun et al., 1989; Yoshida et al., 1990) provide important clues on the active site of this class of inhibitors. Pflugrath and

collaborators (1986) findings almost suggest that the remarkable conserved triplet Trp-Arg-Tyr (Figs.5 and 6), present in all sequenced inhibitors from *Streptomyces* (Ashauer et al., 1983; Murai et al., 1985; Vertesy and Tripier, 1985; Yoshida et al., 1990), could play an important role on the inhibitor binding ability. This hypothesis seems to be supported by previous spectroscopic studies by Goto et al., (1985) which indicate that in the interaction between HAIM-porcine pancreatic amylase tryptophanyl and tyrosyl side chains were involved.

1.5 Significance of α-Amylase Inhibitors

1.5.1 - BIOLOGICAL ROLE

High contents of α-amylase inhibitors found especially in seeds of cereals and legumes have long invited speculation about their physiological role. The most acknowledged hypotheses are that they may act as storage proteins, as regulators of endogenous α-amylases, or as defensive agents against the attacks of animal predators and insect or microbial pests.

Because of their strong structural features (resistance to denaturation by heat extremes of pH and many proteolytic enzymes) as well as rich content of cysteine and cystine, α-amylase inhibitors, together with other enzyme inhibitor from seeds, might be considered a potential class of storage proteins (Richardson, 1991).

Detection of endogenous α-amylase inhibitors in germinated and quiescent wheat, barley, and maize kernels supports the hypothesis that these inhibitors may act as regulators of endogenous α-amylase activity and thereby control starch metabolism in the kernel. Although it is conceivable to speculate that these inhibitors may have a regulatory function during seed development, by inhibiting α-amylase activity during starch granule synthesis, and/or that they might delay starch degradation (during premature sprouting) data supporting this hypothesis are rather limited (Mundy et al., 1983; Weselake et al., 1985; Zawistorwska et al., 1988; Abdul-Hussain and Paulsen, 1989).Inhibition tests between Tendamistat and *Streptomyses tendae* amylase (Vertesy et al., 1984) revealed that enzyme activity is strongly affected by the inhibitor, suggesting a regulatory role of this protein in *Streptomyces*.

Reviews on enzyme inhibitors from plants (Buonocore et al., 1976; Silano, 1986; Garcia-Olmedo, 1987; Richardson, 1991) frequently report a number of insect feeding trials with synthetic diets containing α-amylase inhibitors. These feeding trials have shown that in many cases the development of some pests of stored-cereal grains were

adversely affected, followed by significant mortality rates. However, it is not clear whether these observed affects are a direct consequence of an *in vivo* α-amylase inhibiting activity of those molecules, or due to other unknown factors such as gut osmotic balance changes.

1.5.2 - NUTRITIONAL EFFECTS

Not much is known about the nutritional significance and toxicity of α-amylase inhibitior in the diets of human and domesticated animals. As was mentioned in the beginning of this chapter, some of these inhibitors found in cereal and legume seeds are able to inactivate human salivary and pancreatic amylases. However, as far as human consumption is concerned, it appears that many of them are destroyed by cooking. Even those which still retain their inhibitory activity after drastic food processing (Granum, 1979) seem to produce few nutritional problems for healthy people (Silano, 1986; Buonocore and Silano, 1986). On the other hand, they may have some toxicological effect on infants, who have a lower production of pancreatic α-amylase than adults, and for patients with peptic or gastric disorders.

Barber et al.(1989) and Gomez et al. (1990) found that insect α-amylase inhibitors from barley and wheat flour were the major allergens associated with bakers asthma disease. Amylase inhibtors are also known to induce pancreatic hypertrophy and tissue degeneration in chickens (Macri et al., 1977), and pancreatic hyperplasia in rats and chickens (Granum and Eskeland, 1981).

1.5.3 - APPLICATIONS

Commercial preparation of α-amylase inhibitors from kidney beans (Granum et al., 1983) have been used to decrease the amylolytic digestion of starch in an attempt to produce weight loss (see Richardson, 1991 for references). The ineffectiveness of the so-called "starch-blockers" or slimming pills (Bo-Linn et al., 1982) generated a big scientific and public controversy in the USA, such that in 1982 the US Food and Drug Administration ordered the complete withdrawal of these products from the market.

Streptomyces α-amylase inhibitors such as Tendamistat, HAIM (I, II) and PAIM (I, II) have also been regarded as potential phamacological tools for the treatment of obesity and diabetes (Vertesy et al., 1981; Regitz et al., 1981; Vertesy et al., 1984).

α-Amylase inhibitors have been used for the diagnosis of pancreatic disorders and other forms of hyperamylasemia where it is necessary to differentiate between the activities of both salivary and pancreatic amylases (O'Donnell et al., 1977; O'Donnell and MacGeeney, 1983; Huang and Tietz, 1982; Okabe et al., 1984; Tietz and Shuey, 1984; Nagaraj and Pattabiraman, 1986).

1.6 Thionins

During these studies on the α-amylase inhibitors from sorghum seeds some other small proteins which had inhibitory effects on certain α-amylases were encountered and were subsequently shown to be thionin-like.

Thionins comprise a group of basic low molecular weight (5kDa) and sulphur-rich proteins. This protein family was originally described in the endosperm of wheat (purothionins) (Balls and Hall, 1942) but now includes other proteins such as the viscotoxins and crambins (Garcia-Olmedo et al., 1989).

Thionins may be found in the leaves and seed endosperms of a number of cereals such as *T.aestivum* (purothionins), *H.vulgare, A.sativa, Z.mays and S.cereale* (Balls and Hall, 1942; Garcia-Olmedo et al., 1968; Fernandez de Caleya et al., 1976; Redman and Fisher, 1969; Bekes and Lasztity, 1981; Jones and Cooper, 1980; Hernandez-Lucas et al., 1978; Colilla et al., 1990). Thionins from barley (*Hordeum vulgare*) are also called hordothionins (Mendez et al., 1990; Gausing, 1987; Bohlmann and Apel, 1987).

Viscotoxin was first described in the leaves and stems of mistletoe *(Viscum album)* (Loranthaceae) by Winterfeld and Bijl (1949). Much later, similar toxins, such as phoratoxins from *Phoradendron tomentosum* (Mellstrand and Samuelsson, 1973, 1974; Mellstrand, 1974; Thurnberg, 1983), dendatoxin B from *Dendrophtora clavata* (Samuelsson and Pettersson, 1977), ligatoxin A from *Phoradendron liga* (Thurnberg and Samuelsson, 1982), and leaf thionins from *Pyrularia pubera* (Vernon et al., 1985) were also investigated.

Crambin the other thionin-like cotyledon protein from *Crambe abyssinica* (Van Etten et al., 1965), was found to be a mixture of two variants, whose primary structure were homologous to the purothionins and the viscotoxins (Teeter et al., 1981; Vermeuler et al., 1987).

Thionin related proteins have been also found in tomato (*Lycopersicum esculenta*), mango (*Mangifera indica*), papaya (*Carica papaya*) and walnut (*Juglans regia*) (Daley and Theriot, 1987).

Thionins are among the best studied proteins in terms of structure. They have been suitable models for structural studies and molecular dynamics (Brunger et al., 1986), because of their easy preparation and crystallization, small polypeptide chain, stability and the number of known amino acid sequences available (Fig.9). The x-ray diffraction of highly ordered crambin crystals (Teeter and Hendrickson, 1979), revealed that this protein has the shape of the Greek capital letter gamma (Γ) (Fig.10), where the stem of the Γ is an antiparallel pair of helices and the cross-arm consists of two antiparallel β-strands, an irregular strand and a classic β-turn.

The surface of crambin has an amphipatic character. The six charged groups ($NH_3 1^+$, $Arg10^+$, $Arg17^+$, $Glu23^-$, $Asp43^-$ and $COO46^-$), as well as several other hydrophilic side chains are clearly segregated from what otherwise is largely a hydrophobic molecular surface. The surface of crambin can be understood as comprising three components. The face of the inner bend between the helical stem and the β-arm is a sloping surface that is almost entirely hydrophilic. The other two surface elements, namely the left wall of the helical stem and the underside of the molecule, are primarily hydrophobic. The left-wall surface curves gently around the outer bend while the underside is relatively flat (Hendrickson and Teeter, 1981).

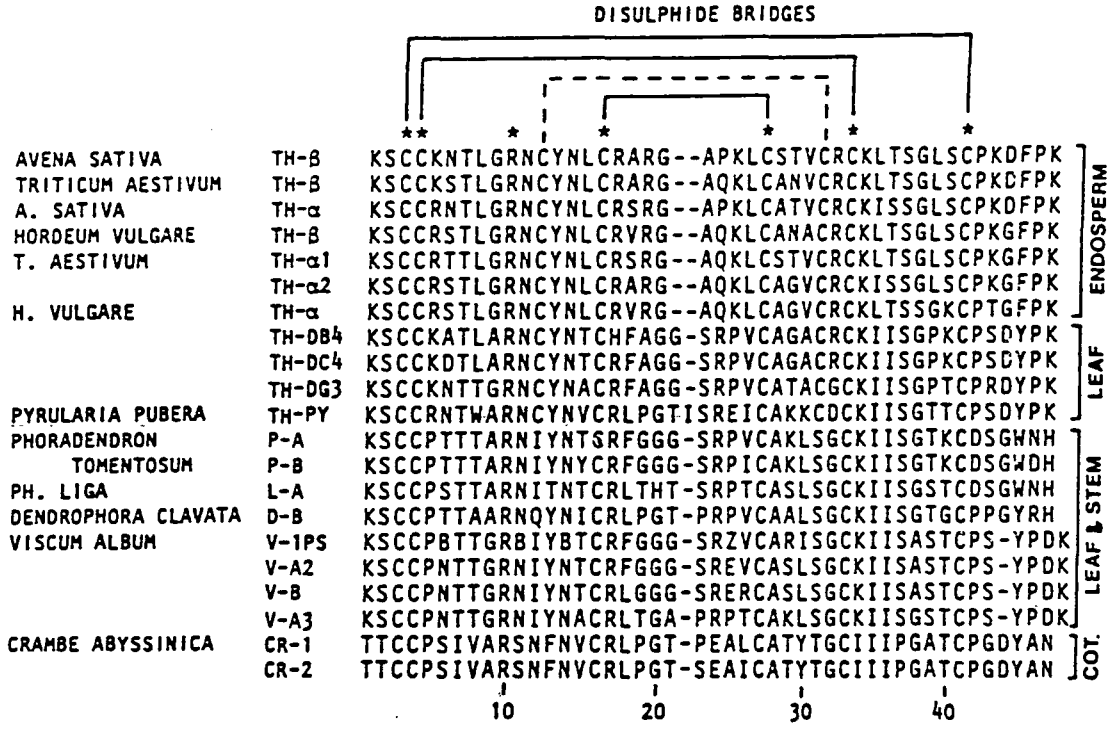


Fig.9: Sequence homologies of thionins.

Alingnment of the currently available amino acid sequences of members of the thionins family. The multiple alignment criteria of Feng and Doolittle (1987) have been followed. Sources for the sequences are the following: *T.aestivum*, wheat endosperm, Ohtani et al. (1975, 1977), Mak and Jones (1976), Jones and Mak (1977) and Jones et al. (1982). *H.vulgare*, barley endosperm, Ozaki et al. (1980), Ponz et al. (1986), and Hernandez-Lucas et al. (1986). *A.sativa*, oats endosperm, Bekes and Latztity (1981). *Pyrularia pubera*, leaves, Vernon et al. (1985). *H.vulgare*, leaves, Gausing (1987), and Bohlman and Apel (1987). *Viscum album*, mistletoe, Samuelsson (1961, 1966, 1974), Samuelsson et al. (1968), Samuelsson et Pettersson (1970), and Samuelsson and Jayarvardene (1974). *Phoradendron tomentosum*, Mellstrand (1974) and Mellstrand and Samuelsson (1973, 1974). *Dendrophtora clavata*, Samuelsson and Pettersson (1977). *Phoradendron liga*, Thurnberg and Sammuelson (1982). *Crambe abyssinica*, Abyssinian cabbge, Teeter et al. (1981) and Vermeulen et al. (1987). Invariant positions are indicated with stars (*). The disulphide bridge indicated with discontinuous line is not common to all sequences. (Taken from Garcia-Olmedo et al., 1989)

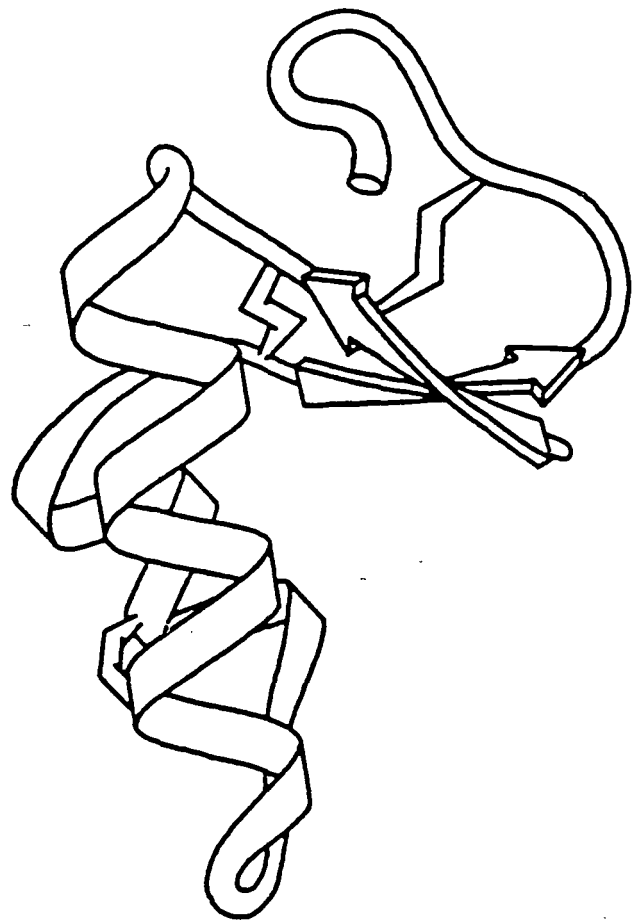


Fig.10: Schematic drawing of the backbone of crambin.

This representation of crambin was drawn by Jane S. Richardson. Arrows depict β-strands. The disulphides are drawn as "lightning flashes" (reproduced from Whitlow and Teeter, 1985). (Taken from Garcia-Olmedo et al., 1989)

All but one of the changed groups are clustered on the face of the inner bend. Six of the 13 other polar side chains also line or rim this surface. These groups are highly interconnected by numerous salt bridges, hydrogen bonds and other interactions including water molecules that link the carboxyl group of Glu 23 to the terminal amino group of Thr1 and the guanidinium group of Arg 17.

Hendrickson and Teeter (1981) explain that unlike the inner-bend surface, or the surfaces of water-soluble proteins in general, the remainder of the crambin molecule is interspersed with hydrophobic groups. The left-wall surface includes eight fully exposed leucine, isoleucine, valine, proline and tyrosine side groups and the underside surface contains seven such exposed groups. There are also exposed polar side groups on these surfaces, but several of these are hydrogen-bonded back to the main chain.

Garcia-Olmedo et al.(1989) have listed a number of methods used to study crambin in solution. The globular structure of the protein obtained by those methods is very much in accordance to that in the crystal, with only slight differences.

α1-Purothionin is another protein from the thionin family which has been well studied structurally. The three-dimensional solution structure of the molecule (Clore et al., 1986) revealed an overall shape of the capital letter L (in reverse I orientation) which is similar to that of crambin, with the longer arm comprising the two helices and the shorter one the mini antiparallel β sheet and the C-terminal residues (35-45). The angle between the long axes of the two helices is approximatly 150 degrees, and the angle between the plane formed by the anti-parallel β-sheet is about 50 degrees. The hydrophobic residues are principally concentrated on the outer surface of the two helices which may present the site of interaction of α1-purothionin with lipid membranes which might explain its haemolytic activity and lyses of a wide variety of mammlian cells (in Clore et al., 1986).

The outer surface of the corner of the I as well as the under surface of the shorter arm are hydrophilic with the exception of two hydrophobic residues (Pro 40 and Phe 43). The inner surface of the I is also mainly hydrophilic with a concentration of positively charged residues.

Biological Activities

A number of *in vivo* activities have been demonstrated for the thionins, among these are: antimicrobial action (Stuart and Harris, 1942; Balls and Harris, 1944; Nore and Ichikama, 1968; Okada and Yoshizumi, 1970, 1973; Fernandez de Caleya et al., 1972; Hernandez-Lucas et al., 1974; Bohlmann et al., 1988); cytotoxic effects on cultured mammalian cells (Nakanishi et al., 1979; Konopa et al., 1980; Carrasco et al., 1981; Vernon et al., 1985); toxic effects on insect larvae (Kramer et al., 1979) and higher animals (Conbon et al., 1942; Sammuelson, 1974); alteration of membrane permeability (Fernandez de Caleya, 1973; Okada and Yoshizumi, 1973; Ohtami et al., 1975, 1977); inhibition of α-amylase (Jones and Meredith, 1982), papain (Balls et al., 1942) and macromolecular synthesis (Nakanishi et al., 1979; Carrasco et al., 1981; Garcia-Olmedo et al., 1983; Mendez et al., 1990).

Biological Role

Besides their toxicity to various organisms, alteration of membrane permeability and inhibition of macromolecular synthesis and certain enzymes, the real biological function of these proteins remains obscure. However, there are indications that they might be involved in the thioredoxin-related reactions (Buchaman et al., 1979; Wada and Buchaman, 1981). Johnson et al.(1987) have reported a thioredoxin system, consisting of a homogeneous preparation of thioredoxin *h* and a partially purified

thioredoxin reductase (NADPH), which effectively reduced wheat purothionin with NADPH as the hydrogen donor. The reduced thionin, in turn, was capable of activating fructose-1,6-bisphosphatase. These results suggest a possible role of thionins as secondary thiol messengers in the redox regulation of enzymes.

There are also indications that thionins may play a role in the protection of plants from pathogens (Fernandez de Caleya et al., 1972; Bohlmann et al., 1988) and cause interference with industrial fermentation (in Garcia-Olmedo et al., 1989).

1.7 Research Objective

Sorghum is the fifth most important cereal grain in the world after wheat, maize, rice and barley. Sorghum belongs to the Tribe Andropogonae of the grass family Poaceae (Harlan and deWet, 1972).

This crop is distributed from the equator to latitudes as temperate as 45° . The United States is the largest producer of sorghum and accounts for almost 31% of the world production followed by India (16%) and Mainland China (10%) (Martin, 1984).

Sorghum grain ranks third after rice and wheat as a cereal for human consumption in Asia. In Africa sorghum is grown primarily for human consumption as a thin porridge or thick paste or for use in beer. In the subhumid zone of Nigeria, which represents approximately half of its land area, sorghum is one of the main crops cultivated (Powell, 1985). In China, sorghum is ground into flour or groats for consumption as bread or porridge, making it an important food in that country. Sorghum in Mexico occupies the second largest area of any crop sown (Barkin and DeWalt, 1988) and constitutes an important food supply for people in that country and of other Central American countries when maize supply is low.

products (Wall and Ross, 1970). The starch is used in wall-boards, oil well-drilling muds, and food products. Sorghum grain is also processed into paste flour, dextrose, dextrose syrup, edible oils, gluten meal, and gluten feed. Alcohol is also produced from sorghum in quantities comparable to those obtained from wheat or maize (Martin, 1984). Especially in Brazil, where alcohol is an important source of energy, sorghum could be an excellent source for this purpose, not only for that country but throughout the world.

Despite the world-wide agricultural, industrial and economic value of this crop, compared with many other cereals there is a relative lack of biochemical data concerning the proteins present in the seeds which may inhibit the α-amylase enzymes of mammals and insects and thus have possible importance in human nutrition and for plant defence. The present investigation of the α-amylase inhibitors in seeds of *Sorghum bicolor* was initiated in an attempt to remedy this lack of information.

MATERIALS AND METHODS

2.1 Materials

2.1.1 CHEMICALS	SUPPLIER
Acetic acid (Glacial) (A.R. grade)	BDH
Acetone	Rathburn Chem
Acetonitrile(HPLC grade)	Rathburn Chem.
Acrylamide (gel grade)	Sigma
Ammonium bicarbonate (A.R. grade)	BDH
Ammonium persulphate (electrophoresis grade)	BDH
Ampholine (pH 3.5-10)	Pharmacia
BAPNA (α-N-benzoyl-DL-arginine-p-nitroanilide-HCl)	Sigma
Bio-gel P6	Bio-Rad
Bis acrylamide	Sigma
Bromophenol blue	Sigma
Butyl acetate (A.R. grade)	BDH
Calcium chloride ($CaCl_2 \cdot 6H_2O$) (A.R. grade)	BDH
Chloroform	BDH
Coomasie Brilliant blue R-250	Sigma
Cyanogen bromide	Sigma
4, 4-Dimethylaminoazobenzene-4'-isothiocyanate (DABITC)	Fluka, Sigma
DABITC - diethylamine standard	Prepared by Mr. J.Gilroy
3,5 - Dinitrosalicylic acid	Sigma

DL-Dithiothreitol	Sigma
Ethanol	BDH
Ethyl acetate	BDH
Glycine	Sigma
Guanidine hydrochloride	Sigma
n-Heptane	BDH
Hexane	BDH
Hydrochloric acid (A.R. grade)	BDH
Iodoacetic acid	BDH
Maltose	Sigma
2-Mercaptoethanol	Sigma
Phosphoric acid (A.R. grade)	BDH
Phenylisothiocyanate (PITC) (sequencer grade)	Rathburn Chem.
p-Nitrophenyl-α-D-Maltotrioside	Boehringer GmbH
Potassium Tartrate (A.R. grade)	BDH
Pyridine (A.R. grade)	Rathburn Chem.
Red Sepharose CL-6B	Pharmacia
Sodium acetate	BDH
Sodium chloride	BDH
Sodium dodecyl sulphate	Sigma
Sodium hydroxide	BDH
Starch	Sigma
TEMED (N, N, N', N',-tetra methylene diamine)	Sigma
Toluene (A.R. grade)	BDH
Trichloroacetic acid (TCA)	Sigma

Trifluroacetic acid (sequencer grade)	Rathburn Chem.
Tris/Trizma	Sigma
4-Vinylpyridine	Sigma
Tributylphosphine	Aldrich Chem. Co.

2.1.2 ENZYMES AND OTHER BIOLOGICAL REAGENTS

α-Amylases (E.C. 3.2.1.1)	
Human salivary	Sigma
Porcine pancreatic	Sigma
Barley	Sigma
S.oryzae	Sigma
Bacillus subtilis	Sigma
Spodoptera litoralis gut extraction	obtained from Dr. A.M.R.Gatehouse
Bovine serum albumin	Sigma
α-Chymotrypsin (E.C. 3.4.21.1)	Sigma
Elastase (E.C.3.4.21.11)	Boehringer GmbH
Myoglobin from horse heart	Sigma
S.aureus V8 protease (E.C.3.4.21.19)	Sigma
Thermolysin (E.C.3.4.24.4)	Sigma
Trypsin (E.C. 3.4.21.4)	Sigma
Molecular weights Kit MW-SDS-702	Sigma

2.1.3 EQUIPMENT

Automatic Pulsed Liquid Phase Protein Sequencer model 477A	Applied Biosystem Ltd.
BioIon 20 Biopolymer Mass analyser	Applied Biosystem Ltd.
Waters Pico-Tag Amino Acid Analyser	Waters
HPLC Chromatography apparatus	Varian
Analytical Reverse Phase column (4.6mmx25cm) of Vydac C_{18}	Technicol Ltd.
Preparative Reverse Phase column (22mmx25cm) of Vydac C_{18} (218 TP 1022)	Technicol Ltd.
Spectrophotometers	LKB 4050
	Cary 105C (NC University)
Microcomputers	Macintosh SE/30 IBM PS2/30

2.2 Purification, and Characterization of α-Amylase Inhibitors from Seeds of *Sorghum bicolor*.

2.2.1 PROTEIN EXTRACTION AND PURIFICATION

Seeds of sorghum (*Sorghum bicolor* (L) Moench, cv. French red) were obtained from Dr. J. Cecil, Overseas Development Natural Resources Institute, Culham, Abingdon. 330g batches of milled seed were defatted with acetone and extracted for 12 h at 4°C with 1.3 L of 0.1M HCl in 0.1M NaCl. After centrifugation (10,000g) for 25 min at 4°C, the pH of the supernatant was adjusted to 7.0 with 1M sodium hydroxide. The proteins which precipitated were removed by centrifugation, and ammonium sulphate was added to the supernatant to give a 60% saturation. The proteins which precipitated were collected by centrifugation, dissolved in 30ml of 0.05M Tris/HCl buffer pH 7.0 containing 0.1M NaCl and dialyzed against the same buffer for 24 h changing the buffer every 8 h. Insoluble proteins were removed by centrifugation and the solution applied to a column (2.5cm x 20cm) of Red Sepharose CL-6B equilibrated in the same buffer. The column was washed with the starting buffer until the absorbance at 280nm reached approximately zero when the proteins bound to the column were eluted with 0.05M Tris/HCl pH 7.0 containing 3M NaCl and collected in fractions of 10 ml. The eluate was dialysed against distiled water for 48 h. and lyophilized. After each run the column was regenerated with 200ml of 6M guanidine followed by 400ml of equilibrating buffer.

About 40mg of protein eluted from the Red Sepharose column were dissolved in 1.5ml of 6M guanidine HCl in 0.1% TFA and applied to a preparative reverse phase HPLC column (22mm x 25cm) of Vydac C_{18} which was eluted with a gradient (0-60%) of acetonitrile in 0.1% aqueous TFA. The individual peaks containing α-amylase inhibitors were rechromatographed on an analytical phase column (4.6mm x 25cm) of Vydac C_{18} (218 TP 54) using an extended gradient (0-45% of acetonitrile over 90 min).

2.2.2 PREPARATION OF α-AMYLASES FROM *Locusta migratoria migratorioides, Periplaneta americana and Sorghum bicolor*

Crude extracts of the α-amylases from the guts of mature larvae (nymphs) of the African migratory locust (*Locusta migratoria migratorioides*) and adult cockroaches (*Periplaneta americana*) were prepared by homogenising the dissected whole guts in 25mM Tris/HCl pH 7.5 containing 1mM $CaCl_2$ at 0°C with a glass to glass manual homogeniser. The extract from four guts was made up to a final volume of 10ml. Lipids were removed by extracting three times with chloroform and the activity of the deffatted extract was determined by using the method of Bernfeld (1955).

100mg of deffatted *Sorghum bicolor* flour was extracted with 1 L of 25mM Tris/HCl pH 7.5 containing 1mM $CaCl_2$ at 0°C for 8 hours. After centrifugation (10,000xg, 1h) the supernatant was collected and found to have amylase activily by the Bernfeld method (1955).

2.2.3 ASSAY OF α-AMYLASE INHIBITORY ACTIVITY

The inhibition of starch hydrolysis by all α-amylases used was determined by following the release of reducing sugars using the dinitrosalicylate reaction as described by Bernfeld (1955) with some buffer modifications.

Assay buffer:

- 25mM Tris-HCl, 1mM $CaCl_2$ pH 6.9 (Human salivary amylase)
- 50mM Glycine/NaOH, 0.1M NaCl pH 9.0 (*S.litoralis* gut amylase)
- 50mM Sodium acetate/acetic acid pH 7.0 (other amylases)

Starch solution:

1% soluble starch w/v was prepared by dissolving 1g of starch in 10ml of distilled water, this solution was boiled gently for 15-30 sec. and then made up to 100ml with distilled water.

Dinitrosalicylate solution:

1g dinitrosalicylic acid was dissolved in 20ml 2N NaOH. The solution was then diluted with 50ml of distilled water, 30g potassium tartrate was added and dissolved.

Assay mixture:

-250µl α-amylase (varying concentrations in assay buffer)

- 250µl inhibitor (varying concentrations in assay buffer)

- 250µl assay buffer

- 250µl starch solution

Total vol. : 1.000µl

The enzyme and the inhibitor were preincubated at 30°C for 25 min. The substrate (250µl starch solution) was added and the reaction incubated at 30°C for 10 min. The reaction was stopped by the addition of 1.0ml ot the dinitrosalicylate solution.

The reaction mixture was boiled for 10 min, allowed to cool, and then 5ml distilled water was added. The reaction was left to stand at room temperature for at least 10 min before the absorbance was measured at 530 nm.

2.2.4 DETERMINATION OF ENZYME ACTIVITY

To determine the enzyme activity of all tested α-amylases under the above assay conditions, maltose standard curves were obtained for the three buffer systems used.

One enzyme unit was assumed to be the amount of enzyme required to produce 1mg of reducing sugar in 3 min (1unit-1mg/3min) in 1ml of a 0.25% starch solution.

2.2.5 COMPARISON OF INHIBITORY ACTIVITY

The relative inhibitory activity of each inhibitor tested was obtained by the comparing their percentage inhibitory activities against the same concentrations of enzyme where the enzyme concentration was fixed such that the absorbance of the product released (after reaction with dinitrosalicylate) was around 0.40 ± 0.05, and the inhibitor concentrations varied at regular intervals. The percentage inhibition of the chosen enzyme at each of the different inhibiotr concentration was taken as the mean value of the results of three assay measurements.

2.2.6 PRELIMINARY KINETIC STUDIES

Curves for the inhibition of human salivary α-amylase by SIα4 were obtained by continuous spectrophotometric measurement of the release of p-nitrophenol by hydrolysis of the artificial substrate p-nitrophenyl maltotrioside. The optical glass cuvette (1cm) contained 1.8μM of human salivary α-amylase, 10mM of the substrate p-nitrophenyl maltotrioside and varying concentrations (0-25μM) of the inhibitor SIα4 in 1ml of 25mM Tris/HCl, 1mM CaCl buffer pH 7.5. The reaction was started by the addition of the enzyme, after all of the reactants had equilibrated at 37°C. Changes in absorption at 405nm were followed in a Cary 105C spectrophotometer. All of the results were obtained without prior pre-incubation of the enzyme with the inhibitor.

2.2.7 ASSAY OF TRYPSIN INHIBITORY ACTIVITY

Assay of trypsin inhibitory activity was performed by using the method of Erlanger et al.(1961) as described by Campos and Richardson (1983). This method is based on the release by trypsin of the coloured product p-nitroaniline from the substrate N-benzoyl-DL-arginine-p-nitroanilide.

Preparation of N-benzol-DL-arginine-p-nitroanilide (BApNA) solution:

50mg of BApNA was dissolved in 1ml dimethylsulfoxide and diluted to 100ml with assay buffer (0.1M Tris-HCl pH 8.0 containing 0.01M $CaCl_2$).

Assay mixture:

20 µl trypsin (0.3mg/ml in 2.5mM HCl)

0.38 ml assay buffer

0.5 ml inhibitor solution

The enzyme and the inhibitor solutions were preincubated for 10 min at 37°C The substrate was added (2ml BApNA solution) and the reactants were incubated for 20 min at 37°C. The reaction was stopped by the addition of 1ml of 10% (v/v) acetic acid. The absorbance of the solution was measured at 410 nm.

2.2.8 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out using a Laemmli system with 20% acrylamide gel solution (Laemmli, 1970). Sigma kit for molecular weight (MW-SDS-70L) and the soybean Bowman-Birk trypsin inhibitor (obtained from Dr M.Richardson) were used as the molecular weight markers. Gels were

stained for 16h with a solution of 0.25% Coomaisie Brilliant Blue R-250 in 50% methanol, 7% acetic acid made up to a final volume of 1 L with distilled water. The destaining solution used was 7% acetic acid, 50% methanol completed to a final volume of 1 L with distilled water.

2.2.9 ISOELECTRIC FOCUSING

The IEF system used was based on Shewry et al.(1988). A gel was prepared by dissolving 22.34g urea (6M) in 30ml of a solution containg 10% acrylamide and 0.5% bisacrylamide in water. To this, 3ml of the 3.5-10 ampholyte were added and then made up to 60ml. 300µl of 10% (w/v) ammonium persulphate and 20µl of TEMED were added as catalysts and the gel poured into a slab equipment (10cm x 8cm x 1mm). The protein sample (1mg/ml) was dissolved in 10mm glycine, 6M urea with Tris-HCl pH 8.0. The anode and cathode buffers were respectively 1M H_3PO_4 and 1M NaOH.

The gel was pre-run for 1 h at 20mA with 30µl of a solution of 1% (w/v) 3.5-10 ampholyte in each slot. Duplicate sets of protein samples (20µl) were loaded into the slots and the gel run for 3h at 25mA. In order to calculate the pH gradient across the gel, half of the gel which contained one of the sets of applied samples was cut into 0.5cm strips each of which was placed in a separate test-tube. Each gel strip was disrupted with a glass rod then mixed with distilled water, left to stand for 15 min and the pH reading taken, then the tubes were sealed with Nescofilm left to stand overnight and the pH reading taken again. The pH values taken from the second reading were used for the pI determination (pH values are affected by the urea present in the gel, hence the readings obtained are only approximate values). The other half of the gel containing one of the duplicate sets of protein samples was fixed with a solution of TCA 12.5% during 48h with four regular changes of the same solution and stained in the same manner as described in the last section.

2.2.10 ESTIMATIONS OF THE MOLECULAR WEIGHT

In addition to the estimations of molecular weight made by SDS-PAGE (see above), the molecular weights of SIα1 and SIα2 were also measured using the plasma desorption time of flight method in the BioIon 20 Biopolymer mass analyser (Applied Biosystems Ltd), according to the instruction of the manufacturer.

Basic Principles of the Instrument and the Technique

The sample, which is deposited on a thin (0.5-1μm) aluminium or aluminized polyester foil, is placed at high potential (10-20kV). The sample molecules are desorbed and eventually ionized by bombardment with primary ions obtained from a 10μC source of ^{252}Cf placed immediately behind the sample foil. ^{252}Cf undergoes spontaneous fission with a rate of approximately 2000 fissions sec^{-1} emitting two colinear fission fragments. A typical pair of fission fragments are $^{106}Tc^{22+}$ and $^{142}Ba^{18+}$ with energies of 104 and 79 MeV respectively. One of the fission fragments pierces the foil and sample and causes desorption of a number of ions which are accelarated towards a grid at ground potential. The ions are then allowed to drift in a field free tube of 18cm length until they reach the stop detector. The flight time for each ion is measured with a time revolution of 1ns. The time measurement is initiated for each fission event by the opposite fission fragment hitting the start detector. Flight time, mass and charge are correlated by the equation $T= C_1 \sqrt{m}/Z + C_2$ where T is the flight time, m the mass and Z the charge of the ion, and $C_1$and C_2 constants. The constants C_1 and C_2 are established for each run, i.e. the spectra are calibrated by entering the time centroids for the peaks corresponding to H^+ and Na^+ in the computer.

The spectra from a number of fission events, usually between $5x10^5$ and 10^6, are accumulated on a computer corresponding to 3-10 minutes accumulation time, and then converted to the mass spectrum by calibrating as described above.

Methods and Sample Preparation

Two methods of sample preparation are currently used. The original method is based on electrospray of a sample solution on to aluminium foil (Brunix and Rudstan, 1961). This method has certain limitations because water-containing solutions are difficult to electrospray, and many proteins are not very soluble in organic solvents. Furthermore, the quality of the spectra obatined by this sample preparation technique is strongly influenced by low molecular weight contaminants in the sample, especially alkali metal ions. For analysis of proteins it is suggested that reduced glutathione should be added to the spray solution (Alai et al., 1986). This results in a noticeable improvement of the spectra and reduced sensitivity for alkali metal contaminants.

An alternative method is based upon first covering the aluminium foil with a thin layer of a material on to which the sample can be adsorbed, followed by washing of the surface. The adsorbing surfaces tested were an ion exchange surface, naphion (Jordan et al., 1985), a hydrophobic surface, polyethylene (Macfarlane et al., 1986) and a protein and nucleic acid binding surface, nitrocellulose (Jonsson et al., 1986). This was the one used for the studies of SIα1 and SIα2. The surface was prepared by electrospraying a 10µl solution of nitrocellulose (19µg/µl) on an aluminized polyester foil. The protein was dissolved in water. 3µl of this solution was then spread out over the surface and left for about 30 sec to allow binding of the protein. The surface was then washed with ultrapure water in order to remove contaminants which do not bind to nitrocellulose, e.g. salts.

2.3 Reduction and S-Carboxymethylation

All five inhibitors were reduced and S-carboxymethylated using a modification of the method of Crestfield et al.(1963) as described by Campos and Richardson (1983).

The protein (10-40mg) was dissolved in 6M guanidine hydrochloride buffered at pH 8.6 with 0.6M Tris-HCl (up to 2ml). Ten (10)µl of 2-mercaptoethanol/10mg protein was added and the mixture left standing at room temperature, under a constant stream of nitrogen for 3 h. Then 100-300µl of iodoacetic acid (0.268g/ml in 1M NaOH) was added and the reaction kept in the dark for 30 min. The reduced and S-carboxymethylated protein was recovered by dialysis against distilled water in the dark using dialysis membrane with a 2kDa cut-off value. The dialysed solution was then frozen with liquid nitrogen and lyophilised.

Samples for automated sequencing were S-pyridylethylated by using the vapour phase method of Amons (1987). Samples of the protein (2-10nmol) were dried on the sequencer glass fibre discs previously treated with TFA.The solvent was allowed to evaporate completely in a current of air at 40°C. S-pyridylethylation was performed in an ampoule which had a second constriction in its centre. The lower part of the ampoule was filled with a freshly prepared mixture of 100µl H_2O, 100µl pyridine, 20µl 4-vinylpyridine and 20µl tributylphosphine. The protein-(or peptide-) coated filter disk was placed into the upper part of the ampoule above the central constriction. This constriction still allowed a free diffusion of reagent vapors, but prevented the filter disk from being in direct contact with the liquid reagent mixture. The vapor-phase reaction was conducted for 2h at 60°C under an atmosphere of argon. Subsequently, the filter disk was removed from the ampoule, and washed three times in each of the following solvents: n-heptane; n-heptane: ethyl acetate (2:1, v/v); ethyl acetate, and dried. The filter disk was then wetted with 30µl of a solution of polybrene (50mg/ml H_2O), dried and loaded into the sequencer.

2.4 Chemical Cleavage with Cyanogen Bromide (CNBr)

The CNBr cleavage was performed by using the method of Steers et al.(1965) as described by Campos and Richardson (1983). The reduced and S-carboxymethylated SIα4 and SIα5 were separately dissolved in 1ml of 70% (v/v) formic acid containing 10µl of 2-mercaptoethanol and a 20-fold molar excess (relative to the expected number of methionine residues) of CNBr added. The reactions were kept in the dark for 24 h at room temperature. After this time the reaction was diluted 10-fold with distilled water, frozen and lyophilised. At this concentration of CNBr, normally the only peptide bonds cleaved are those involving the C-terminals of methionine but Ozals and Gerhard (1977) have reported the anomalous cleavage of tryptophanyl peptide bonds by an excess of CNBr in the presence of 70% formic acid.

2.5 Enzyme Hydrolyses

Samples (1-5mg) of the reduced and S-carboxymethylated α-amylase inhibitors (SIα 1-5) were digested separately with trypsin, chymotrypsin, *S.aureus* V-8 protease, elastase, and thermolysin.

2.5.1 TRYPSIN (E.C. 3.4.21.4)

The protein was dissolved in a minimal volume of 0.2 M N-ethylmorpholine buffer pH 8.5 and trypsin (TPCK-treated) added to give a 2% (w/v) enzyme/substrate ratio. The solution was incubated for 3-7 h at 37°C. The reaction was either stopped by freezing and lyophilization or by direct application onto a gel filtration column.

Trypsin catalyses the hydrolysis of peptide bonds on the carboxyl side of lysine and arginine, except where the following residue is a proline. In prolonged digestions some cleavage may be observed after tyrosine, leucine and phenylalanine (Allen, 1981).

2.5.2 CHYMOTRYPSIN (E.C. 3.4.21.1)

The digestion conditions were the same as for trypsin. Cleavage with chymotrypsin is less specific than with trypsin; cleavage usually occurs on the carboxyl side of leucine, tyrosine, phenylalanine, tryptophan and methionine (except where the following residue is proline) but may also occur at other sites.

2.5.3 *Staphylococcus aureus* (STRAIN V-8) PROTEASE (E.C. 3.4.21.19)

The protein was dissolved in a minimal volume of 0.1M sodium phosphate buffer pH 7.8 and incubated for 16 hours at 37°C with 2% (w/v enzyme/substrate) of the enzyme. The reaction was stopped by lyophilisation.

If the digestion is performed in 50-100mM sodium-phosphate buffer pH 7.8 the V-8 protease cleaves specifically on the carboxyl side of glutamyl and aspartyl-peptide bonds. The rate of digestion is low if glutamic acid or aspartic acid are within three residues of either the N- or C-terminus of a peptide containing lysine or arginine and the Glu-Pro and Asp-Pro peptide bonds are not cleaved (Austen and Smith, 1976; Croft, 1980; Drapeau, 1977). If the buffer is changed to either 0.1M NH_4HCO_3 (pH 7.8) or 0.1M ammonium acetate (pH 4.0), cleavage only occurs at glutamyl residues.

2.5.4 THERMOLYSIN (E.C. 3.4.24.4)

The conditions of digestion were the same as those used with trypsin, except that the buffer contained $CaCl_2$.

Thermolysin catalyses the hydrolysis of peptide bonds involving the amino group of the hydrophobic amino acids leucine, isoleucine, methionine, phenylalanine, tryptophan, and valine. Other sites, at the amino side of alanine, tyrosine and threonine may also be cleaved (Matsubara, 1965).

2.5.5 ELASTASE (E.C. 3.4.21.11)

The sample was dissolved in 0.1M ammonium bicarbonate pH 8.05 and up to 3% (w/w) of elastase (in the same buffer) was added. Incubation conditions were similar to those for trypsin (i.e. 37°C for 6-7 h.) (Aitken et al., 1989). Elastase catalyses the hydrolysis of peptide bonds involving amino and carboxilic groups of serine, cysteine, alanine and hydrophobic amino acids.

2.6 Purification of Peptide Mixtures

Two methods were employed to purify peptides produced by chemical cleavage or enzymic digestion. These were gel filtration and reverse-phase high performance liquid chromatography (HPLC).

2.6.1. GEL FILTRATION

Samples were dissolved in a minimal volume (0.5-1ml) of 0.1M ammonium bicarbonate and applied to a Bio-Gel P-6 column (1 x 200cm) equilibrated and eluted with the same buffer. Fractions of 1ml were collected at a flow rate of 4ml/h and peptides were detected by measuring the absorbance at 280nm and/or 230nm. Further purification was achieved by chromatographing the eluted peaks on reverse-phase HPLC.

2.6.2 REVERSE-PHASE HPLC

Reverse-phase HPLC was carried out using a VYDAC C_{18} analytical column (218 TP 54)-(25cm x 4.6mm) using variable gradients of acetonitrile in 0.1% trifluoroacetic acid (TFA) . Samples for injection were dissolved in 100µl 6M guanidine hydrochloride in

0.1% TFA. Peptides were detected by measuring the absorbance of the eluting solvent at 214nm. Peaks were collected manually and lyophilised.

2.7 Amino Acid Analyses

Samples of the reduced and S-carboxymethylated proteins and the peptides derived from them were hydrolyzed with 5.6M HCl containing 0.02% (v/v) cresol at 108°C for 24 hours. After drying *in vacuo* over solid NaOH the amino acids in the hydrolyzates were derivatised with PITC and analysed by HPLC using the Water Pico-Tag method (Milipore Waters Chromatography Corp., 1984 - Operators Manual N^{o} 88140).

2.8 Sequence Determination

The native and S-alkylated proteins were sequenced either employing manual and/or automatic methods.

2.8.1 DABITC-PITC DOUBLE COUPLING METHOD

Proteins and peptides were manualy sequenced using the DABITC-PITC double coupling method (Chang et al., 1978) as described by Yarwood (1989).

Samples of the peptide or protein (5-10nmol) were placed in a glass tube (0.5 x 3.0cm) fitted with a ground glass stopper and dissolved in 80µl of 50% (v/v) aqueous pyridine). 40µl of the DABITC solution (2.8mg/ml in pyridine) was added, the tube flushed with nitrogen, the contents mixed by agitation on a vortex and incubated at 52°C for 50 minutes. 10µl of PITC was then added, the mixture flushed again with nitrogen and incubated at 52°C for 20 minutes. The excess of reagents and by-products were

removed by extracting the mixture three times with a 2:1 (v/v) solution of heptane/ethyl acetate. The upper (organic) phase was separated in each case by centrifugation, removed by vacuum aspiration and discarded. The aqueous phase was dried down in vacuo over solid NaOH. 50µl of anhydrous TFA was added, the mixture was flushed once more with nitrogen and incubated for 15 min at 52°C. The TFA was removed by drying under vacuum. The extraction of the DABTZ-amino acid (4-N, N-dimethylaminobenzene-4'-thiazolinone-amino acid) was carried out by adding to the residue 50µl of water and 200µl of butyl acetate. After mixing on a vortex for about three seconds, the contents were centrifuged for approximately 1 min. The upper phase (butyl acetate) was removed, and the peptide in the aqueous phase was dried down in vacuo over solid NaOH and subjected to the next degradation cycle.

The butyl acetate extract was dried down in vacuo over solid NaOH and the residue was dissolved in 50µl of 50% aqueous TFA. Conversion of the thiazolinones of the amino acids into the thiohydantoins was carried out by heating at 80°C for 10 minutes. The sample was dried again under vacuum over solid NaOH and redissolved in a suitable volume (2-5µl) of 95% (v/v) ethanol.

The identification of the DABTH-amino acid (4-NN-dimetylaminobenzene-4'-thiohydantoin-amino acid) was done by thin layer chromatography (TLC). 4-NN-dimethylaminobenzene-4'-thicarbamyl (DABTC)-diethylamine was used as a marker and spotted at the bottom left corner of a 3 x 3 cm polyamide sheet. The DABTH-amino acid was spotted on top of the standard.

The first dimensional separation was run using an acetic acid/water (1:2, v/v) solution and after drying the sheet in a stream of warm air the second dimension separation with a toluene/n-hexane/acetic acid solution (2:1:1, v/v/v). After TLC the dried sheets were exposed to HCl vapours which led to the temporary formation of the characteristic red/pink, brown and blue colours of the DABTH-amino acids. The DABTH amino acids were identified by comparing the position of the coloured spots with

the standard (Fig.11) and reference to a map of the mobilities of the DABTH derivatives of the amino acids.

2.8.2 AUTOMATIC EDMAN DEGRADATION

Proteins and peptides (5 nmol) were sequenced using a model 477A automatic pulsed liquid phase protein sequencer (Applied Biosystems Ltd) employing a standard Edman degradation sequenator programme.

2.9 Determination of Disulphide Bonds

Intact native SIα1 (3mg) and SIα5 (5mg) were digested with trypsin and *S.aureus* V8 protease (SIα1), and chymotrypsin (SIα5). The peptides produced were separated by HPLC and sequenced as described previously.

2.10 Qualitative Method for Cysteine Analyses

A qualitative method for cysteine and cystine analysis was adapted from Cavins and Friedman (1970), and Inglis et al. (1976) to detect the presence of any of these residues in the tested protein.

Three equal amounts of the same protein (samples *a*, *b* , *c*) were dissolved in 200µl of 6M guanidine-HCl in 0.6M Tris-HCl pH 8.6. 2mM of DL-Dithiothreitol was added to sample *c* and incubated under nitrogen for one hour at room temperature. Then 15µl of 4-Vinylpyridine was added to samples *b* and *c*; the samples were incubated for 45min, under nitrogen, in the dark at room temperature. Sample *a* was used as the control.

The proteins were purified by HPLC as previously described. The lyophilized samples were dissolved in distilled water and U.V. absorption scan (320 nm to 220 nm) was taken to detect any change in the spectrum of the protein, caused in particular by the presence of S-β-4-pyridilethyl cysteine residues which absorb specifically at 254 nm.

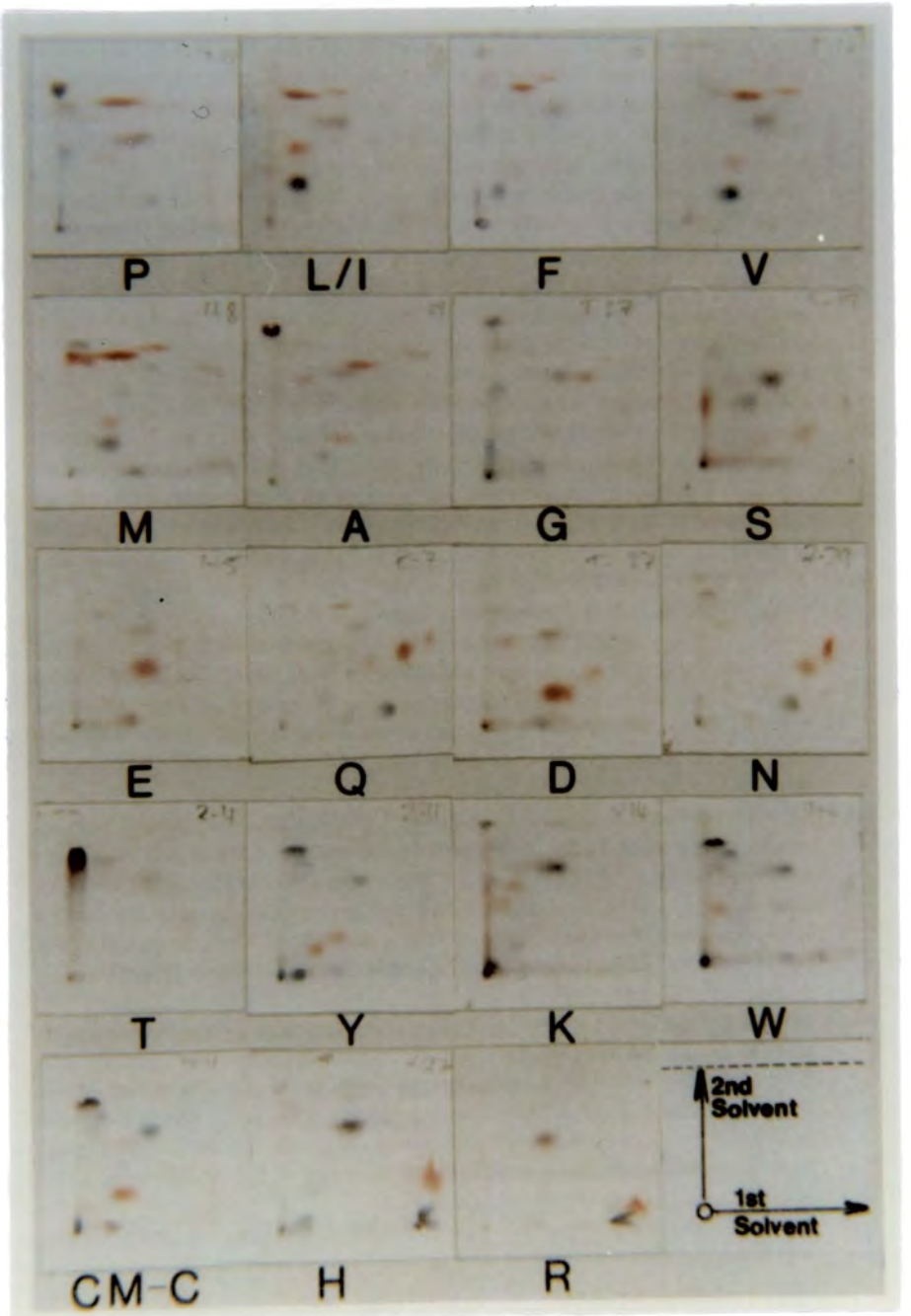


Fig 11: Chromatography of the DABTH-derivatives of amino acids, obtained by means of the DABITC/PITC double-coupling sequencing method. Solvent 1: glacial acetic acid:water (1:2); Solvent 2: toluene:n-hexane:glacial acetic acid (2:1:1 by vol.). DABTH-derivatives are identified by the single letter amino acid code.(Taken from Yarwood, 1989)

2.11 Protein Structure Analyses

2.11.1 PREDICTIONS OF SECONDARY STRUCTURE

The Garnier et al. (1978) computer prediction method was used for the determination of the secondary structures of SIα4 and SIα5. This method is presented as an algorithm based on properties derived from a database and results in a four state prediction: α-helix (H), extended chain (E), reverse chain (T) or random coil (C).

2.11.2 MOLECULAR WEIGHT CALCULATIONS AND ESTIMATIONS

The molecular weight of all five proteins (SIα1 - 5) and pI estimations were obtained from their respective amino acid sequences using the Mount and Conrad Sequence Analysis Package (Mount and Conrad, 1988) which was kindly provided by Dr N.Harris.

2.11.3 HYDROPHOBIC PLOTS

Hydrophobicity plots of SIα4, SIα5 and other related proteins were obtained by using the Kyte and Doolittle method (1982) and the computer software of Pearson and Lipmann (1988). This computer based method progressively evaluates the hydrophilicity and hydrophobicity of a protein along its amino acid sequence. For this purpose, a *hydropathy scale* has been composed wherein the hydrophilic and hydrophobic properties of each of the 20 amino acid side-chains is taken into consideration. The scale is based on an amalgam of experimental observations derived from the literature. The programme uses a moving-segment approach that continuously determines the average hydropathy within a segment of predetermined length as it advances through the sequence. The consecutive scores are plotted from the amino to the carboxy terminus. At

the same time, a mid point line is printed that corresponds to the grand average of the hydropathy of the amino acid compositions found in most of the sequenced proteins.

2.11.4 SEQUENCE ALIGNMENTS

The amino acid sequence alignments were obtained by using the CLUSTAL programme (Higgins and Sharp, 1988, 1989). The method is based on first deriving a phylogenic tree from a matrix of all pairwise sequence similarity scores, obtained using a fast pairwise alignment algotithm. Then the multiple alignment is achieved from a series of pairwise alignments of clusters of sequences, following the order of branching in the tree.

2.11.5 SEQUENCE IDENTITIES AND THEIR SIGNIFICANCE

The programmes FASTA, ALIGN and RDF2 (Pearson and Lipmann, 1988) were used to evaluate the percentage of sequence identities of the five sorghum inhibitors (SIα1-5) between themselves and with related proteins, and the biological significance of the obtained identities.

The FASTA and ALIGN programmes were used to search and locate all identities or groups of identities between two amino acid sequences giving the highest scoring alignment between the two studied sequences. RDF2 was an additional programme used for evaluating the statistical significance of the obtained alignments, which was done by comparing one sequence with the randomly permuted versions of the potentially related sequence. The similarity scores determined by FASTA do not follow a normal distribution from which one can calculate a probability (*P* value). As a result, the significance of a

similarity is frequently expressed not as a *P* value but as a *Z* value where: Z = (similarity score - mean of random scores)/(standard deviation of random scores).

Regarding the *Z* values of initial and optimazed score, it has been suggested by the authors that the following guidelines apply:

Z > 3 possibly significant

Z > 6 probably significant

Z > 10 significant

RESULTS AND DISCUSSION

3.1 Purification and characterization of the α-amylase inhibitors from *Sorghum bicolor*

The method used for the extraction of the α-amylase inhibitors from seeds of sorghum was essentially the same as used by Kutty and Pattabiraman (1986a) for this species and from other cereals except that the ammonium sulphate fraction collected was 0-60%.

Affinity chromatography on Red-Sepharose (procion red HE-3B ligand) has proved to be a useful step in the purification of protein inhibitors of α-amylases from a number of cereal seeds (Kutty and Pattabiraman, 1986a, 1986b, 1987; Ary et al., 1989; Richardson, 1991). The reason why the α-amylase inhibitors from cereal seeds bind to the procion red ligand is yet unknown (Turner, 1981; Lowe and Pearson, 1984). Figure 12 shows the results of a typical affinity chromatography of the 60% ammonium sulphate fraction on Red-Sepharose. In some cases peak 1 (unbound material) was found to contain low levels of α-amylase inhibitory activity which was thought to be due to saturation of the ligand. In these instances the unbound material was subjected to a further passage through the affinity column. Trypsin inhibitory activity was also detected in the unbound material (peak 1) which was in accord to the findings of Kumar et al (1978), who investigated proteinase inhibitors in grains of sorghum. Elution of the bound α-amylase inhibitors was achieved with 3M NaCl (peak 2), however subsequent washing of the column with 6M guanidine in the elution buffer released small quantities of α-amylase inhibitory material together with pigments thought likely to be tannins. No trypsin inhibition was found in either of these two eluted materials.

When the inhibitor material eluted by 3M NaCl was examined by SDS PAGE (Fig. 13) a small number of bands were observed. The proteins were further fractioned by preparative reverse phase HPLC. This yielded six major peaks (Fig. 14) of inhibitory activity against human salivary α-amylase. The inhibitors were coded SIα1, SIα2, SIα3,

according to their order of elution. Each of these inhibitors was then further purified by rechromatography on analytical reverse phase HPLC using an extended gradient. This combination of methods yielded the six different inhibitors in a pure form (as confirmed by subsequent N-terminal amino acid sequencing).

Under SDS-PAGE conditions SIα1, SIα2, SIα3 showed the greatest mobility, migrating even further than the 8kDa marker used (Fig. 13) and suggesting that these inhibitors had an approximate molecular weight of 5 kDa. This result was then confirmed by time of flight desorption mass spectroscopy which revealed that SIα1 and SIα2 had molecular weights of 5372.3 and 5217.5 respectively.

SIα4 and SIα5 showed mobilities comparable to the marker α-Lactalbumin (14.2 kDa) with SIα5 migrating further than SIα4. Both inhibitors were considered to have a molecular weight of approximatly 14 kDa (Fig. 13). These results are different from those reported for the human salivary α-amylase inhibitor from sorghum by Kutty and Pattabiraman (1986a, 1987). These workers stated that they had purified a 21 kDa inhibitor from salivary amylases which behaved as a 10 kDa molecular weight protein during SDS-PAGE.

SIα6 migrated much less than the other inhibitor. Its mobility was comparable to the molecular weight marker carbonic anhydrase (29kDa) (Fig. 13).

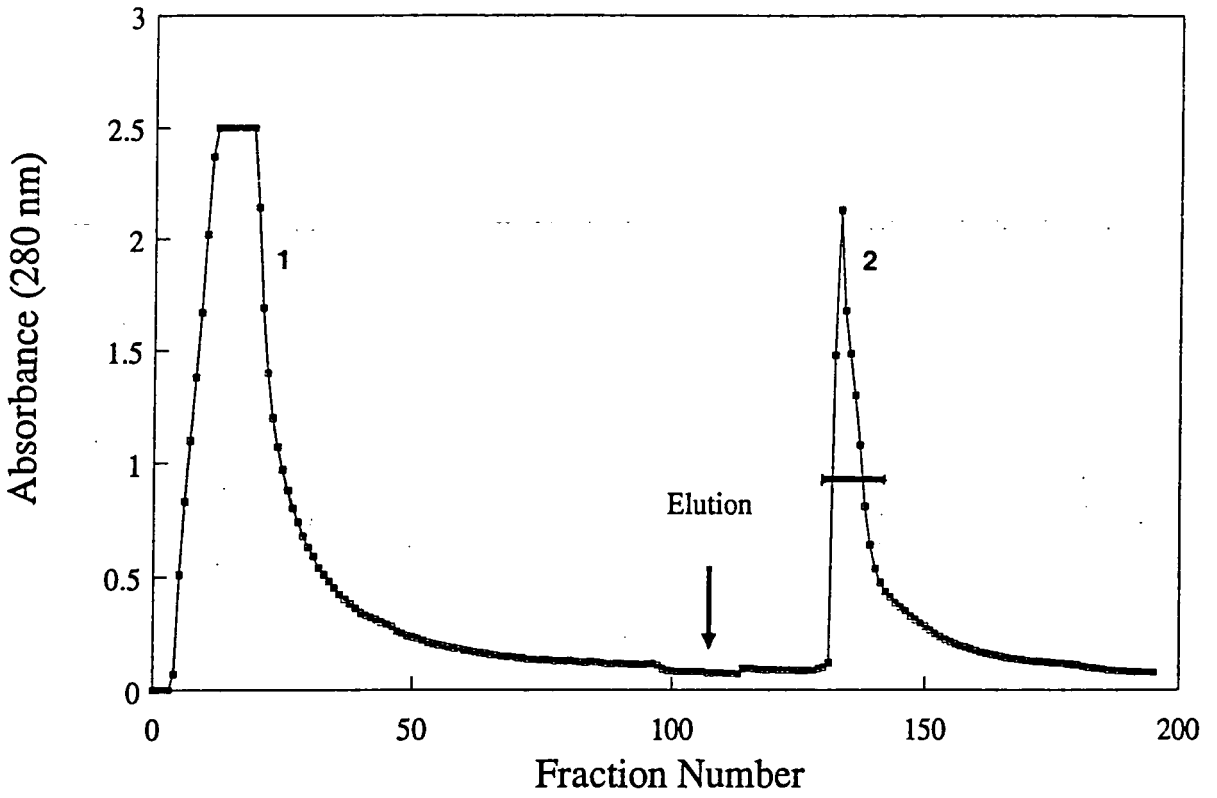


Fig.12: Affinity chromatography of 60% ammonium sulphate precipitate from extract of sorghum seeds on Red-Sepharose CL-6B (2.5 x 20cm) column equilibrated and washed with 0.05M Tris-HCl buffer pH 7.0, containing 0.1M NaCl. Peak 1 contained unbound material. The arrow (↓) indicates the start of the elution with buffer containing 3M NaCl. The main fractions containing inhibitors of α-amylase (peak 2) which were collected are shown by the bar (⊢⊣)

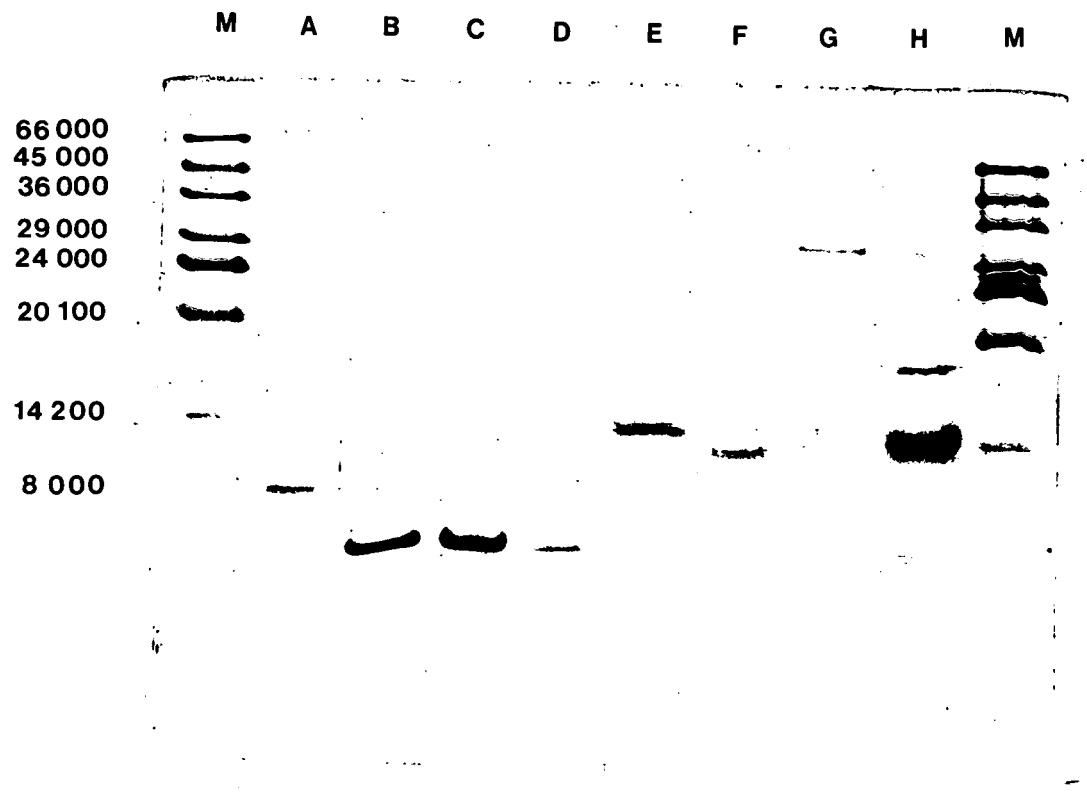


Fig.13: SDS-PAGE of the sorghum inhibitors (SIα1-6). (M) Sigma molecular weight markers; (A) Bowman-Birk trypsin inhibitor from soybean; (B) SIα1; (C) SIα2; (D) SIα3; (E) SIα4; (F) SIα5; (G) SIα6; (H) Eluted material from Red-Sepharose (peak 2, Fig.12).

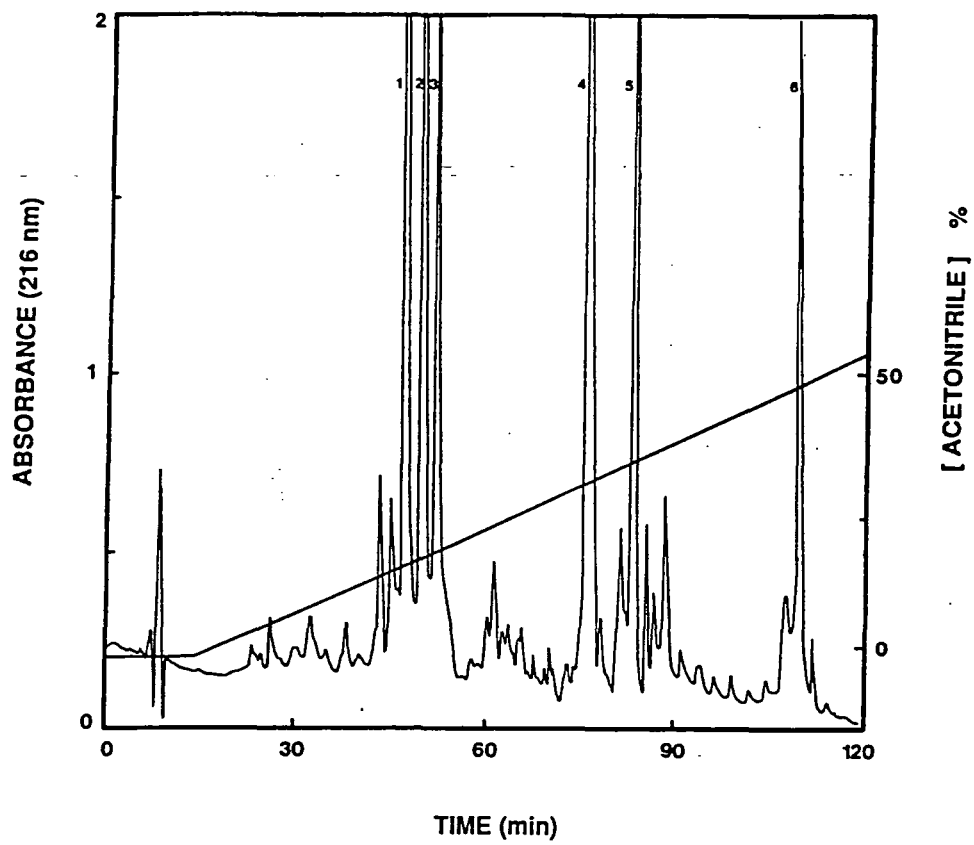


Fig.14: Preparative reverse-phase HPLC separation of sorghum α-amylase inhibitors obtained from affinity chromatography on Red-Sepharose (Fig. 12, peak 2). The proteins were dissolved in 1.5ml of 6M guanidin HCl in 0.1% aqueous TFA and applied to the reverse-phase HPLC column (22mm x 25cm) of Vydac C_{18} (218.TP 1022, Tecnicol ltd) which was eluted with a gradient of acetonitrile (thiker solid line) in 0.1% aqueous TFA. The numbered peaks (1-6) contained inhibitors of α-amylase.

Isoelectric focusing of SIα5 gave a single band with a pI in the region of 8.85. However, the other inhibitors failed to focus under the same experimental conditions which probably indicated a very basic nature of the polypeptide chain of these inhibitors. This was subsequently confirmed by the results of amino acid sequencing (See section 3.6). A similar phenomenon was also observed by Campos and Richardson (1983) with the Ragi bifunctional trypsin/α-amylase inhibitor.

3.2 Specificity of the Inhibitors

Assays of the inhibitory activity of the eluted material from Red-Sepharose affinity chromatography and all six reverse-phase HPLC purified proteins (SIα1-6) revealed clear activity of these substances against human salivary α-amylase (see Fig. 15-19). These results are in accord with those of Chandrasekher et al. (1981) who were the first to detect inhibitors of human salivary α-amylase from sorghum seeds, and also with the work of Kutty and Pattabiraman (1986a, 1986b, 1986c). In addition to this, all of these 6 proteins were also observed to be active against at least one α-amylase from the guts of some insects.

The 14kDa inhibitors (SIα4 and SIα5) showed activity against the amylases from guts of adult cockroach, and the larvae of locust and *Spodoptera litoralis* (Fig. 16-18). It was observed that 15μg (~$1.2x10^{-6}$M) of SIα5 inhibited 93% of human salivary amylase (2.8μunit) (Fig. 15); 69% of cockroach gut amylase (3.9μunit) (Fig. 16); 57% of locust gut amylase (2.4μunit) (Fig. 17); and 51% of *S.litoralis* larvae gut amylase (2.0μunit) (Fig. 18). 15ug (~$1.1x10^{-6}$M) of SIa4 inhibited 76% of human salivary amylase (2.8μunit) (Fig. 15); 69% of cockroach gut amylase (3.9μunit) (Fig. 16); 47% of locust gut α-amylase (2.4μunit) (Fig. 17); and about 30% of *S.litoralis* larvae gut amylase (2.0μunit) (Fig. 18). The amylase residual activity curves (Fig. 15-18) indicate that SIα5 is apparently more active than

SIα4 for the same tested enzymes. SIα4 and SIα5 also showed very weak inhibition (below 30%) against α-amylases from *A.oryzae* and *Bacillus* spp, but no detectable inhibition under the assay conditions used was found towards the porcine pancreatic, barley and sorghum amylases. Inhibitor SIα6 also show a similar inhibitory pattern as SIα4 and SIα5 but no amylase residual activity curve was done

These results represent an additional confirmation of the numerous previous reports that cereals contain inhibitors particularly effective against amylases from insects (Silano et al., 1975; Buker, 1987b, 1988; Ary et al., 1989).

SIα1, SIα2, SIα3 were found to be active against amylases from locust and cockroach guts, *A.oryzae* and human saliva. It was observed that 300μg (~$5.6x10^{-5}$M) of SIα1 and SIα2 inhibited about 80% and 70% of locust gut amylase (2.8μunit) (Fig.19). The amylase residual activity curves of SIα1 and SIα2 indicate that SIα1 is about 10% more active than SIα2 for all inhibitor concentrations tested. With these inhibitors, maximum inhibition of locust gut amylase was achieved with molar concentrations of approximately 47 times higher than the ones observed for SIα4 and SIα5. The shapes of the amylase residual activity curves of SIα1 and SIα2 also differ from those of the SIα4 and SIα5. This difference is more clear in the lower inhibitor concentration intervals where the curves assume a semi-parabolic form (Fig. 19). These features strongly suggest that SIα1, SIα2 and SIα3 may inhibit α-amylase in a quite different manner to the SIα4 and SIα5. It was not possible to obtain amylase residual activity curves of SIα1, SIα2 and SIα3 with other enzymes because the limited amount of these proteins available after the purification step and the high concentration of inhibitor needed for each experiment.

All the curves shown in the figures 15 to 19 were obtained from the data of the percentage residual activity of each tested α-amylase after 30 min preincubation with a given amount of a chosen inhibitor. For each set of residual activity values the best available curve was fitted by hand and also by using non-linear regression. The adjusted curves obtained by non-linear regression in all cases but one (Fig. 19) failed to represent

more accurately the initial intervals of the residual amylase activity which have been observed to be almost linear in the majority of previously reported cases (O'Donnell and McGeeney, 1976; Buonocore et al., 1980, 1984; Baker, 1988).

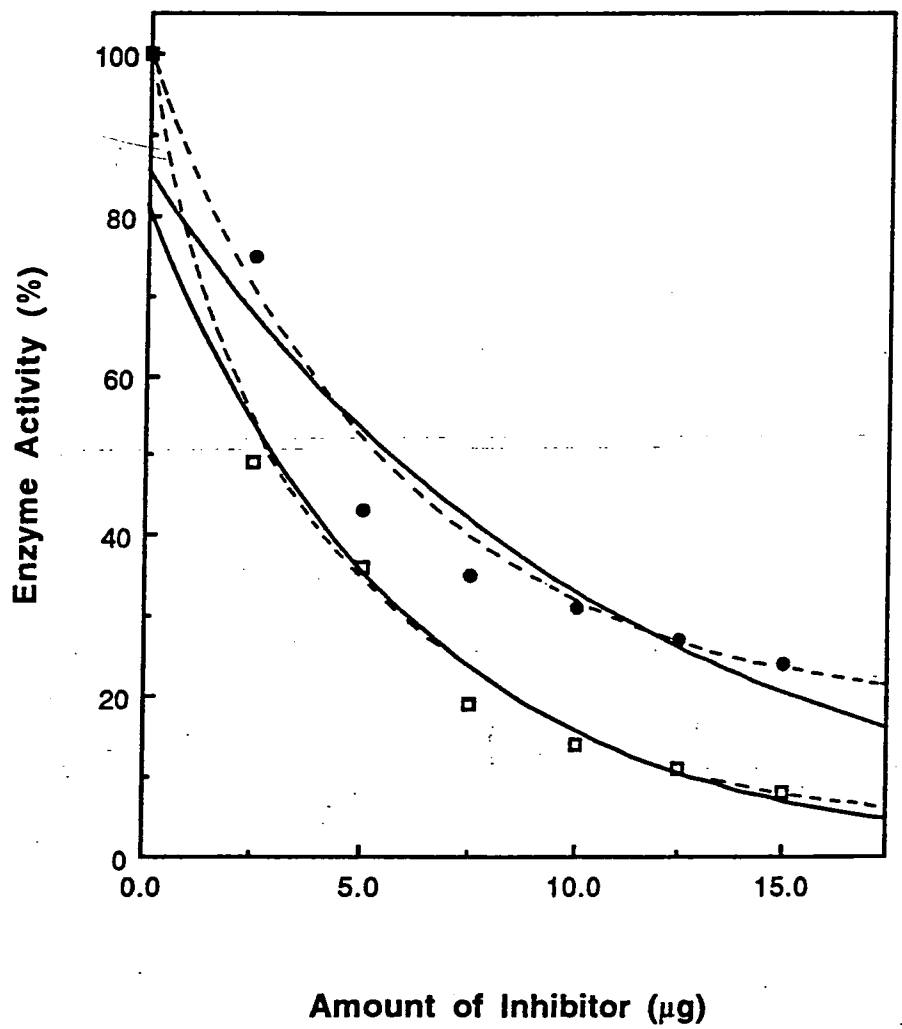


Fig.15: Inhibition of human salivary α-amylase by different amounts of SIα4 (●) and SIα5 (□). Enzyme activity (2.8 µunit). Curves fitted by hand (---); Curves fitted by non-linear regression (—).

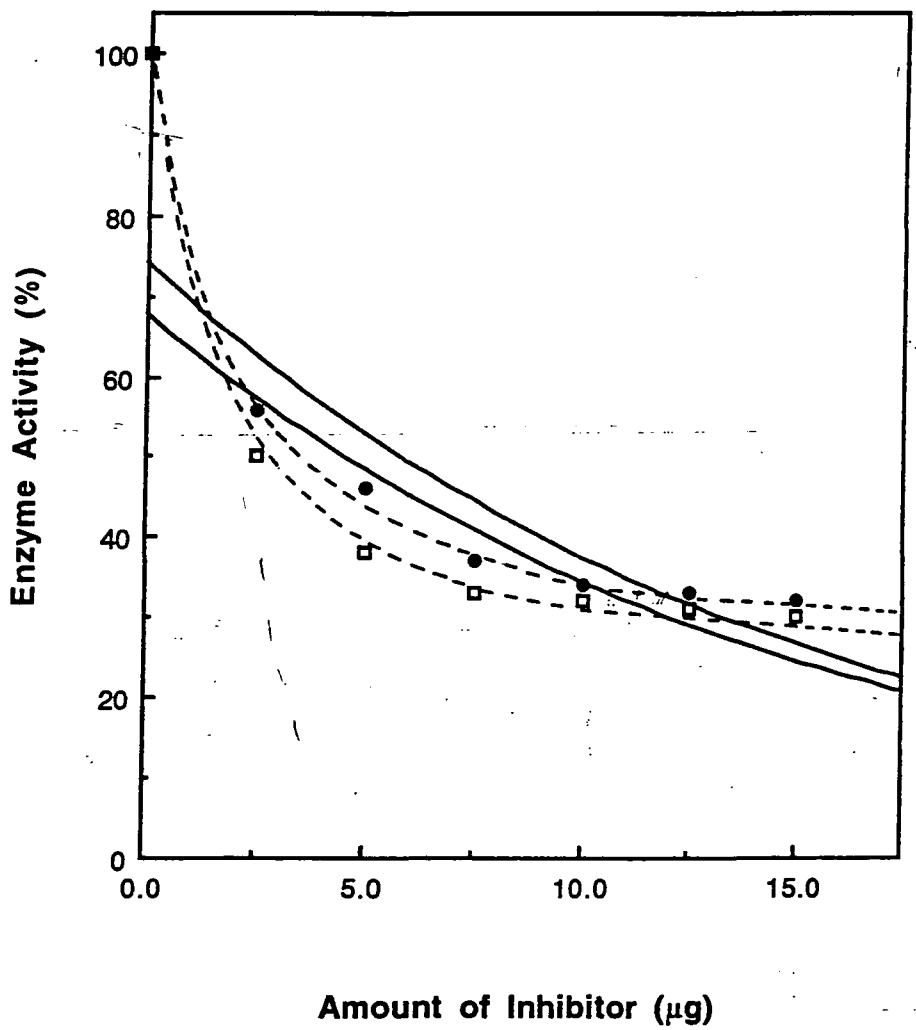


Fig.16: Inhibition of cockroach (*Periplaneta americana*) gut amylase by different amounts of SIα4 (●) and SIα5 (□). Enzyme activity (3.9 μunit). Curves fitted by hand (--); Curves fitted by non-linear regression (—).

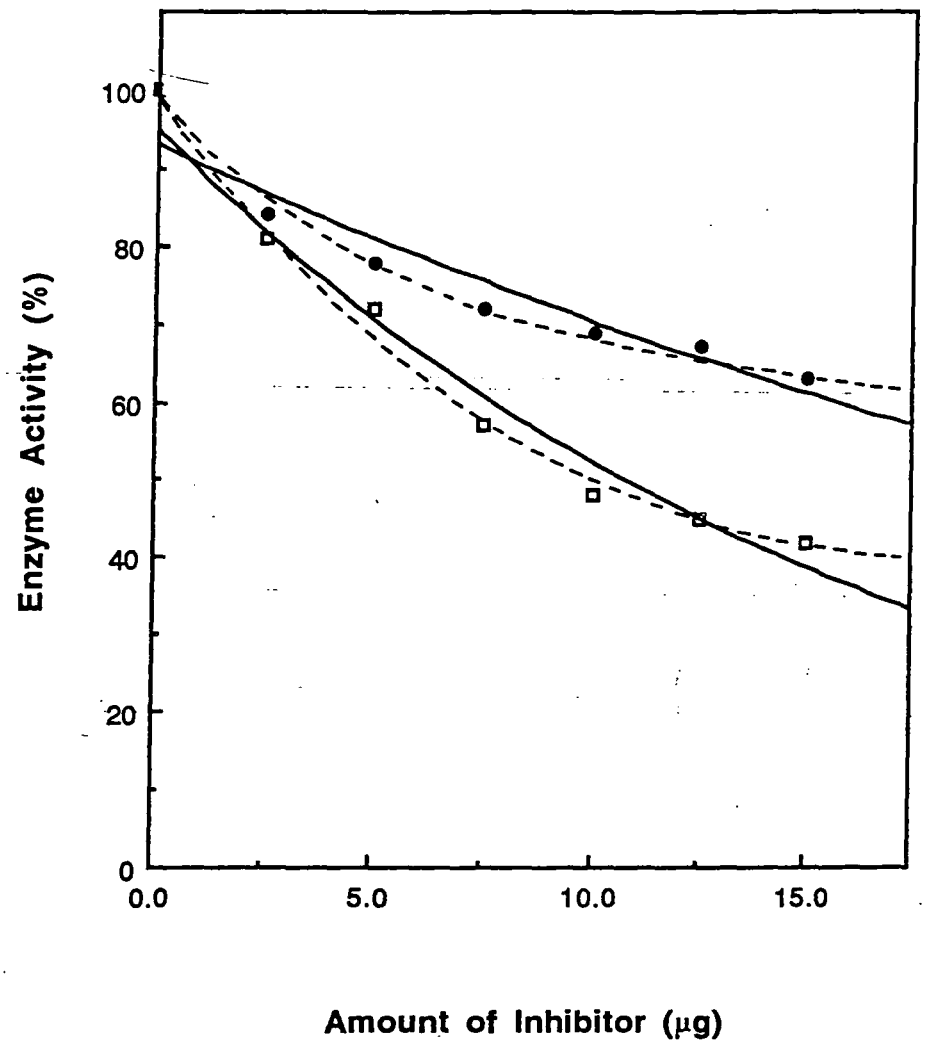


Fig.17: Inhibition of locust gut amylase (*Locusta migratoria, migratorioides*) by different amounts of SIα4 (●) and SIα5 (□). Enzyme activity (2.4 µunit). Curves fitted by hand (---); Curves fitted by non-linear regression (—).

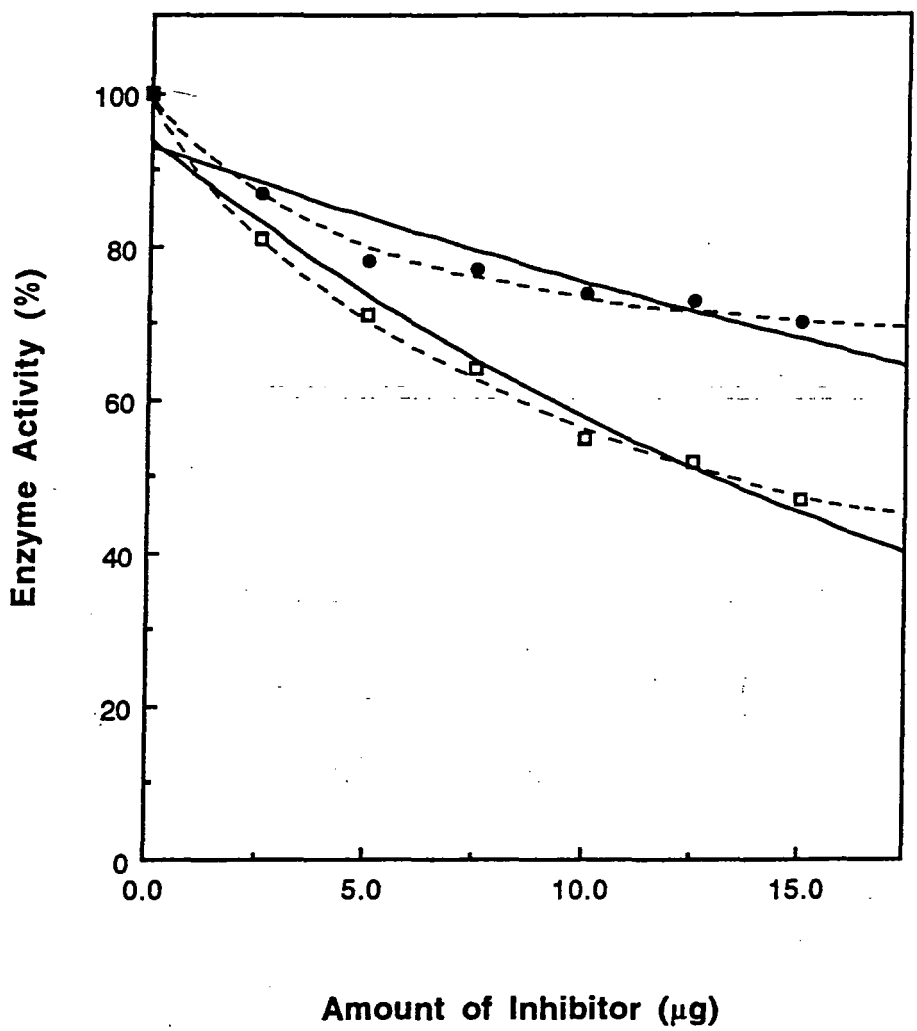


Fig.18: Inhibition of *S.litoralis* larvae gut amylase by different amounts of SIα4 (●) and SIα5 (□). Enzyme activity (2.0 µunit). Curves fitted by hand (--); Curves fitted by non-linear regression (—).

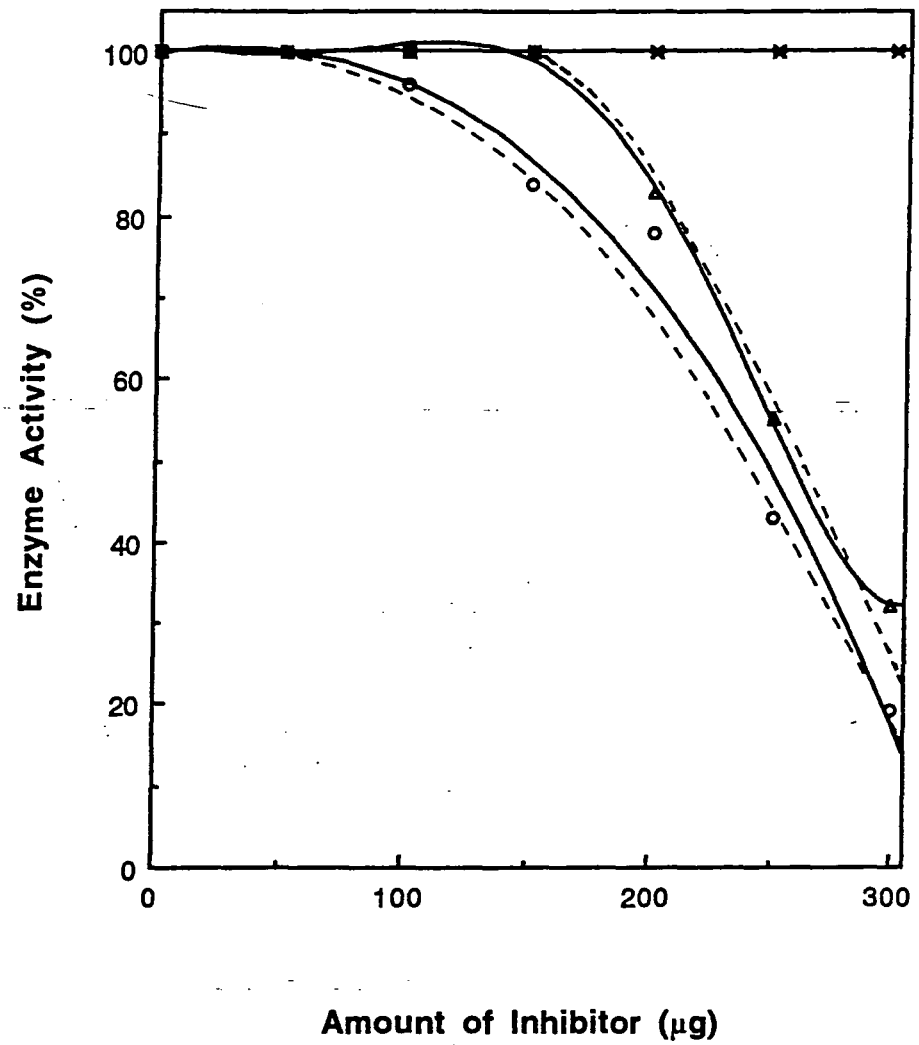


Fig.19: Inhibition of locust gut amylase (*Locusta migratoria, migratorioides*) by different amounts of SIα1 (O), SIα2 (▲) and BSA (*). Enzyme activity (2.4 µunit). Curves fitted by hand (---); Curves fitted by non-linear regression (—)

3.3 Preliminary Kinetic Studies

Preliminary studies of the continuous progress curves ([P] vs t) generated from the hydrolysis of p-nitrophenyl-α-D-maltotrioside by human salivary α-amylase in the presence and absence of the inhibitor SIα4 showed that the enzyme activity was progressively reduced when this inhibitor was present (Fig. 20). The concentration of 10mM of p-nitrophenyl-α-D-maltotrioside was previously chosen (based on Wilcox and Whitaker, 1984) for all the experiments and three different SIα4 concentrations (5μM, 15μM, 25μM) were tested against 1.8μM of human salivary α-amylase. This enzyme concentration was chosen after a series of experiments using different concentrations (0.5μM - 5.0μM) of human salivary amylase with 10mM of p-nitrophenyl-α-D-maltotetraside. The molar absorptivity of p-nitrophenol (product of the reaction) in standard buffer (5mM phosphate buffer, pH 6.9, containing 0.25M NaCl and 1mM $CaCl_2$) was determined to be $9000cm^{-1}$ at 20°C. Because of the preliminary nature of these experiments this extinction coefficient value was used without the necessary corrections to calculate the approximate product concentration [P] during the reactions. The three different inhibitor concentrations tested against a fixed 1.8μM α-amylase yielded three curves which showed similar shapes to those observed in the red kidney bean α-amylase inhibitor/porcine pancreatic α-amylase progress curves (Wilcox and Whitaker, 1984). These shapes are typical of slow binding and slow, tight-binding inhibition (Williams and Morrison, 1979; Morrison 1982; Longstaff and Gaffney, 1991). Because the inhibitor concentrations used could be assumed as "comparable" to the enzyme concentration (Morrison, 1982), these results suggest that slow, tight-binding inhibition might be occuring between SIα4 and human salivary α-amylase. However, these results alone are insuficient to give a more complete understanding of the type of kinetics involved in this case. The reason why SIα4 was used instead of any other of the sorghum inhibitors was because of its easier solubility than SIα5 and its higher yields during the purification step.

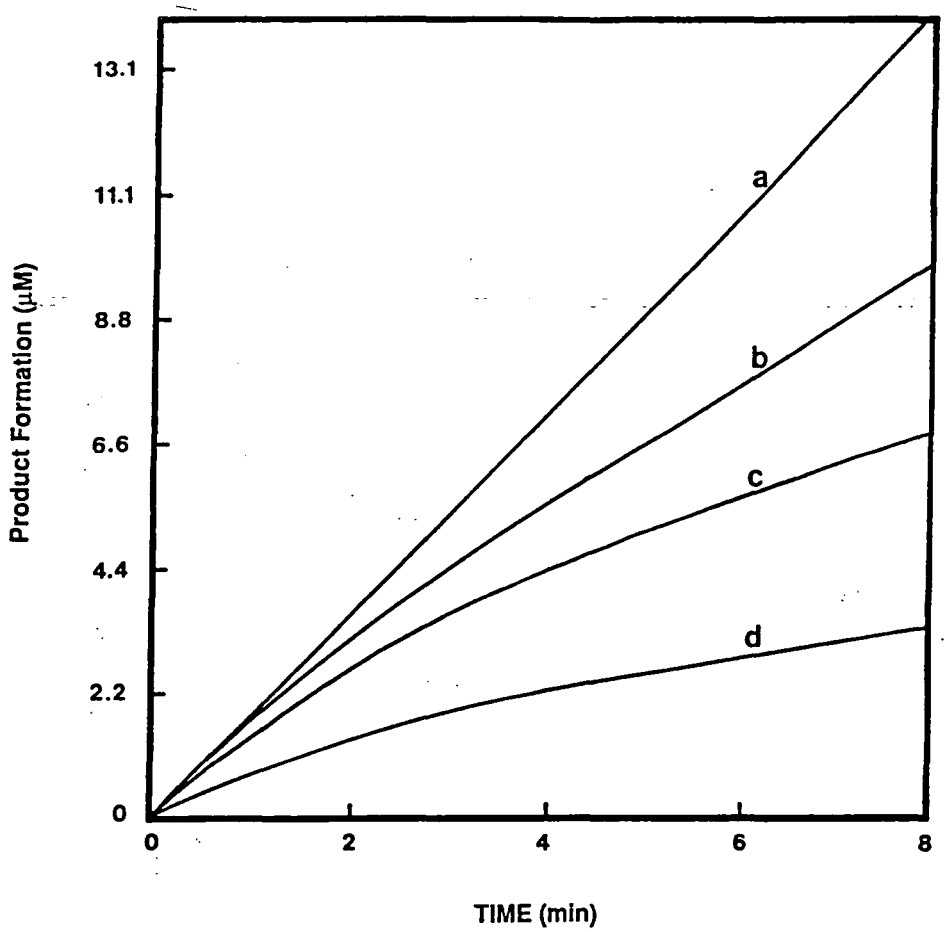


Fig 20: Continuous progress curves [**P**] *vs* **t** generated from the hydrolysis of p-nitrophenyl-α-D-maltotetraoside by human salivary α-amylase (1.8 μM) in the presence and absence of SIα4 at pH 6.9 at 37°C. (a) No inhibitor; (b) [I] = 5μM; (c) [I] = 15μM; (d) [I] = 25μM.

3.4 Purification of the Peptides from Enzymatic Digestion and Cyanogen Bromide (CNBr) Cleavage of Inhibitor Proteins

Peptides produced by enzymatic digestion (see Material and Methods, section 2.6) of reduced and S-carboxymethylated SIα4 and SIα5 were initially size fractionated by gel filtration on a BioGel P-6 column (Figs 21-26). Each peak from each gel filtration was further purified by a reverse phase chromatography using a Vydac C_{18} analytical column (two of them are shown in the figures 27 and 28). The major peaks were collected manually frozen with liquid nitrogen, lyophilized and stored an 0°C for amino acid sequencing. This peptide purification procedure proved to be one of the most important steps for the complete determination of both the SIα4 and SIα5 sequences. Cyanogen bromide cleavage of SIα4 and SIα5 produced no detectable peptide from SIα4 and just one from SIα5 which was separated by reverse phase HPLC on C_{18} analytical column using an extended gradient of acetonitrile (0-60%).

Peptides produced from reduced and S-carboxymethylated SIα1, SIα2 and SIα3 were separated only by reverse phase HPLC on Vydac C_{18} analytical columns using the same extended gradient of acetonitrile used for the CNBr peptides. However, in these cases, this single step purification proved highly satisfactory in providing peptides for sequencing with vary few contaminations. This may be related to the small size of the proteins and hence a smaller number of peptides to be separated.

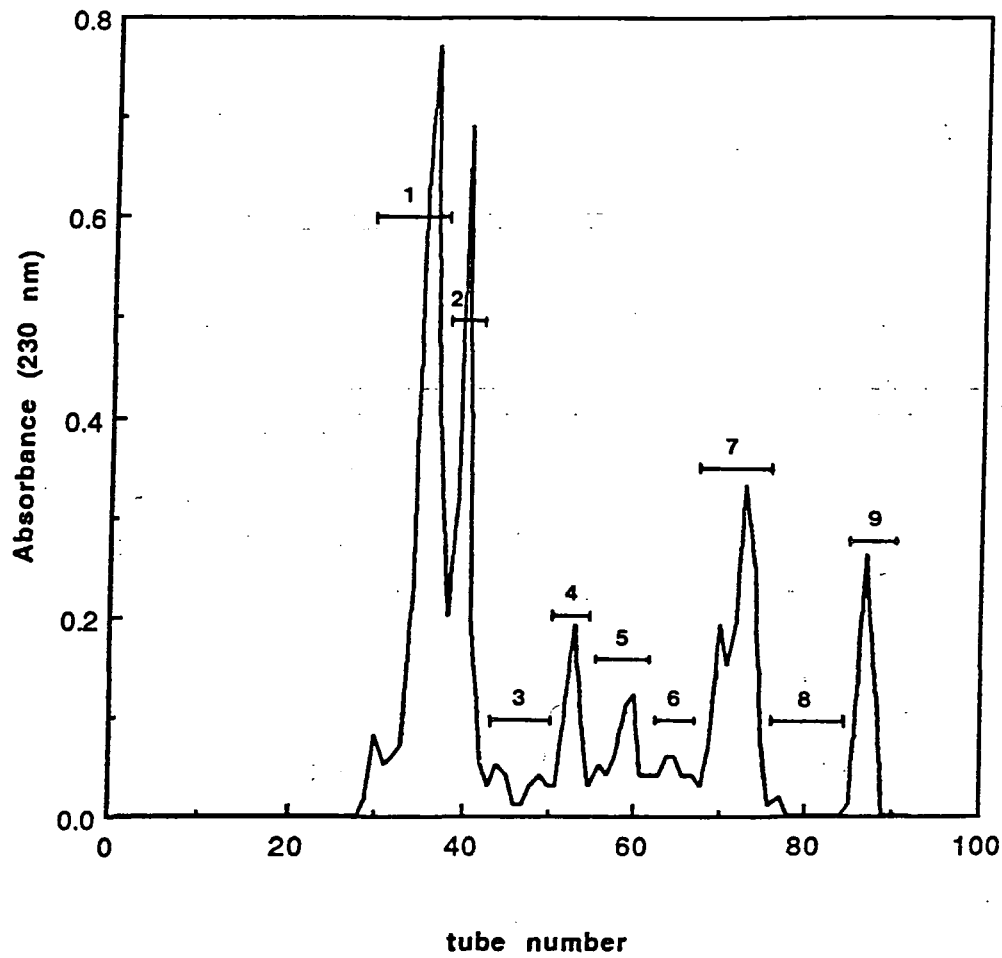


Fig 21: Gel filtration of the SIα4 after enzymatic digestion with trypsin on a Bio-Gel (1 x 200cm) column in 0.1 NH_4HCO_3 (flow rate 4ml/h, fraction size 1 ml). (⟼) indicates fractions pooled.

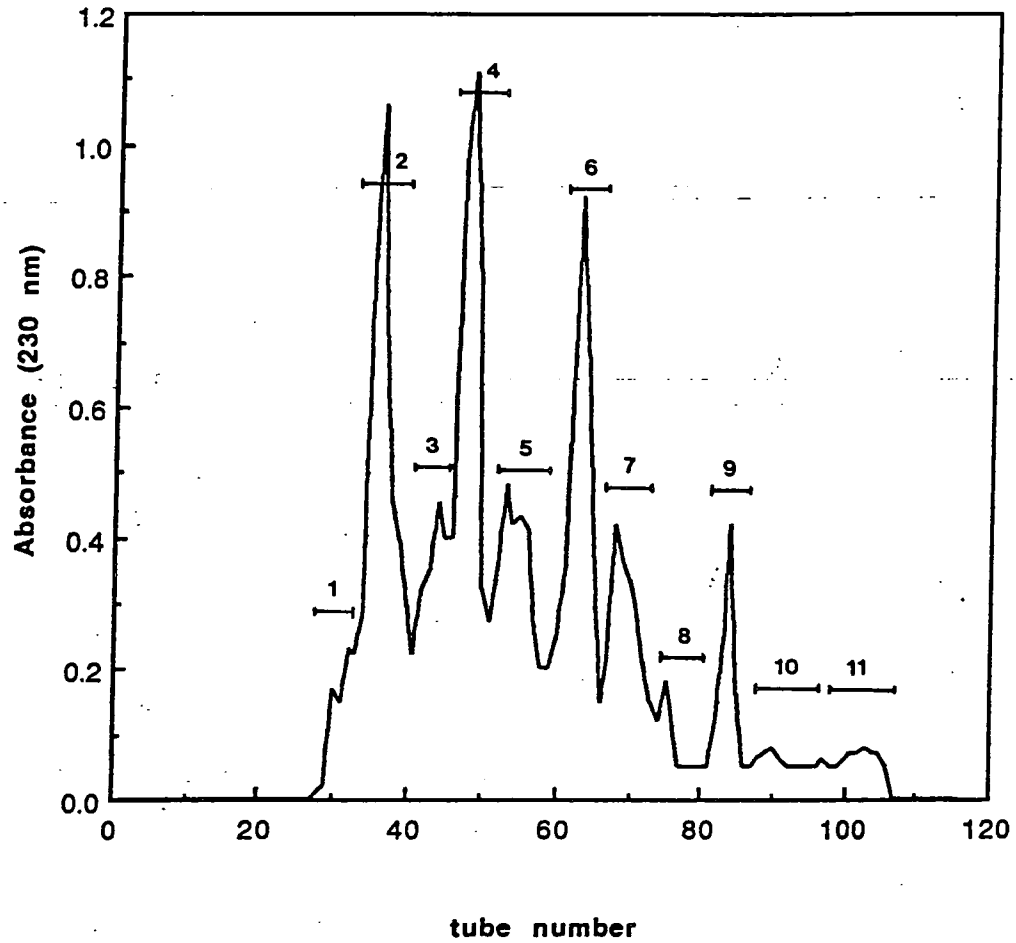


Fig 22: Gel filtration of the SIα4 after enzymatic digestion with chymotrypsin on a Bio-Gel P-6 (1 x 200cm) column in 0.1M NH_4HCO_3 (flow rate 4ml/h, fraction size 1ml). (⟼) indicates fractions pooled.

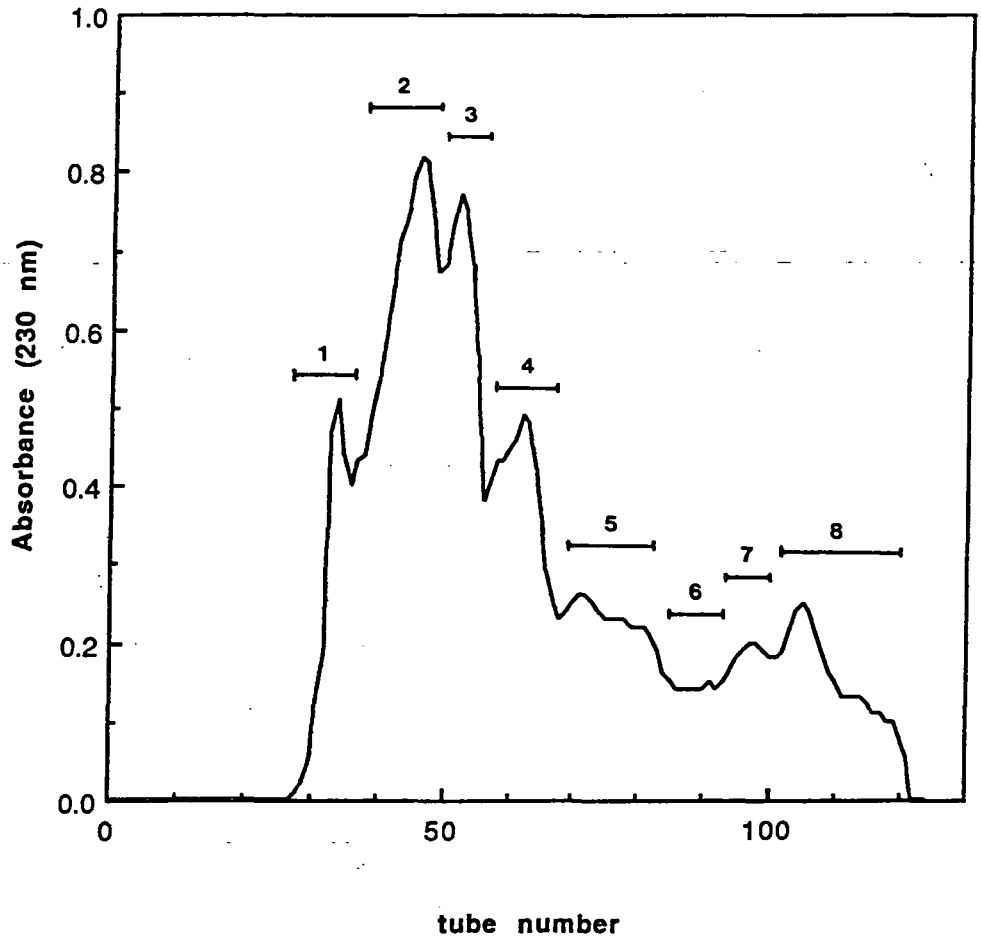


Fig 23: Gel filtration of SIα4 after enzymatic digestion with *S.aureus* V8 protease on a Bio Gel P-6 (1 x 200cm) column in 0.1M NH_4HCO_3 (flow rate 4ml/h, fractions size 1 ml). (⟼) indicates fractions pooled.

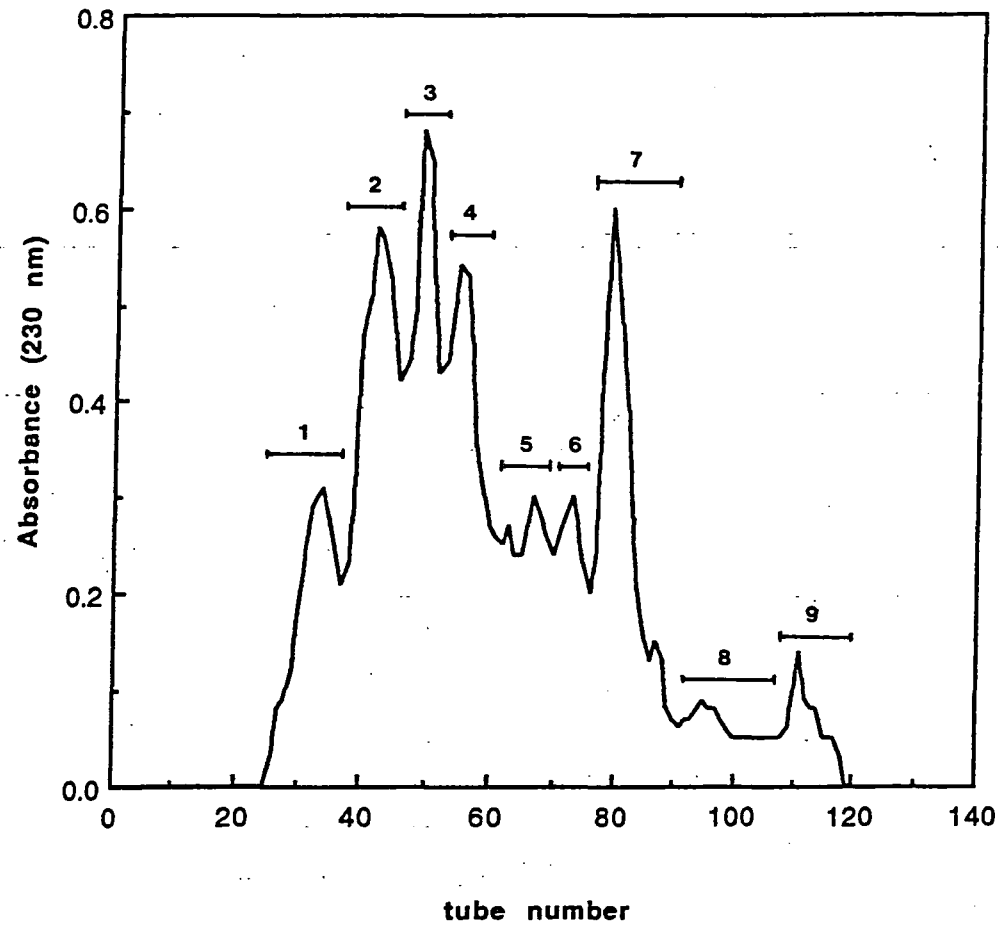


Fig 24: Gel filtration of SIα4 after enzymatic digestion with elastase on a BioGel P-6 (1 x 200cm) column in 0.1M NH_4HCO_3 (flow rate 4ml/h, fractions size 1ml). (⊢⊣) indicates fractions pooled.

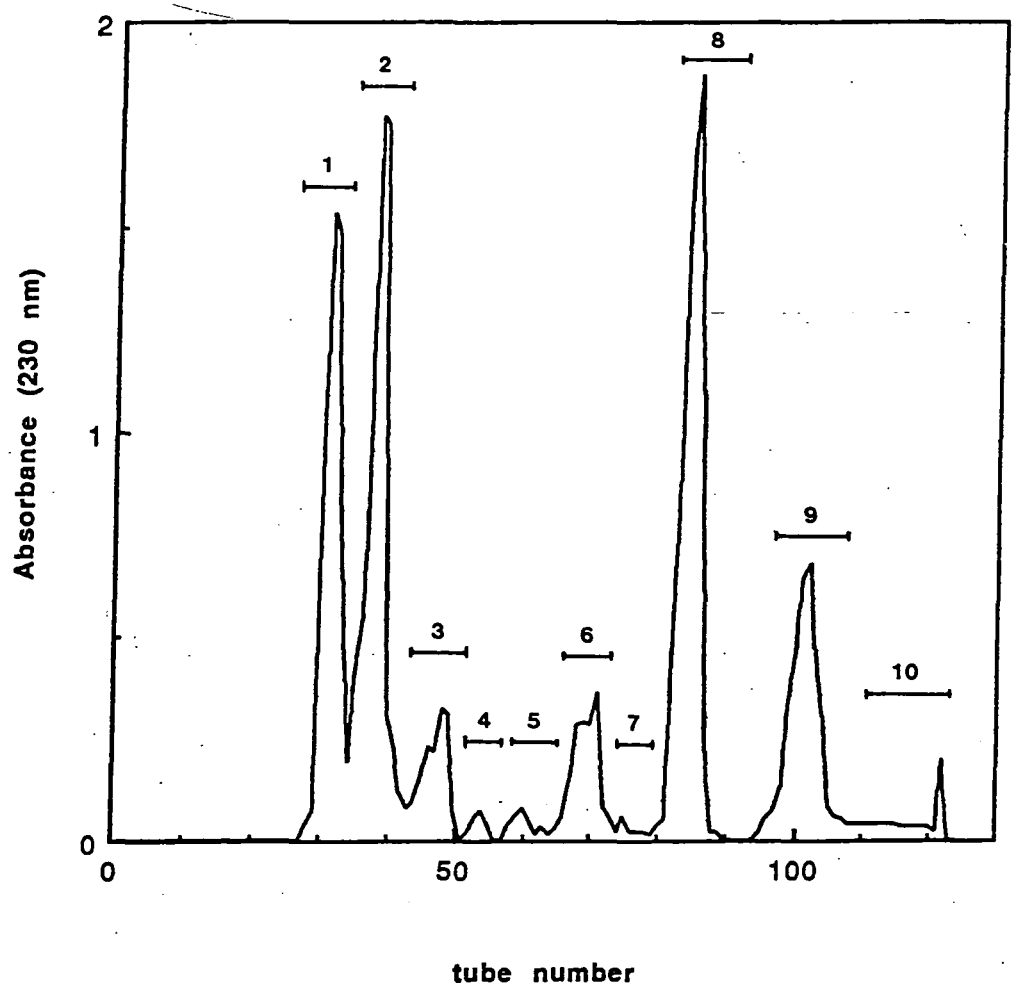


Fig 25: Gel filtration of SIα5 after digestion with trypsin on BioGel P-6 (1 x 200cm) column in 0.1M NH_4HCO_3 (flow rate 4ml/h, fractions size 1ml). (⟷) indicates fractions pooled.

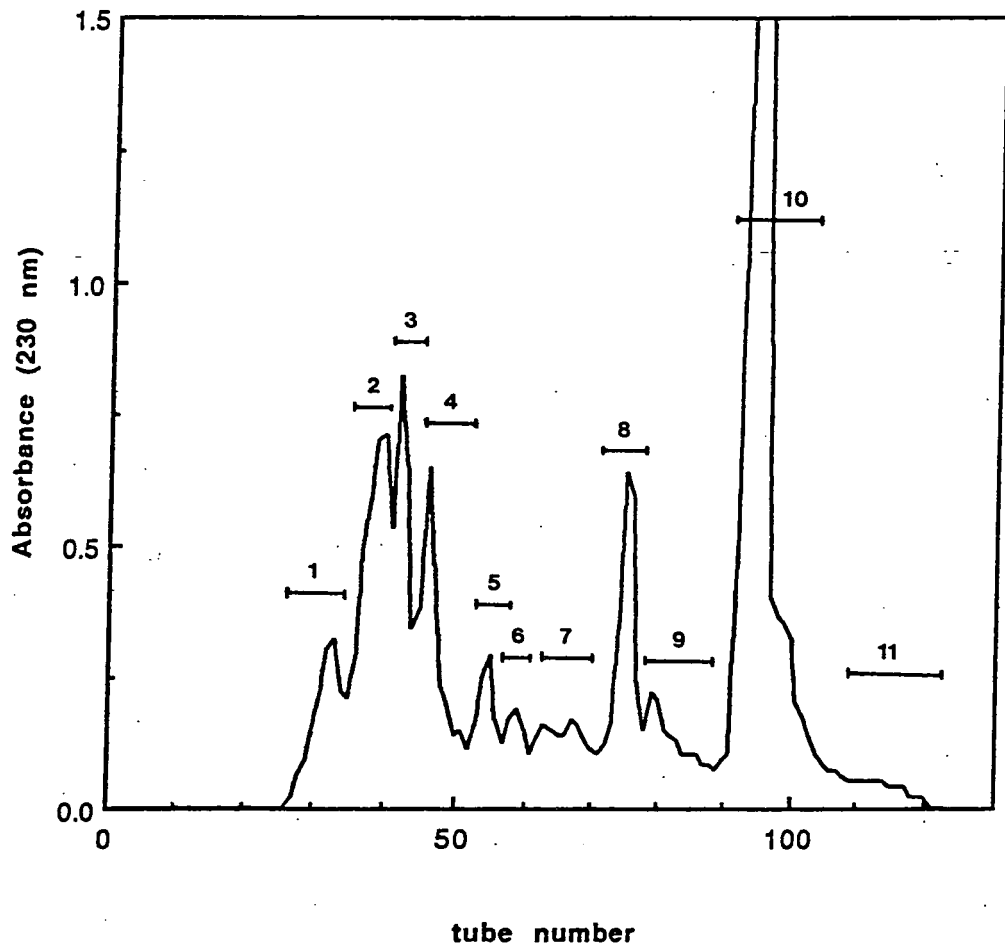


Fig 26: Gel filtration of SIα5 after enzymatic digestion with chymotrypsin on a BioGel P-6 (1 x 200cm) column in 0.1M NH_4HCO_3 (flow rate 4ml/h, fractions size 1ml). (⊢⊣) indicates fractions pooled.

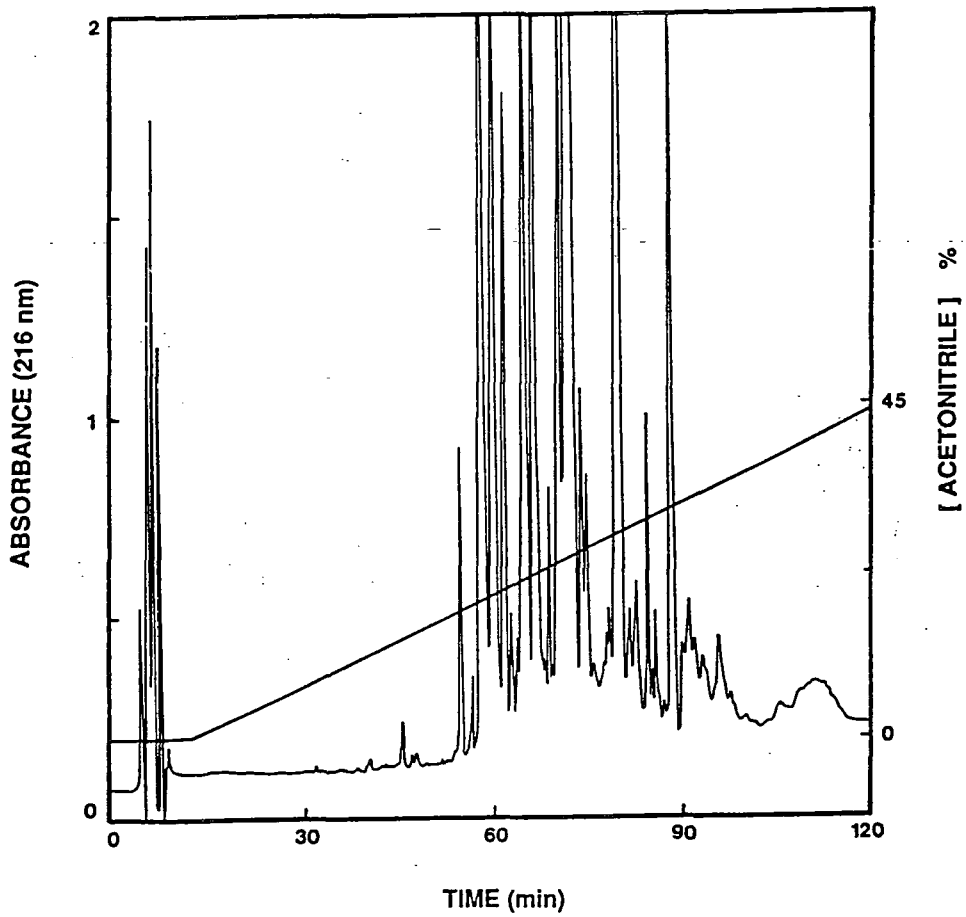


Fig 27: Reverse-phase HPLC separation of the peptides (Pool 1, Fig.21) obtained by the enzymatic digestion of SIα4 with trypsin. The peptides were dissolved in 400µl distilled water, applied to the analytical column (4.6mm x 25cm) of Vydac C_{18} equilibrated in 0.1% aqueous TFA and eluted with a gradient of acetonitrile (thicker solid line) in 0.1% TFA at a flow rate of 1.0ml/min.

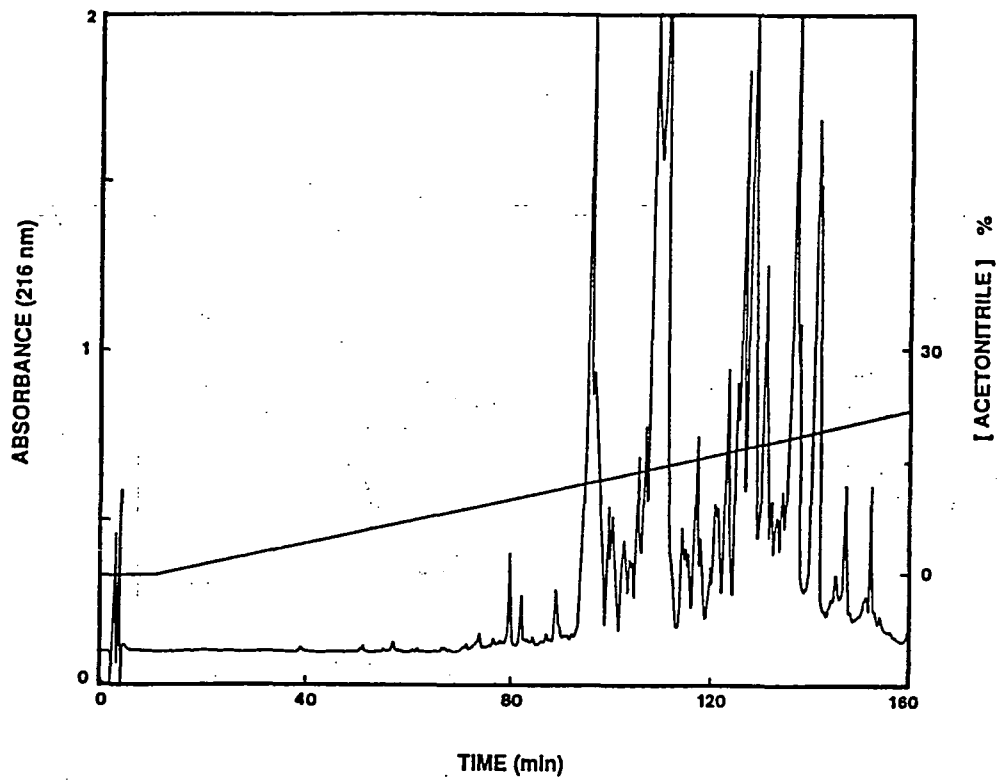


Fig 28: Reverse-phase HPLC separation of the (Pool 2, Fig.22) peptides obtained by the enzymatic digestion of SIα4 with chymotrypsin. The peptides were dissolved in 400ml distilled water,applied to the analytical column (4.6mm x 25cm) of Vydac C_{18} equilibrated in 0.1% aqueous TFA and eluted with a gradient of acetonitrile (thicker solid line) in 0.1% TFA at a flow rate of 1.0ml/min.

3.5 Amino Acid Analyses of Peptides and Proteins

All five proteins and their most highly purified enzymatic peptides were hydrolysed and analysed as described in Materials and Methods (section 2.7). The amino acid compositions of SIα4 and SIα5 are displayed in Table 2. These results showed an overall agreement with the amino acid sequence results (Table 2) except for the cysteine residues which were consistently low, probably due to problems with the standard for that particular amino acid.

Amino acid compositions of SIα1, SIα2 and SIα3 could not be determined in spite of many attempts. The problem was caused by the appearence in the HPLC separations of the PITC-amino acids of a large peak of unknown material which interfered with the integration of the other peak areas. Manual inspection of the peaks suggested values for the amino acids which were entirely consistent with the values calculated from the sequences. The amino acid composition of SIα6 has not been determined yet.

3.6 Amino Acid Sequence Analyses

The complete amino acid sequences of the five inhibitors SIα1, SIα2, SIα3, SIα4 and SIα5 were determined by manual and automated sequencing of the intact proteins and peptides derived from them following enzymic digestion and/or chemical cleavage. It was clear from the sequences obtained that the proteins SIα4 and SIα5 were closely related to one another and quite different from the inhibitors SIα1, SIα2 and SIα3, which form a second group. For this reason, the results and discussion for these two groups will be treated separately.

3.6.1 AMINO ACID SEQUENCES OF SIα4 AND SIα5

The sequences of SIα4 and SIα5 were obtained entirely by manual sequencing of both the native and reduced and S-carboxymethylated protein samples and their respective enzymatic and CNBr peptides (Tables 3,5,6,7,9,11,12,13 and Figs 29,30 and 31). Amino acid analyses of some of the enzymic peptides were used to confirm their respective sequencing results as well as to distinguish leucine from isoleucine residues (Tables 4,8,10,14). The molecular weight of SIα4 was calculated from its amino acid sequence as 12,485 daltons. This sequence (Fig. 29) was found to be composed of 118 amino acid residues of which 15 (12.7%) were proline, 13 (11.0%) arginine, 13(11.0%) alanine, 11 (9.3%) glycine, 10 (8.5%) leucine and 10 (8.5%) cysteine. Five aromatic residues were located in this sequence: 2 (1.7%) tyrosine and 3 (2.5%) phenylalanine. In certain peptides phenylalanine occurred as microheterogeneity in place of the methionines normally found in positions 68 and 86. No tryptophan was found in the SIα4 sequence.

SIa5 was found to have a molecular weight of 12,761 daltons. The amino acid sequence of SIα5 was composed of 116 residues (Fig. 30) of which 14 (12.1%) were leucine, 11 (9.5%) proline, 11 (9.5%) cysteine, 10 (8.6%) glycine and 10 (8.6%) threonine. Of the nine aromatic residues found in the sequence of SIα5, 5 (4.3%) were tyrosine, 2 (1.7%) phenylalanine and 2 (1.7%) tryptophan. No glutamine was found in this sequence.

Both sequences showed a very low content of lysine (1 residue in each sequence) which is in accord with what has been observed previously for the 0.19, 0.28 and 0.53 wheat α-amylase inhibitors (Richardson and Kashlan, 1981; Maeda et al., 1983b, 1985a). On the other hand, the relatively low content of residues such as asparagine (2 residues in SIα4 and 3 residues in SIα5), aspartic acid (5 residues in SIα4 and 1 residue in SIα5), glutamic acid (3 residues in SIα4 and 7 in SIα5) when compared with the much higher compositions of these amino acids (sometimes as high as 40% of the total amino acid content) from the Brazil nut, Conglutin δ, SFA8, 2S storage proteins (Ampe et al., 1986;

Gayler et al., 1990; Kortt et al., 1991) suggest significant structural differences (apart from existence of two subunits in the 2S proteins) between these two classes of proteins which have been regarded as structurally related (Kreis et al., 1985; Shewry and Tatham, 1990).

TABLE 2: Amino acid compositions of SIα4 and SIα5

Amino Acid	SIα4 Analysis[a]	SIα4 Sequence	SIα5 Analysis[a]	SIα5 Sequence
Asp	7.01	5	4.74	1
Asn		2		3
Thr	7.77	8	8.45	9
Ser	4.48	5	6.95	5
Glu	7.53	3	9.72	7(1)
Gln		3		0
Pro	13.26	15	11.49	11
Gly	10.76	11	9.85	10
Ala	12.81	13	8.60	7
Val	8.04	7	6.74	8
Cys	6.58	10	6.24	11
Met	3.35	(3)	3.01	4
Ile	3.09	3(1)	3.54	3(1)
Leu	10.14	10	13.71	14(1)
Tyr	2.54	2	4.52	5
Phe	3.36	2(4)	2.58	2
Lys	2.05	1	2.90	2
His	0.99	1	0.82	1
Arg	14.39	13	10.73	10
Trp	ND	0	ND	2
Total	118	118	115	116

a Residue per mol

( ) Heterogeneous residue

TABLE 3: Peptides obtained from the Tryptic digestion of SIα4

Peptide	Acetonitrile(%)	Sequence
T1-7	21	AAECN(L/I)PT(L/I)PGGGCHSLNSPR
T1-8	23	AAECN(L/I)PT(L/I)PGGGCH
T1-13	27	CAA(L/I)GF
T1-14	28	TVDVTACAPG(L/I)A(L/I)PAPP(L/I)PTCR
T1-16	31	CAA(L/I)GFMMDGVDAP(L/I)QDFR(a) CAA(L/I)GFF(L/I)DGVDAP(L/I)QDFR(b)
T2-8	25	TCG(L/I)GGPYGPVDPSPV(L/I)
T2-11	27	F(L/I)DGVDAP
T2-16	33	CAA(L/I)GFMMDGVDAP(L/I)QDFR
T3-10	26	TCG(L/I)GGPYGPVDPSPV(L/I)KQR
T4-3	6	CCR
T4-4	16	ELAAVPSR
T5-2	6	GCTR
T5-3	8	EMQR
T6-4	16	S(L/I)NSPR
T7-3	8	(L/I)TR
T7-4	11	TFAR
T7-5	11	TFARPR

TABLE 4:Amino acid composition of Tryptic peptides used in the sequence analysis[a] of SIα4

Amino Acid	T1-7	T1-16	T3-3	T3-4	T3-16	T4-4	T6-4	T7-3	T7-5	T7-6
Asx	2	4	2	-	1	-	1	-	-	-
Thr	1	-	-	1	1	-	-	1	1	-
Ser	2	-	1	-	1	1	2	-	-	1
Glx	1	-	-	-	-	1	-	-	-	-
Pro	3	1	3	1	4	1	1	-	1	-
Gly	3	2	1	3	4	-	-	-	-	-
Ala	2	3	-	-	-	2	-	-	1	-
Val	-	1	2	-	2	1	-	-	-	1
Cys	2	1	-	1	1	-	-	-	-	-
Met	-	2	-	-	-	-	-	-	-	-
Ile	1	-	-	-	-	-	-	-	-	1
Leu	2	2	1	1	2	1	1	1	-	-
Tyr	-	-	-	1	1	-	-	-	-	1
Phe	-	2	-	-	-	-	-	-	1	-
Lys	-	-	1	-	1	-	-	-	-	-
His	1	-	-	-	-	-	-	-	-	-
Arg	1	1	1	-	-	1	1	1	2	1
Totals	21	19	12	8	18	8	6	3	6	6

a Residue per mol

TABLE 5: Peptides obtained from the Chymotryptic digestion of SIα4

Peptides	Acetonitrile (%)	Sequence
C2-3	18	AAECN(L/I)PGGGCH
C2-10	20	TRAAECN(L/I)PT
C2-11	20	TVDVTACAPG
C2-18	23	ACAPGLA(L/I)PA
C2-20	25	MMDGVDA
C2-23	26	GFMM
C2-24	26	(L/I)DGVDAP
C3-3	14	AAVPSR
C3-6	18	GPVDPSPV
C4-7	15	RCAA
C4-9	16	GPVDPSPVLKQRC
C4-10	17	TCG(L/I)GGP
C4-12	18	A(L/I)PAPP
C5-2	3	GCTR
C5-4	8	AAVPSR
C5-13	17	A(L/I)PAPP(L/I)PTCRTF
C5-15	18	RTCG(L/I)GGP
C6-3	6	RCCRE
C6-8	9	AAVPSR
C6-10	14	CR
C7-5	7	NSPR
C7-6	8	GPVDPSPV
C8-3	6	AVSR
C8-6	14	QR(L/I)

TABLE 6: Peptides obtained from the V8 protease digestion of SIα4

Peptide	Acetonitrile (%)	Sequence
V2-11	22	CN(L/I)PT(L/I)
V2-13	23	(L/I)NSPR
V2-15	24	GFMMDGV
V2-19	27	(L/I)AAVPSRCR
V2-28	28	RCRCAA(L/I)GFF(L/I)
V3-2	21	CN(L/I)PT(L/I)
V3-7	25	AVSR(L/I)TRA
V3-10	26	VTACAPG(L/I)A(L/I)PAPP(L/I)PTCR
V3-13	26	VTACAPG(L/I)A(L/I)PAPP(L/I)PT
V3-19	28	GVDAP(L/I)QDF
V3-25	31	R(L/I)Y
V4-8	22	CN(L/I)PT(L/I)
V4-12	25	TCG(L/I)GGPYGPV
V4-14	29	VTACAPG(L/I)A(L/I)PAPP(L/I)P
V4B-17	13	GGGCHS(L/I)NSPR
V4B-18	14	GGGCHS(L/I)NSPR
V4B-20	15	GGGCHS(L/I)NSPR
V5-5	14	RCCR
V5-9	14	FRGCTR
V5-14	15	RCCR
V6-10	16	MQR
V8-3	6	KQR
V8-11	14	FRGCTR
V8-13	14	FRGCTR

TABLE 7: Peptides obtained from the elastase digestion of SIα4

Peptide	Acetonitrile (%)	Sequence
E2-3	16	FRG
E2-18	23	ECN(L/I)PT(L/I)PGG
E2-23	26	GFMMDG
E2B-25	13	SRCRC
E3-20	25	RTCG(L/I)GG (a) SRLTR (b)
E3-21	24	CG(L/I)GGPYGPV
E3-22	25	(L/I)A(L/I)PAP
E4-9	17	QDFRGCT
E4-10	18	PAPP(L/I)PT
E4-14	19	RAAECN(L/I)PT(L/I)
E4-15	19	(L/I)KQRCCR
E4-15	20	(L/I)KQRCCREL
E4-17	21	(L/I)KQRCCR
E4-18	22	(L/I)PAPP(L/I)PT
E4-19	24	GGPYGPVDPS
E4-20	25	ACAPG
E4-23	27	LA(L/I)PAP
E4-24	32	DGVDA
E5-10	14	AAVP
E5-12	15	TVDV
E6-9	24	REM
E7-10	14	YAV
E7-13	17	YAV
E7-15	18	S(L/I)NSPR

TABLE 8: Amino acid compositions of Chymotryptic, V8 and Elastase peptides used in the SIα4 sequence analysis[a]

Amino Acid	C2-20	C4-7	C4-9	C8-6	V2-22	V3-13	V3-19	V4-14	E4-10	E4-15
Asx	3	-	1	-	1	-	2	-	-	-
Thr	-	-	-	-	2	1	-	2	1	-
Ser	-	-	1	-	-	-	-	-	-	-
Glx	1	-	1	1	-	-	1	-	-	1
Pro	1	-	3	-	2	5	1	6	4	-
Gly	1	-	1	-	1	1	1	1	-	-
Ala	1	2	-	-	3	4	1	5	1	-
Val	1	-	2	-	2	1	1	1	-	-
Cys	-	1	1	-	1	1	-	1	-	2
Met	2	-	-	-	-	-	-	-	-	-
Ile	-	-	-	1	1	1	-	1	-	-
Leu	1	1	1	-	1	2	1	2	1	1
Tyr	-	-	-	-	-	-	-	-	-	-
Phe	1	-	-	-	-	-	-	-	-	-
Lys	-	-	1	-	-	-	-	-	-	1
His	-	-	-	-	-	-	-	-	-	-
Arg	1	1	1	1	-	-	-	-	-	1
Total	13	5	13	3	14	16	8	19	7	6

a Residue per mol

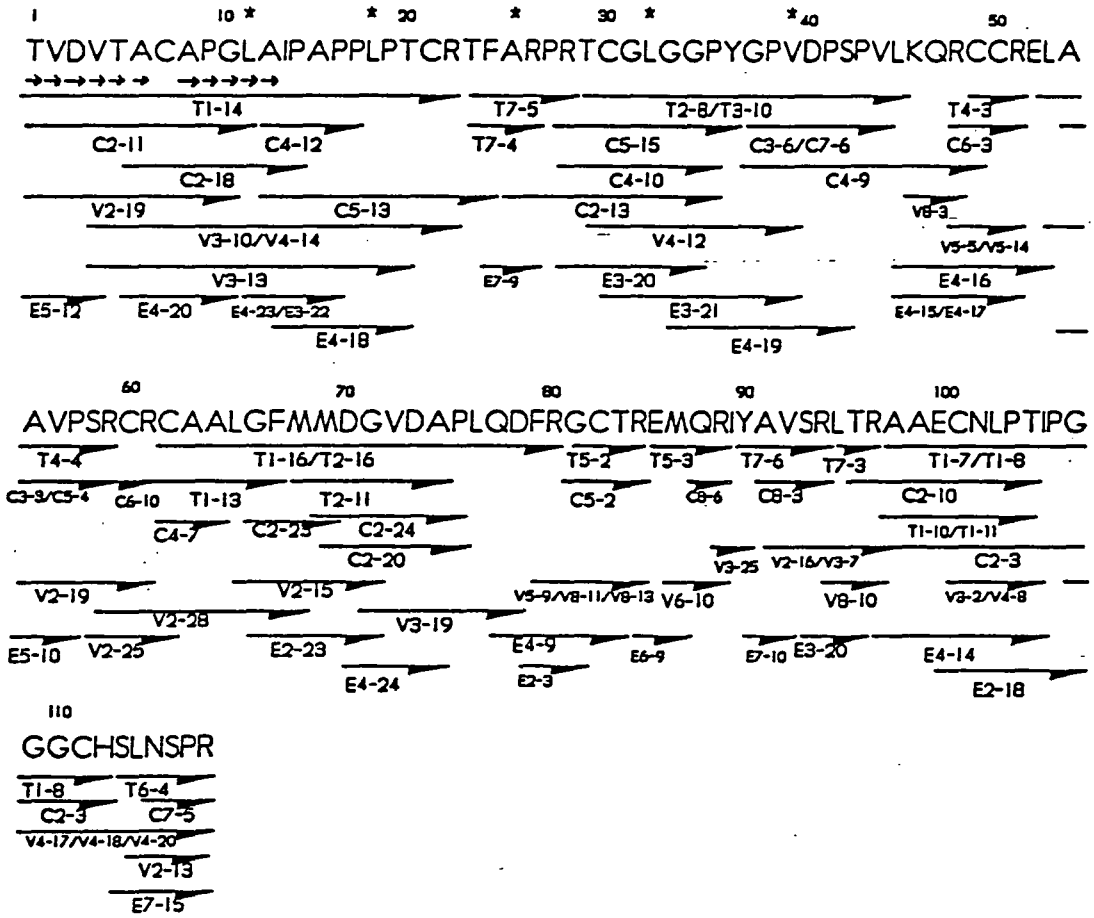


Fig.29: Amino acid sequence of Slα4. Smaller arrows (→) indicate residues determinated by manual sequencing method on native Sla4. Bigger arrows (⟶) indicate peptides obtained from digestions with trypsin (T), chymotrypsin (C), *S.aureus* V8 protease (V) and elastase (E) which were also sequenced by the manual DABITC/PITC method. (*) indicates repeating heptads of hydrophobic residues.

Table 9: Peptides obtained from tryptic digestion of SIα5

Peptides	Acetonitrile(%)	Sequence
T1-13	25	EMRPTCSWGG
T1-14	17	TA(L/I)DSMMTGYEMR
T2-2	23	PFCYA(L/I)GAEGT
T2-5	24	ERCCSE(L/I)E
T2-6	27	PFCYA(L/I)GAEGTTT
T2-7	28	PFCYA(L/I)GAEGTTT
T2-14	36	ANWCEPG(L/I)V(L/I)P(L/I)NP(L/I)PSCR
T3-5	25	PTCSWGG(L/I)
T3-6	28	TALDSMMTGYEMR
T3-7	29	TALDSMMTGYEMR
T3-9	31	ACGVS(L/I)GPVVP(L/I)PV(L/I)
T5-15	30	RACGVS(L/I)GPVVP(L/I)PV
T6-4	16	CGA(L/I)R
T6-6	19	T(L/I)VCYR
T9-4	14	T(L/I)HGR
T9-11	17	TYMVRR
T9-12	15	T(L/I)HGR

TABLE 10: Amino acid composition of Tryptic peptides used in the SIα5 sequence analysis[a]

Amino Acid	T2-5	T2-6	T2-7	T2-14	T3-7	T3-9	T5-15	T6-4	T6-6	T9-4	T9-9	T9-11
Asx	-	1	1	2	1	-	-	-	-	-	--	-
Thr	-	3	3	1	2	-	-	-	-	1	1	1
Ser	1	-	-	1	1	1	1	-	-	-	-	-
Glx	2	1	1	1	1	-	-	-	-	-	-	-
Pro	-	-	-	4	-	3	3	-	-	-	-	-
Gly	-	2	2	1	1	2	2	1	-	1	-	-
Ala	-	2	2	1	1	1	1	1	-	-	-	-
Val	-	-	-	1	-	4	4	-	1	-	1	1
Cys	2	1	1	2	-	1	1	1	1	-	-	-
Met	-	-	-	-	3	-	-	-	-	-	1	-
Ile	-	-	-	1	-	1	1	-	1	-	-	-
Leu	1	1	1	3	1	2	2	1	-	1	-	1
Tyr	-	1	1	1	1	-	-	-	1	-	1	-
Phe	-	-	-	-	-	-	-	-	-	-	-	-
Lys	1	-	-	-	-	1	1	-	-	-	-	-
His	-	-	-	-	-	-	-	-	-	1	-	-
Arg	-	-	-	2	1	-	1	1	1	1	1	2
Totals	7	12	12	21	13	16	17	5	5	5	5	5

a Residue per mol

Table 11: Peptides obtained from chymotryptic digestion of SIα5

Peptide	Acetonitrile(%)	Sequence
C2-3	16	A(L/I)GAEGTTT
C2-20	26	CRCGA
C2-23	28	TFAPT(L/I)VC
C2-29	36	ANW
C2-30	37	RCC
C3-3	12	A(L/I)GAEGTTT
C3-17	28	CEPG(L/I)V(L/I)P(L/I)
C3-18	30	RECN(L/I)RT
C4-13	19	EK(L/I)VPY
C5-5	21	DSMMTG
C5-12	19	EMRPTCS
C5-18	22	RTALDSMM
C6-13	17	GRPF
C6-15	18	NP(L/I)PSCRT
C6-17	19	HGRPFC
C8-4	8	MVRR

Table 12: Peptides obtained from V8 digestion of SIa5

Peptide	Acetonitrile(%)	Sequence
V8-5	8	TA(L/I)D
V8-8	9	CN(L/I)R
V8-10	10	CGA(L/I)R
V8-11	10	RCCS
V8-12	13	SMMTGYE
V8-14	14	TYMVR
V8-15	15	ANWCE
V8-17	15	(L/I)VPYCR
V8-20	17	K(L/I)VPYCR
V8-22	19	FAPT(L/I)VCYRE
V8-25	21	MRPTCSWGG(L/I)
V8-26	23	T(L/I)HGRPFCYA(L/I)GAE
V8-29	24	PTCSWGG(L/I)(L/I)T
V8-32	26	PG(L/I)V(L/I)P(L/I)NP(L/I)PSCR
V8-33	26	CN(L/I)RT(L/I)HGRPFCYA(L/I)GA
V8-34	27	RACGVS(L/I)GPV
V8-36	30	ANWCEPG(L/I)V(L/I)P(L/I)NP(L/I)PSCR

Table 13: Peptides obtained from Elastase digestion of SIα5

Peptides	Acetonitrile(%)	Sequence
E2-6	16	SE(L/I)EK(L/I)
E2-7	17	SE(L/I)EK
E2-15	21	HGRPFY
E2-17	27	(L/I)KERC
E3-10	17	A(L/I)DSM
E3-12	18	CRTYM
E3-14	20	ANWCEPG(L/I)V
E3-24	28	(L/I)KERC
E4-4	15	(L/I)GAEG
E4-6	16	GYEM
E4-7	17	PYCR
E4-10	21	CN(L/I)R
E5-6	12	CRC
E5-9	13	CGA
E5-13	17	TFAPT
E5-15	21	ACGV
E5-16	23	(L/I)P(L/I)NP(L/I)PS
E5-21	27	GG(L/I)(L/I)
E5-22	33	WGG(L/I)(L/I)TFA
E6-11	29	S(L/I)PVVP(L/I)PV

TABLE 14: Amino acid composition of Chymotryptic and Elastase peptides used in the SIα5 sequence analysis[a]

Amino Acid	C3-3	C4-13	E5-22
Asx	1	-	-
Thr	3	-	1
Ser	-	-	-
Glx	1	1	1
Pro	-	1	1
Gly	1	-	2
Ala	2	-	1
Val	-	1	-
Cys	-	-	-
Met	-	-	-
Ile	-	-	-
Leu	1	1	2
Tyr	-	1	-
Phe	-	-	1
Lys	-	1	-
His	-	-	-
Arg	-	-	-
Totals	9	6	9

a Residue per mol

CNBr-3: G-G-(L/I)-(L/I)-T-F-A-P-T-(L/I)-V

N-terminal: A-N-W-C-E-P-G-L-V-I-P-L-N-P-L-P-S-C-R-T-Y-M-V-R-R-A-C-G-V-S-I-G-P-V-V-P-L-P-V-L

Fig.30: CNBr peptide and N-terminal sequence of SIα5 using manual and automatic methods respectively.

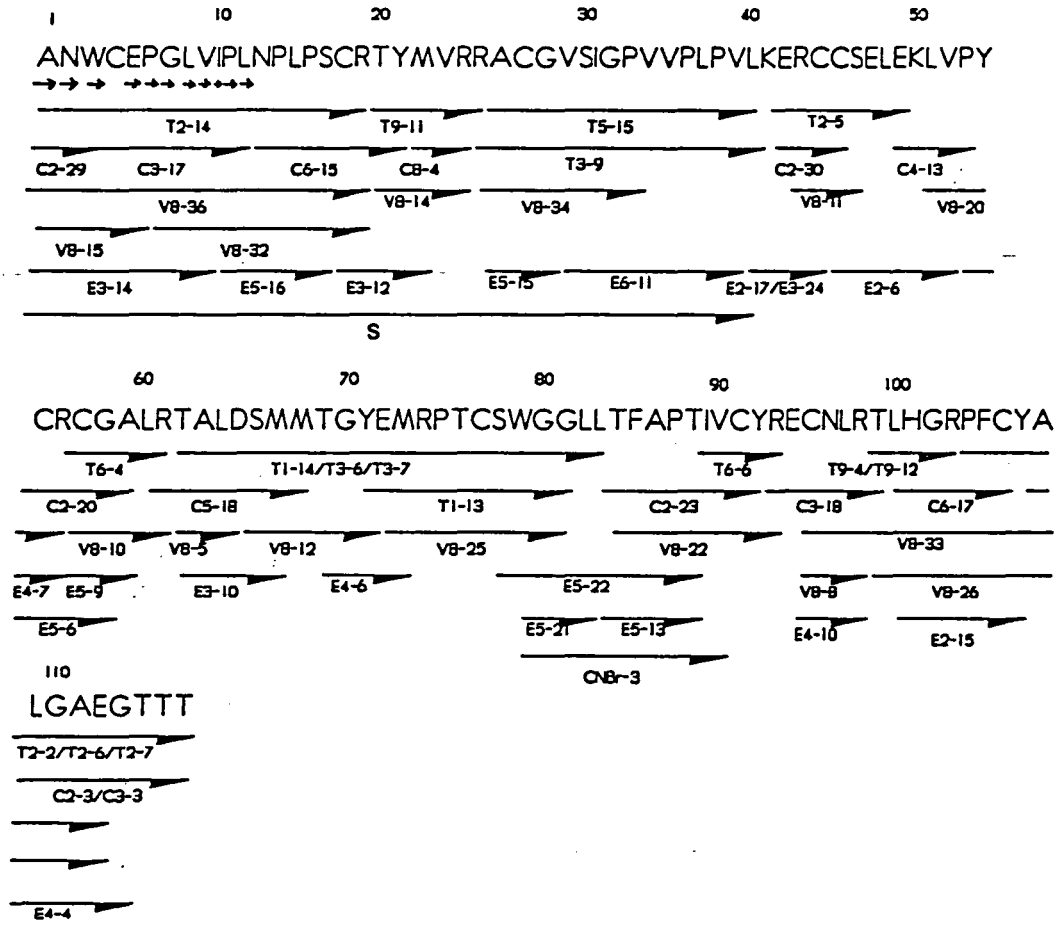


Fig.31: Amino acid sequence of SIα5. Smaller arrows (→) indicate residues determined by manual sequencing methods of native SIα5. Bigger arrows (⟶) indicate peptides obtained from digestions with trypsin (T), chymotrypsin (C), *S.aureus* V8 protease (V), elastase (E) and cyanogen bromide (CNBr), which were sequenced by the manual DABITC/PITC method. (S) indicates residues determined by automatic degradation of reduced and S-carboxymethylated SIα5.

Sequence alignment of SIα4 and SIα5 using CLUSTAL, FASTA and RDF 2 programmes (Figs. 32 and 33 and Table 15) revealed that these proteins show a statistically significant homology of approximatly 35% between themselves. SIα4 and SIα5 exhibited the greatest percentage of sequence identity with the ragi bifunctional trypsin/α-amylase inhibitor (Campos and Richardson, 1983) (40-42%) but also showed higher homology (33-39%) with the maize, barley and rye trypsin inhibitors (Mahoney et al., 1984; Odani et al. 1983a ; Lyons et al. 1987) than with the other cereal inhibitors of α-amylase (Maeda et al.,1983b; Kashlan and Richardson, 1981; Maeda et al., 1985a) and CM-proteins (Halford et al., 1988) (21-27%).

Hydrophobicity plots of SIα4 and SIα5 and some of the above mentioned proteins suggested that the two inhibitors from sorghum are more structurally related to the other α-amylase and trypsin inhibitors than to the 2S storage proteins (Figs 34, 35). Examination of these plots of hydrophobicity show that SIα5 contains the most hydrophobic regions. This fact correlates well with the observation that SIα5 was the least soluble of the two and had only approximately 33% of the solubility of SIα4 (1.5mg/ml) in distilled water at room temperature.

Secondary structure predictions of SIα4 and SIα5 (Fig. 36, 37) revealed a high percentage of ordered structures, specially α-helix. In fact, it was predicted that SIα4 would have 43% of helix, 23% extended chain and 33% of coil. For SIα5 the prediction was 50% of helix, 16% extended chain and 33% coil. These results appear to be similar to those reported for the homologous trypsin and α-amylase inhibitors from rye (Lyons et al., 1987;), α-amylase inhibitors (0.19, 0.28 and 0.39) from wheat (Silano et al., 1973; Petrucci et al., 1976, 1978) and the trypsin inhibitor from maize (Mahoney et al., 1984).

All these findings strongly suggest that the α-amylase inhibitors SIα4 and SIα5 are members of the cereal superfamily (Table 1, Fig. 1).

The molecular weights of SIα4 and SIα5 calculated from their amino acid sequences (12,485 and 12,761) were close to the values estimated from their mobilities

on SDS-PAGE (13-14 kDa) (section 3.1). These results suggest that neither protein contained any significant quantity of covalently attached carbohydrate. Also preliminary experients in which SDS-PAGE gels were stained for carbohydrate by the Schiff-reagent yielded only negative results for SIα4 and SIα5.

```
1       TVDVTACAPGLAIPAPPLPTCRTFARPRTCGLGGPYGPVDPSPVLKQRCCRELAAVPSRC
2       A---NWCEPGLVIPLNPLPSCRTYMVRRACGV--SIGPVVPLPVLKERCCSELEKLVPYC
        .   . *.***.**  ***.***.  .*.**.  . *** * ****.***.**. . . *

1       RCAALGFMMDGVDAPLQDFRGCTREMQRIYAVSRLTRAAECNLPTIPGGG-CHSLNS---
2       RCGALRTALDSMMTGYEMRPTCSWGGLLTFAPT-IVCYRECNLRTLHGRPFCYALGAEGT
        **.**   .*.. .  .  ..*...   ..* . ..  ****.*..* . *..*..

1       PR
2       TT
```

Fig.32: Amino acid sequence alignment of SIα4 (1) and SIα5 (2) using CLUSTAL. (*) Match across all sequences; (·) Conservative substitutions.

```
1      TVDVTACAPGLAIPAPPLPTCRTFARPRTCGLGGPYGPVDPSPVLKQRCCRELAAVPS-R
2      A---NWCEPGLVIPLNPLPSCRTYMVRRACGV--SIGPVVPLPVLKERCCSELEKLVP-Y
3      SGPWM-CYPGQAFQVPALPACRP-LLRLQCN-----GSQVPEAVLRD-CCQQLAHIS-EW
4      SGPWSWCNPATGYKVSALTGCRA-MVKLQCV-----GSQVPEAVLRD-CCQQLADINNEW
5      SGPWM-CYPGQAFQVPALPGCRP-LLKLQCN-----GSQVPEAVLRD-CCQQLADIS-EW
6      SVG-TSCIPGMAIPHNPLDSCRWYVAKRACGVGPR----LATQEMKARCCRQLEAIPA-Y
7      SVG-GQCVPGLAMPHNPLGACRTYVVSQICHVGPR----LFTWDMKRRCCDELLAIPA-Y
8      -FG-DSCAPGDALPHNPLRACRTYVVSQICHQGPR----LLTSDMKRRCCDELSAIPA-Y
9      SAG-TSCVPGWAIPHNPLPSCCWYVTSRRCGIGPR--PRLPWPELKRRCCRELADIPA-Y
              * *.       .* .*          *                 ..  ** .*  .

1      CRCAALGFMMDGVDA-P----------LQDFRGCTREMQRIYAVSRLTRAAECNLPTI--
2      CRCGALRTALDSMMT-G----------YEMRPTCSWGGLLTFA-PTIVCYRECNLRTL--
3      CRCGALYSMLDSMYKEHGAQEGQAGT--GAFPRCRREVVK---LTAASITAVCRLPIVVD
4      CRCGDLSSMLRSVAQELGVREGKE-----VLPGCRKEVMK---LTAASVPEVCKVPIP-N
5      PRCGALYSMLDSMYKEHGVSEGQAGT--GAFPSCRREVVK---LTAASITAVCRLPIVVD
6      CRCEAVRILMDGVVT-P--SGQHEGRLLQDLPGCPRQVQRAFA-PKLVTEVECNLATI--
7      CRCEALRILMDGVVT-Q--QGVFEGGYLKDMPNCPRVTQRSYA-ATLVAPQECNLPTI--
8      CRCEALRIIMQGVVT-W--QGAFEGAYFKDSPNCPRERQTSYA-ANLVTPQECNLGTI--
9      CRCTALSILMDGAIP-PGPDAQLEGA-LEDLPGCPREVQQGFA-ATLVTEAECNLETI--
        **...    . .                      . *          .      *.. .

1      ---PGGG-CHSLNSP---R
2      ---HGRPFCYALGAEGTTT
3      ASGDGAYVCKD-----VAA
4      PSGDRAGVCYG---DWAAY
5      ASGDGAYVCKD-----VAY
6      ---HGGPFCLSLL-G---A
7      ---HGSPYCPTLQAG----
8      ---HGSAYCPELQPG---Y
9      ---S---------------
```

Fig.33: Sequence alignment of SIα4 (1) and SIα5 (2) and with other members of the Cereal Superfamily using CLUSTAL. (3) 0.19 wheat α-amylase inhibitor (Maeda, et al., 1983a); (4) 0.28 wheat α-amylase inhibitor (Kashlan and Richardson, 1981); (5) 0.53 wheat α-amylase inhibitor (Maeda et al, 1985a); (6) Bifunctional trypsin/α-amylase inhibitor from ragi (Campos and Richardson, 1983); (7) Rye trypsin inhibitor (Lyons et al., 1987); (8) Barley trypsin inhibitor (Odani et al., 1981); (9) Maize trypsin inhibitor (Mahoney et al., 1984).(*) Match across all sequences; (·) conservative substitutions

TABLE 15: Percentage identities* of SIα4 and SIα5 between themselves and with other members of the Cereal Superfamily**.

	SIα4	SIα5	RBF	MTI	BTI	RTI	0.19W	0.28W	0.53W	CMe	CMd
SIα4	-	**22.68**	**36.06**	**28.60**	**25.73**	**23.57**	**17.69**	**12.76**	**7.84**	**28.55**	**17.58**
SIα5	35.2	-	**32.29**	**27.61**	**40.28**	**29.75**	**14.23**	**9.87**	**9.35**	**23.08**	**6.77**
RBF	42.2	40.5	-	**53.05**	**42.75**	**42.57**	**18.93**	**15.26**	**14.53**	**38.16**	**17.77**
MTI	39.4	33.9	52.2	-	**35.47**	**40.48**	**15.32**	**10.53**	**13.07**	**48.31**	**15.65**
BTI	37.5	33.6	54.9	46.8	-	**98.90**	**11.63**	**9.06**	**16.53**	**82.00**	**21.46**
RTI	39.4	38.9	61.5	49.2	76.2	-	**17.13**	**16.49**	**17.13**	**50.87**	**20.33**
0.19W	27.3	27.1	24.4	23.7	26.0	28.7	-	**44.71**	**89.07**	**21.03**	**9.00**
0.28W	21.5	24.2	22.7	20.6	22.7	25.0	53.2	-	**56.00**	**10.14**	**4.88**
0.53W	25.8	25.6	24.4	23.7	23.6	27.9	94.4	55.6	-	**16.83**	**5.82**
CMe	32.2	28.2	46.9	40.5	82.1	64.6	24.0	19.1	22.0	-	**27.29**
CMd	26.7	22.8	31.9	33.1	35.0	35.6	24.2	18.7	23.0	33.9	-

* Figures in bold type represent the *Z* values (Statistical signficancies) obtained by the computer RDF2 programme (Pearson and Lipmann, 1988). Values in normal type are the % identities of the sequences.

** Members of the Cereal Superfamily used: Bifunctional trypsin/α-amylase inhibitor from ragi **[RBF]** (Campos and Richardson, 1983); Maize trypsin inhibitor [MTI] (Mahoney et al., 1984); Barley trypsin inhibitor **[BTI]** (Odani et al., 1983a); Rye trypsin inhibitor **[RTI]** (Lyons et al., 1987); 0.19 wheat α-amylase inhibitor **[0.19W]** (Maeda et al., 1983b); 0.28 wheat α-amylase inhibitor **[0.28W]** (Kashlan and Richardson, 1981); 0.53 wheat α-amylase inhibitor **[0.53W]** (Maeda et al. 1985a); Barley CMe-Protein **[CMe]** (taken from the US. Nacional Biomedical Research Foundation Databank, 1990); Barley CMd-protein **[CMd]** (Halford et al., 1988).

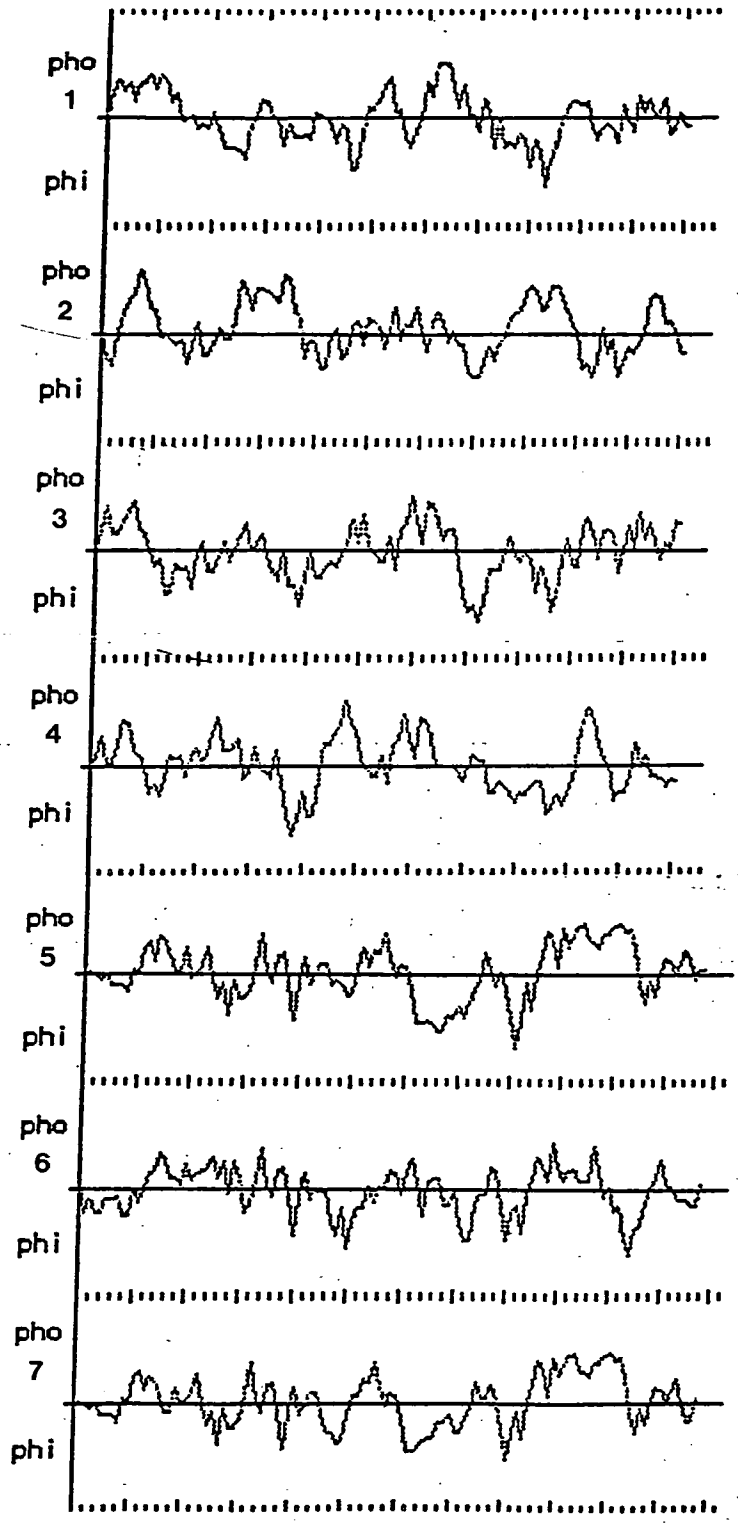


Fig.34: Hydrophobicity plots of SIα4 (1), SIα5 (2), and other members of the Cereal Superfamily using Kyte and Doolittle (1982) alghorithm. (3), Ragi bifunctional trypsin/α-amylase inhibitor (Campos and Richardson, 1983); (4) Rye trypsin inhibitor (Lyons, et al., 1987); (5) 0.19 wheat α-amylase inhibitor (Maeda et al., 1983a); (6) 0.28 wheat α-amylase inhibitor (Kashlan and Richardson, 1981); (7) 0.53 wheat α-amylase inhibitor (Maeda et al., 1985).

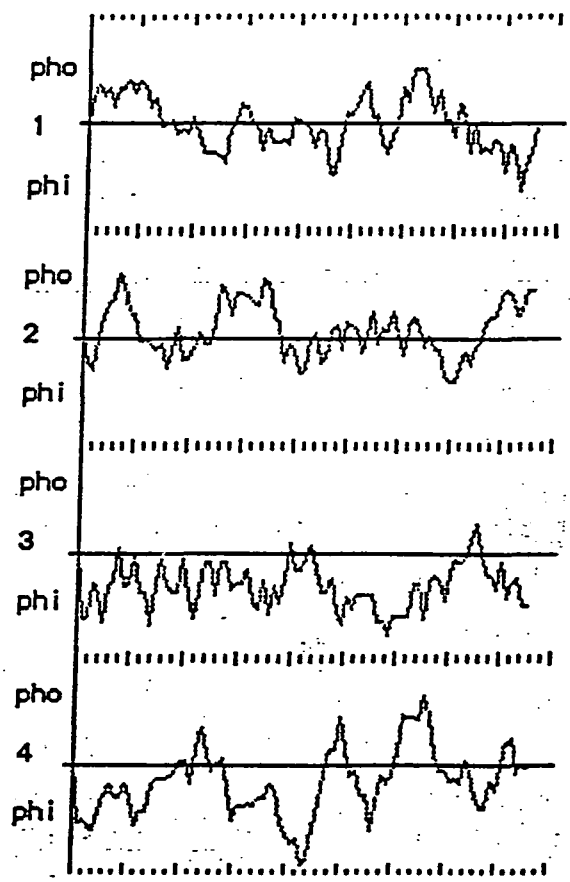


Fig.35: Hydrophobicity plots of SIα4 (1), SIα5 (2), Conglutin δ [3] (Gayler et al., 1990), and SFA8 [4] (Kortt et al., 1991) 2S storage proteins, using Kyte and Doolittle (1982) algorithm.

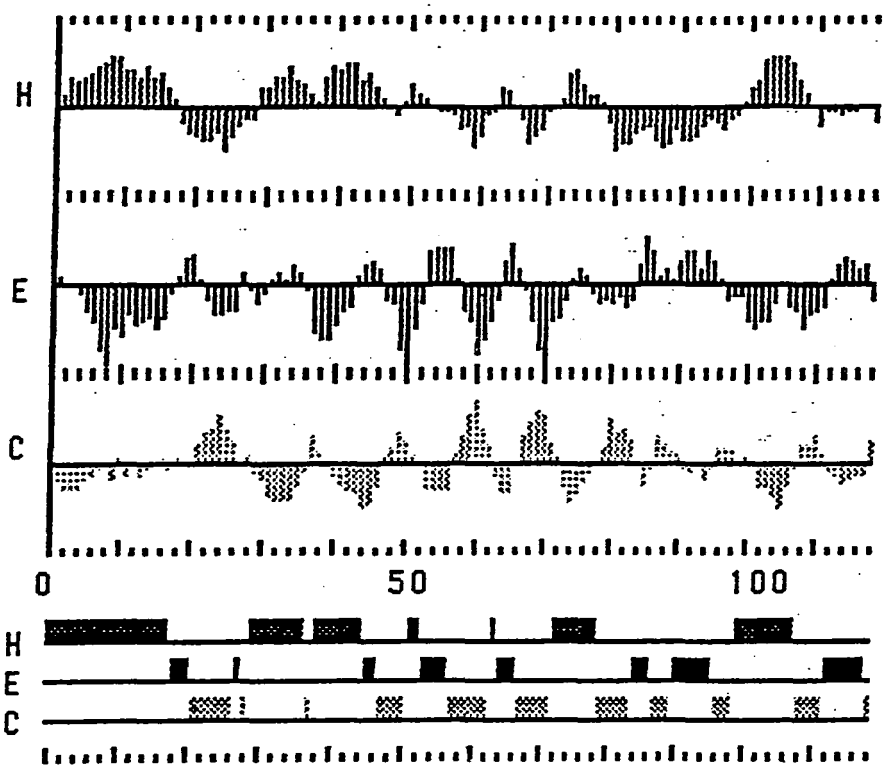


Fig.36: Secondary structure predictions of SIα4 using Garnier et al., (1978) algorithm. 43% of helix (H); 23% of extended (E); 33% of coil (C).

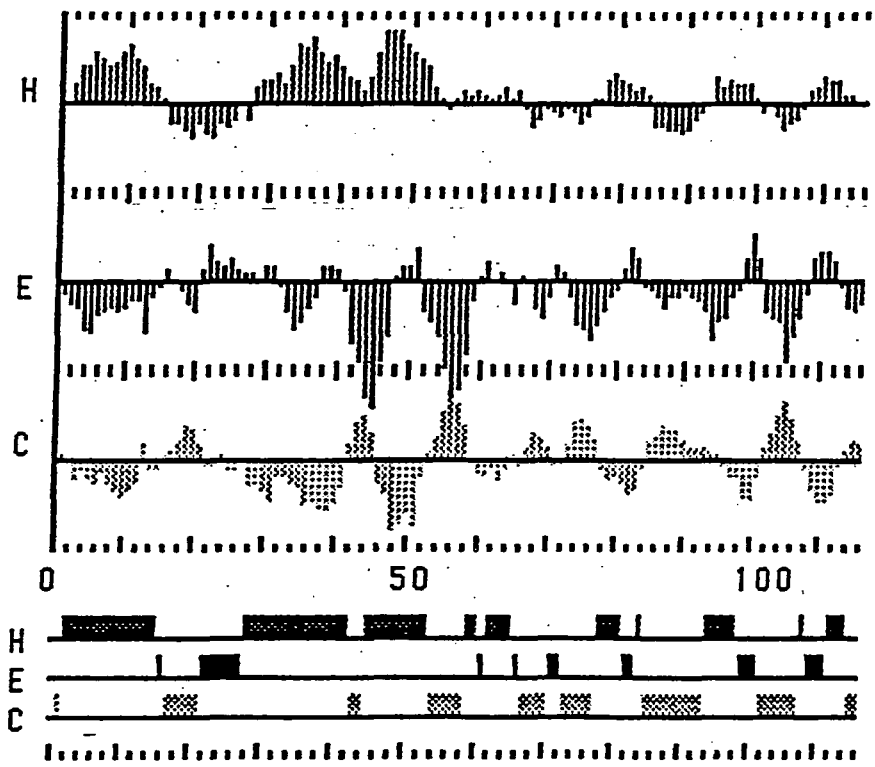


Fig.37: Secondary structure predictions of SIα5 using Garnier et al., (1978) algorithm. 50% helix (H); 16% extended (E); 33% coil (C).

3.6.2 ISOELETRIC POINT ESTIMATIONS

The isoelectric points of SIα4 and SIα5 were predicted from their respective amino acid sequences by using the Mount and Conrad sequence analysis package (Mount and Conrad, 1988). SIα4 and SIα5 have an estimated pI of 8.73 and 8.71 respectively. These estimations appear to be closely similar to the isoelectric focusing results reported in the section 3.1, in particular with the obtained pI value for SIα5 which was 8,85.

3.7 N-Terminal Sequence of SIα6

Preliminary investigations on the amino-terminal of SIα6 yielded the first five residues which were sequenced manually using only the native form of that protein. the first five residues were identified as follow:

Leu/Ile - Gly - Val - Leu/Ile - Val

3.8 Preliminary Studies on the Location of the Disulphide Bonds of SIα5

U.V spectra from 320 to 220nm were taken from samples of SIa5 in its intact form and after incubation with 4-vinylpyridine, in the presence and absence of DTT (Fig. 38). The spectrum of the sample *a* (native SIα5) was considered to be a typical protein spectrum with a major peak in the region of 280-270nm. The spectrum of sample *b* (SIα5 incubated with 4-vinylpyridine only) revealed a slight increase in absorption around the 254nm region when compared to the spectrum of the native protein. The spectrum of the sample *c* (SIα5 incubated with 4-vinylpyridine in the presence of DTT) showed a large peak in the 254nm region. Since S-β-4-pyridilethyl cysteine has a specific absorbance at 254nm, these results indicate that some S-pyridylethylation occurred in the sample *b* (which had no reducing agent), but that most of the S-alkylation happened when SIα5 was in contact with DTT. These results also suggest that most of the eleven cysteines present in the sequence of SIα5 were in the oxidized form (i.e. present as cystine).

A preliminary digestion of native SIα5 with chymotrypsin yielded a few peptides which were manually sequenced. After many sequencing cycles, only two of these peptides were considered suitable for amino acid sequence analysis (Fig. 39). In each peptide two different amino acid residues were identified in most of the sequencing cycles, however on two occasions only one amino acid residue was identified for each one of these peptides producing a gap of one residue in one of the sequences. The resulting peptide sequences were compared with the complete amino acid sequence of SIα5 and their respective positions located in it. This procedure revealed that the one residue gap in the peptide sequences corresponded to a cysteine residue in the complete SIα5 sequence. These results strongly suggest that the Cys4 is linked with Cys77, and Cys27 is linked with Cys91. However, further studies are needed to confirm these findings and to determine the three other possible disulphide bonds.

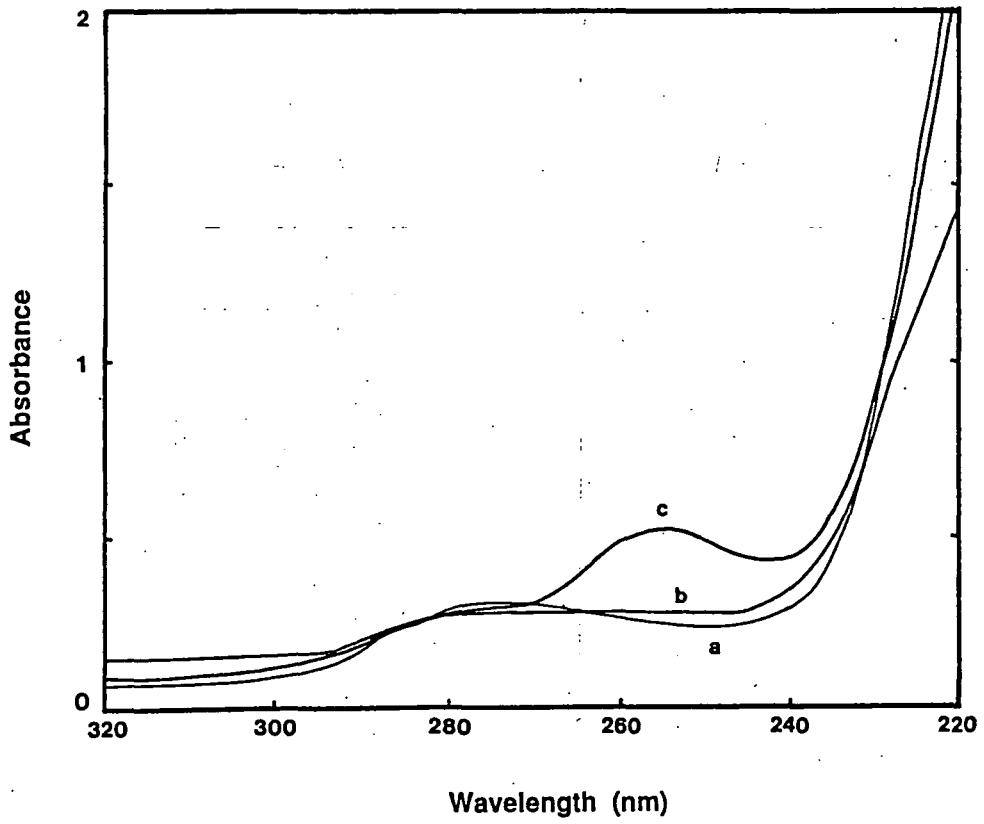


Fig.38: U.V spectrum of SIα5. (a) 0.3mg of native SIα5 in 1ml of distilled water; (b) 0.3mg of SIα5 after treatment with 4-vinylpyridine, in 1ml of distilled water; (c) 0.3mg of SIα5 after treatment with 4-vinylpyridine in the presence of DTT, in 1ml of distilled water. Each spectrum was taken at room temperature.

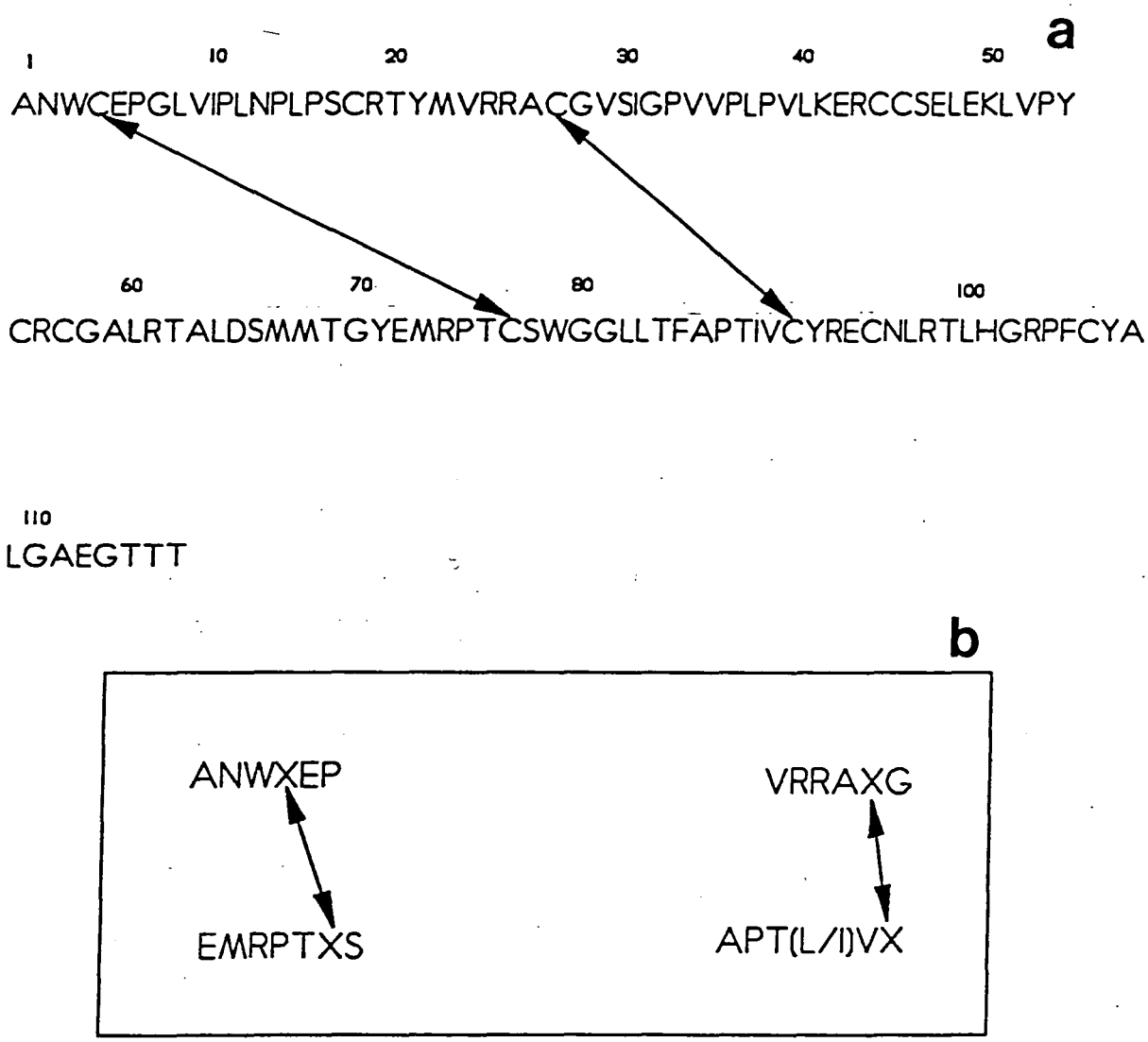


Fig.39: Localization of disulphide bonds in the SIα5 sequence. (a) Location of the disulphide bonds in the amino acid sequence of SIα5, indicated by arrows. (b) In the box, peptides showing evidence for intramolecular -S-S- bonds (X indicates cysteine residue which could not be seen with the sequencing method used).

3.9 Amino Acid Sequences of SIα1, SIα2 and SIα3

The amino acid sequences of SIα1, SIα2 and SIα3 determined by both automated and manual sequencing methods (Tables 16 - 19) are shown in the Figure 40. The sequences were in good agreement with the results of the amino acid analyses, and the molecular weight values of 5369, 5310 and 5384 calculated from the respective SIα1, SIα2 and SIα3 sequences corresponded well with the estimates od 5.000-5.300 made from SDS-PAGE and plasma desorption mass spectroscopy.

The sequences of all three proteins are notable for their high contents of cysteine residues (8 in each) and basic amino acids such as arginine and lysine. These proteins are very poor in proline (1 residue in SIα1 and none in SIα2 and SIα3), threonine (1 residue in SIα1 and none in SIα2 and SIα3) and tyrosine (1 residue in SIα1 and none in SIα2 and SIα3).

Estimation of the isoelectric points of these three protein from their amino acid sequences using the Mount and Conrad sequence analysis package, showed basic pI values (8.14 for SIα1, 8.25 for SIα2, 8.43 for SIα3) which correlates well with the fact that these proteins failed to focus when subjected to isoelectric focusing probably due to very basic pI values.

The three proteins are clearly homologous (Fig. 41, Table 20) with the greatest identity (87.5%) occuring between SIα2 and SIα3, whilst SIα1 has only 40.8 identity with SIα2 and 36.7% with SIα3. They also showed striking similarities with the γ1- and γ2-purothionins from wheat endosperm (Colilla et al., 1990), and with the γ-hordothionin from barley (Fig. 41, Table 20) which was found to inhibit translation in cell-free systems derived from mammalian as well as non-mammalian cells by affecting the polypeptide chain initiation process (Mendez et al., 1990). It has been suggested that the γ-purothionins and γ-hordothionin (Colilla et al., 1990; Mendez et al., 1990) show homology with the β and α purothionins (Jones and Mak, 1977; Ohtani et al., 1977; Hase

et al., 1978), α-hordothionin (Ozaki et al., 1980) and a number of other thionins such as crambin (Teeter et al., 1981). However, inspection of the Z values in the Table , shows that there is no significant relationship (i.e. Z values in the range of -0.61 to 2.89) between the first group (containing SIα1, SIα2 and SIα3, the γ-purothionins and γ-hordothionin) and the other thionins that allow these proteins to be called thionins. However, other typical thionin features such as low molecular weight, high content of cysteine residues, basic pI's and above all some similar biological activities (inhibition of macromolecular synthesis and α-amylase) suggest that these two groups might be related, and therefore may be considered as putative members of a new thionin family.

As was mentioned before (see Introduction), the inhibition of α-amylase by thionins appears to be via competition for calcium ions (Jones and Meredith, 1982) which are essential for the activity of the majority of the α-amylases (Payan et al., 1980; Matsuura et al., 1984; Buisson et al., 1987). This fact could explain the unusual inhibition results obtained when SIα1, SIα2 and SIα3 were tested against locust gut α-amylase (see section 3.2).

However perhaps one of the most curious results was the surprising similarity found between SIα1, SIα2, SIα3 and a region of a potato trypsin inhibitor precursor (Stiekema et al., 1988) (Fig. 41, Table 20).

Table 16: Peptides obtained from tryptic digestion of SIα1

Peptide	Acetonitrile (%)	Sequence
T13	12	CH(L/I)R
T14	18	RVCMG
T16	19	CQKAC
T17	23	YCR
T22	27	(L/I)CSNECV
T25	29	SQHHSFPC(L/I)SDR
T32	32	EEGGWTAGR

Table 17: Peptides obtained from chymotryptic digestion of SIα1

Peptide	Acetonitrile(%)	Sequence
C14	14	TAGR
C16	16	GKSQHH
C25	18	CRCQKAC
C28	18	RVC
C29	22	CH(L/I)RY
C31	25	SFPC(L/I)SDR
C34	28	CSNECVKEEGG

Table 18: Peptides obtained from thermolysin digestion of SIα1

Peptide	Acetonitrile (%)	Sequence
H15	15	(L/I)SDR
H16	17	AGRCH
H19	17	RVC
H22	20	CSNEC
H26	22	GKSQHH
H27	23	SFPC
H29	29	(L/I)RYCRCQKAC
H36	31	VKEEGGWT

Table 19: Peptides obtained from chymotryptic and thermolysin digestions of SIα2

Peptides	Acetonitrile (%)	Sequence
C2-1	15	RQC
C2-3	17	RVC
C2-16	17	GKSAG
C2-27	20	RDQNCAQVC
C2-30	24	GGGNCDGV
C2-36	30	QCW
H2-19	22	VMRQC
H2-21	31	RQCW

Fig.40: Amino acid sequences of SIα1, SIα2 and SIα3. Arrows (⟿) indicate residues determined by automatic degradation of native (SIα1) or S-pyridylethylated (SIα2 and SIα3) protein samples. Solid lines (—) indicate peptides obtained from digestions with trypsin (T), chymotrypsin (C) and thermolysin (H) which were sequenced by the manual DABITC/PITC method

```
1     RVCMGKSQHHSFPCISDRLCSNECVKEEGGWTAGRCH--LRYCRCQK-C-
2     RVCMGKSAGFKGLCMRDQNCAQVCLQE--GWGGGNCDGVMRQCKCIR-CW
3     RVCRRRSAGFKGLCMSDHNCAQVCLQE--GWGGGNCDGVIRQCKCIR-CW
4     RICRRRSAGFKGPCVSNKNCAQVCMQE--GWGGGNCDGPLRRCKCMR-C-
5     KICRRRSAGFKGPCMSNKNCAQVCQQE--GWGGGNCDGPFRRCKCIRQC-
6     KVCRQRSAGFKGPCVSDKNCAQVCLQE--GWGGGNCDGPFRRCKCIRQC-
7     RHCESLSHRFKGPCTRDSNCASVCETE--RFSGGNCHGFRRRCFCTKPC-
      . *   *   .  *  .  *.  *  *   ...*.*.   * * *   *
```

Fig 41: Amino acid sequence alignments for SIα1(1), SIα2(2), and SIα3(3) with the γ-hordothionin(4) from barley endosperms (Mendez et al., 1990), the γ1(5) and γ2(6) -purothionins from wheat (Colilla et al., 1990) and a region of a precursor of a trypsin inhibitor(7) from potato (Stiekema et al., 1988) using CLUSTAL. Match across all sequences (*); Conservative substitutions (.)

TABLE 20: Percentage identities* of SIa1, SIa2 and SIa3 between themselves and with other thionin-like proteins**

	SIα1	SIα2	SIα3	γPur1	γPur2	γHord	POTP	αPur1	αPur2	αHord	Cram
SIα1	-	**12.30**	**15.23**	**9.50**	**12.01**	**11.92**	**9.58**	-0.61	-0.17	-0.15	-0.15
SIα2	40.08	-	**21.39**	**20.43**	**24.50**	**20.96**	**16.28**	0.26	0.99	0.83	-0.28
SIα3	36.7	87.5	-	**22.76**	**18.56**	**24.31**	**12.31**	0.62	1.30	1.10	2.20
γPur1	30.6	72.3	80.9	-	**32.86**	**27.87**	**10.65**	2.89	1.52	0.96	0.69
γPur2	36.7	76.6	83.0	89.4	-	**28.59**	**12.55**	1.33	1.25	1.18	1.10
γHord	36.7	68.1	76.6	87.2	83.0	-	**11.19**	0.82	1.68	1.28	1.40
POTP	20.5	29.9	28.6	31.6	30.3	32.9	-	0.22	1.40	2.65	0.77
αPur	16.0	15.7	18.9	23.1	23.1	19.2	18.4	-	**18.35**	**27.48**	**13.33**
αPur1	10.4	18.9	20.8	25.0	23.1	21.2	19.7	88.9	-	**21.64**	**19.77**
αHord	13.5	20.8	20.8	25.0	23.1	21.2	19.7	86.7	88.9	-	**12.71**
Cram.	14.3	10.2	12.2	12.2	12.8	10.6	11.8	34.8	34.8	30.4	-

* Values above the diagonal represent the *Z* values (Statistical significancies) obtained by the computer RDF2 programme (Pearson and Lipmann, 1988). Values below the diagonal are the % identities of the sequences.

** (γPur1 and γPur2): γ1- and γ2-purothionin from wheat (Collila et al., 1990); (γHord): γ-hordothionin from barley (Mendez et al., 1990); (POTP): A trypsin inhibitor precursor from potato (Stiekema et al., 1988); (αPur1 and αPur2): α1- and α2-purothionins from wheat (Ohtani et al., 1977; Hase et al., 1978); (αHord): α-hordothionin from barley (Ozaki et al., 1980); (Cram): Crambin from Crambe abyssinica (Teeter, et al., 1981).

3.10 Calcium Binding Sites and Model Building

Another fact that supports the hypothesis that SIα1, SIα2 and SIα3 might inhibit α-amylases through competition for calcium ions is that in the amino acid sequence of these proteins calcium binding motifs such as **EF-hand** loops and probably others (da Silva and Reinach, 1991; Heinzmann and Hunziker, 1991) are present (Fig. 42). However, it is not clear whether these sequence motifs are structurally relevant to the observed inhibition (section 3.2).

To verify the possible existence of structurally functional calcium binding sites in these proteins three dimensional structure predictions of SIα1, SIα2 and SIα3 by molecular modelling are under investigation at the Biomolecular Model Building Unit of the U.C.L (University College of London).

Preliminary structure diagrams of SIα1 (Fig. 43) were obtained by using the molecular coordinates of crambin (Hendrickson and Teeter, 1981), Scorpion Neurotoxin (Fontecilla-Camps et al., 1980) and bovine trypsin inhibitor (Huber et al., 1974). The crambin/SIα1 diagram was found to be the most appropriate from all three diagrams for the next model building procedures.

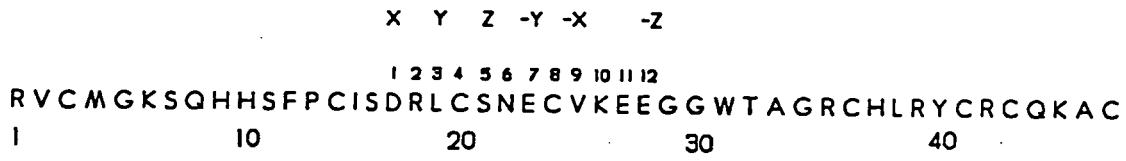


Fig.42: Identification of an **EF-Hand** motif in the amino acid sequence of SIα1. Numbers (1 - 12) indicate the 12 residues involved in the calcium-binding loop of **EF-hand**. *X, Y, Z, -Y, -X* and *-Z* indicate the positions that coordinate the metal ion.

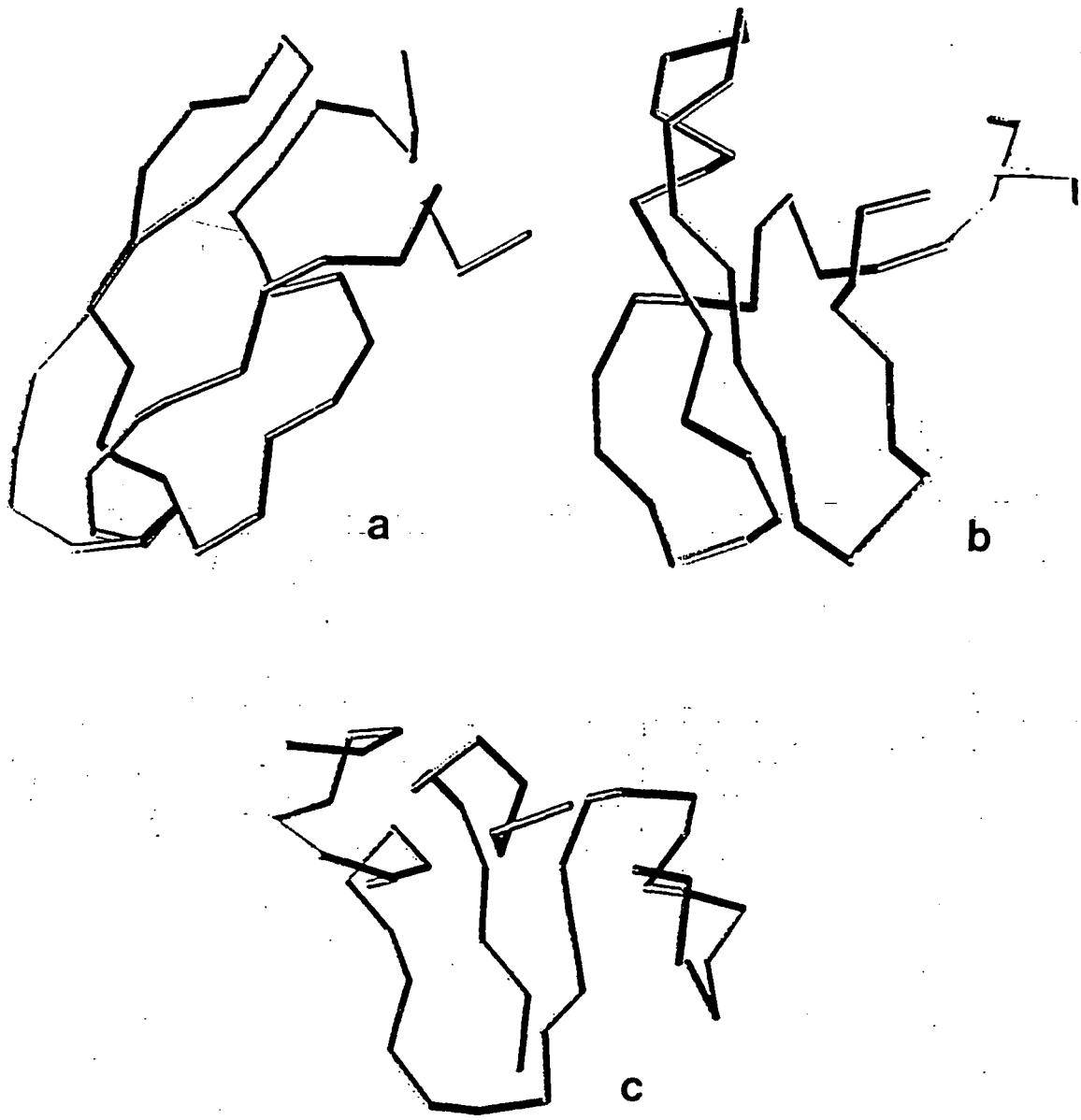


Fig.43: Three-dimensional diagrams of the preliminary models of SIα1. (a) Bovine trypsin inhibitor/SIα1; (b) Scorpio neurotoxin/SIα1; (c) Crambin/SIα1.

3.11 Location of the Disulphide Bonds of SIα1

Assuming that all the cysteines (8) in the SIα1 sequence were involved in forming four disulphide bridges as has been reported for the homologous γ-hordothionin and γ-purothionins (Colilla et al., 1990; Mendez et al., 1990), peptides derived from the trypsin and *S.aureus* V8 protease digestions of native SIα1 (Figs. 44, 45, 46) were purified by HPLC and manually sequenced.

Most of the peptides obtained from a five hour tryptic digestion (Fig 44) showed high levels of contamination after at least five complete sequencing cycles (except T1-9 and T1-10, Table 21). Peaks 25 and 26 (Fig. 44) were found to be SIα1 partially digested. These peaks were further digested for 9 hours with trypsin and *S.aureus* V8 protease (using phosphate buffer which also allows cleavage at the C-terminal of aspartic acid residues) respectively. The peptides obtained from these procedures were purified by HPLC (Fig. 45 and 46) and their amino acid sequences are shown on the Table 21 . Using the same alignment and analysis procedures described in the section 3.8, a complete localization of the four disulphide bonds of SIα1 was possible (Fig 47). The cysteines are linked in the following order: Cys3-Cys47; Cys14-Cys43; Cys20-Cys41 and Cys24-Cys36.

These results revealed that SIα1 (Fig 47) has a similar pattern of the disulphide bonds to those observed in the crambin-related thionins, but because none of the cysteines can be aligned to those of the crambin group the bonds are shifted towards the C-terminal of SIα1, whereas in the crambin related thionins their overall locations are near to their N-terminal end. These results contradict what has been previously suggested for the γ-hordothionin (Mendez et al., 1990) in that four of its cysteines could be aligned to those of the crambin related thionins resulting in a possible formation of two disulphide bonds (i.e. Cys3-Cys34; Cys14-Cys20).

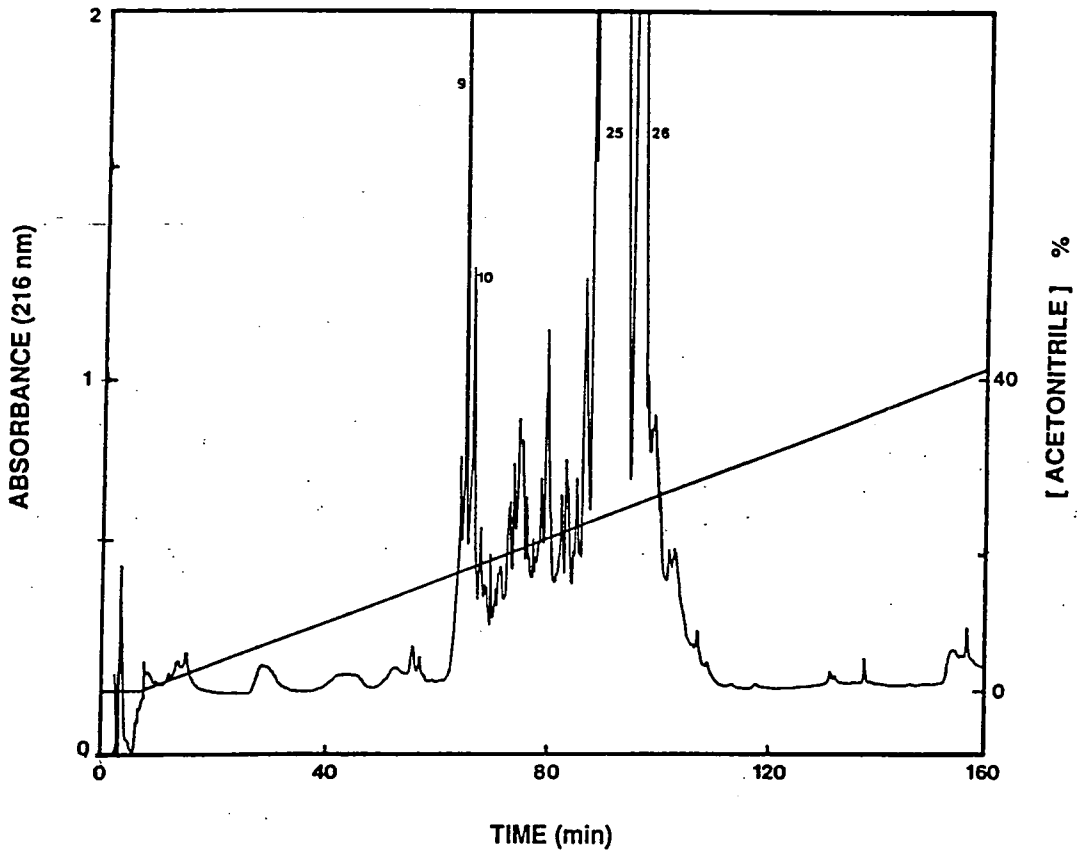


Fig.44: Reverse-phase HPLC of the fragments obtained after 5h tryptic digestion of native Slα1. All other experimental conditions as in Fig. 27.

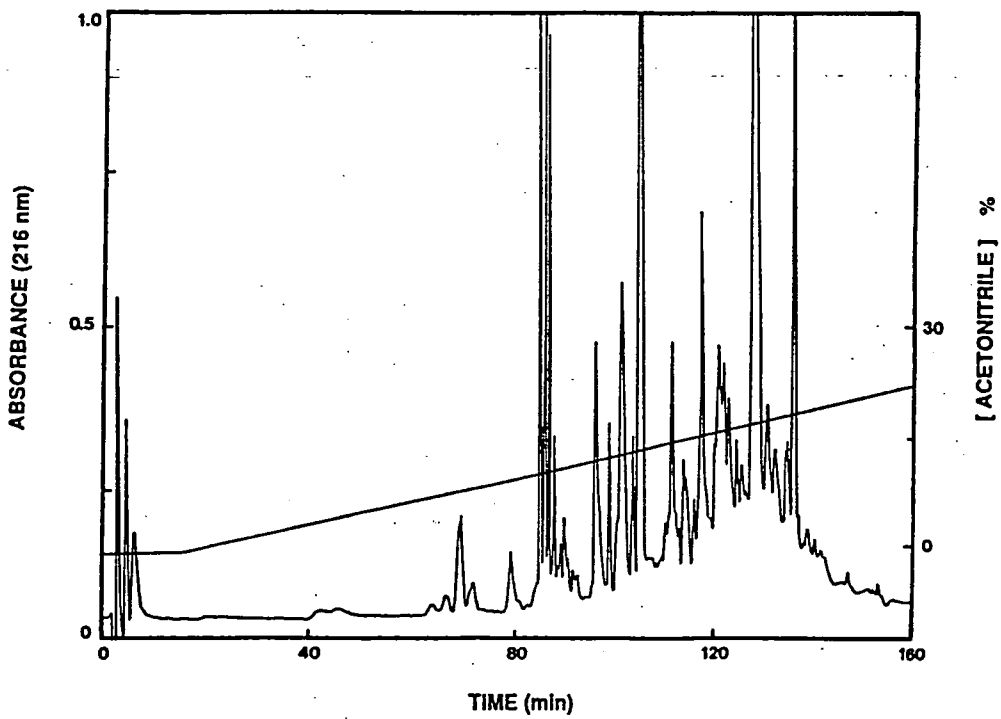


Fig.45: Reverse-phase HPLC of the peptides obtained after 9 hour as tryptic digestion of SIα1, peak 25 (Fig. 44). All experimental conditions as in Fig. 27.

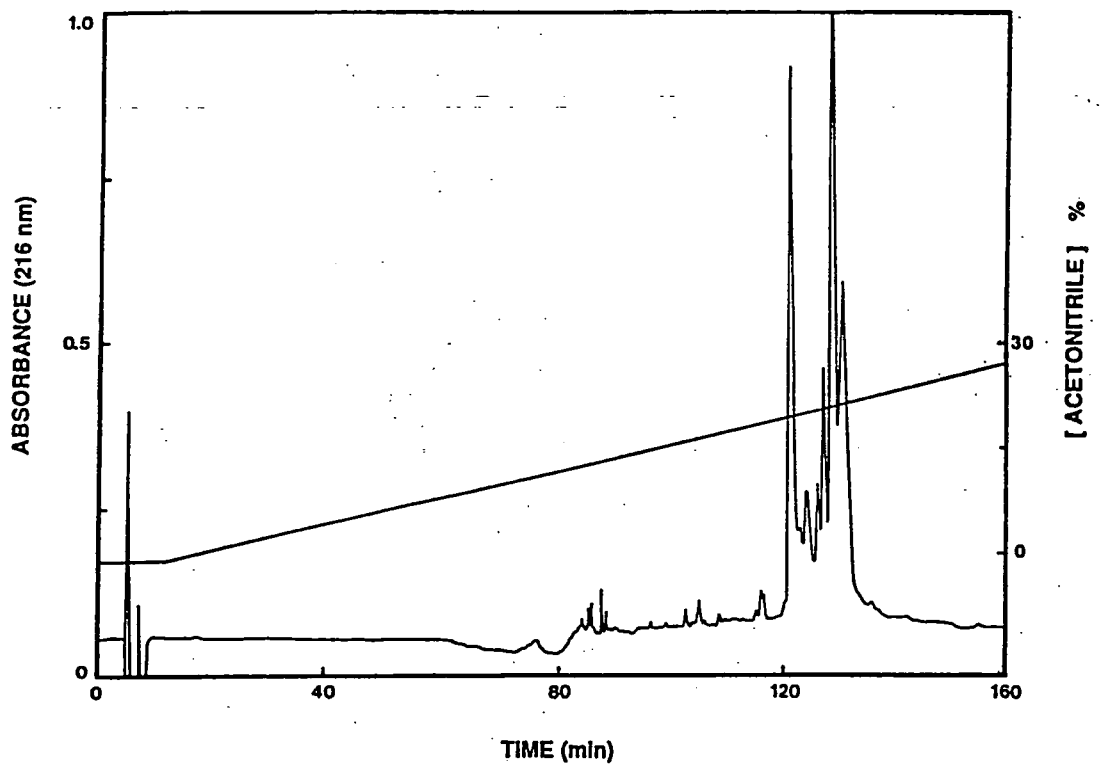


Fig. 46: Reverse-Phase HPLC of the peptides obtained after 9 hours of V8 digestion of SIα1, peak 26 (Fig. 44). All other experimental conditions as in Fig. 27.

Table 21: Peptides obtained from tryptic (T1, T2) and V8 (V) digestions of native SIa1

Peptide	Acetonitrile(%)	Sequence
T1-9	15	RV**X**MG (a) A**X** (b)
T1-10	16	(L/I)**X**SN (a) Y**X**R (b)
T1-25	23	SQHHSFP**X**(L/I)S **X**Q
T2-8	14	RV**X**MG (a) A**X** (b)
T2-9	15	(L/I)**X**SNE (a) Y**X**R (b)
T2-13	16	SQHHSFP**X**(L/I)SDR (a) **X**Q (b)
T2-14	17	EEGG
T2-15	17	Y**X**R (a) (L/I)**X**S (b)
T2-17	18	**X**V (a) **X**H(L/I)R (b)
T2-18	19	(L/I)**X**SN (a) Y**X**R (b)
V-12	23	**X**V (a) GGWTAGR**X** (b)
V-14	24	**X**V (a) GGWTAGR**X**H(L/I)R (b)

X Cysteine residue that could not be seen with the sequencing method used.

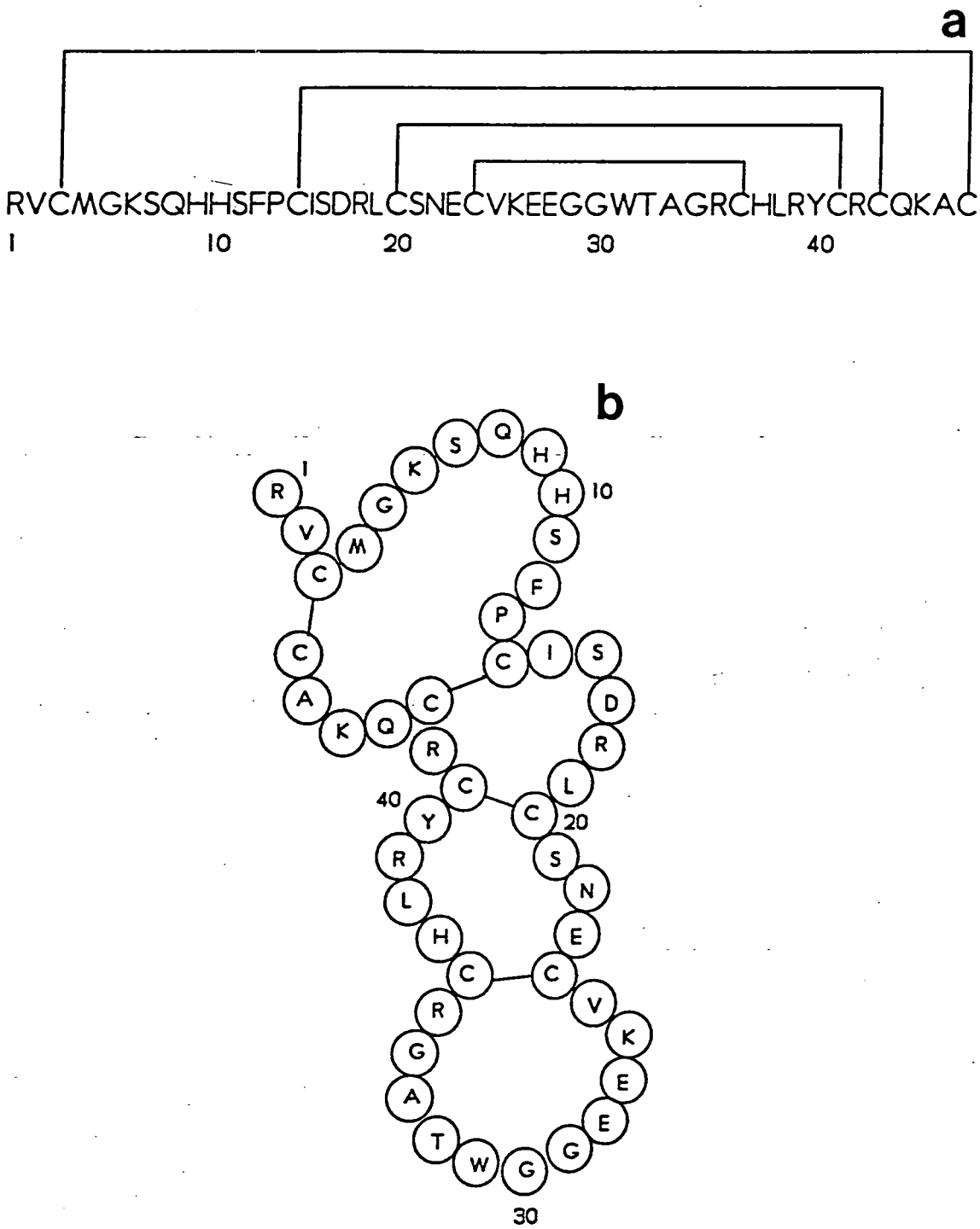


Fig.47: Localization of the disulphide bonds in the amino acid sequence of SIα1. (a) Disulphide bonds indicated by solid lines (——); (b) Covalent representation of SIα1.

3.12 Final Considerations

During the many stages in the preparation of this work, the author took notice of the general idea that α-amylase inhibitors, specially those from legumes and cereals, are regarded as important substances potentially involved in the protection of plants against pathogens and/or predators because of their intrinsic abilities to inhibit α-amylases from several different sources (see Introduction).

This idea appears to have received some degree of support from the results obtained by feeding trials of some insects (Gatehouse et al., 1986, 1990a; Ishimoto and Kitamura, 1988, 1989). In these trials only insects of the bruchid family (Coleoptera: Bruchidae) have been tested and have proved to be highly susceptible to α-amylase inhibitors which appear to interfere with the normal development of the larvae and inflict high mortality rates (Gatehouse et al.,1986; Ishimoto and Kitamura, 1989). However, there is no evidence that indicates that these symptoms are related to the *in vivo* action of the inhibitors against the α-amylase from these insects (Gatehouse et al., 1990b). A slow rate formation, which is reversible when in contact with the substrate (starch in most of the cases) as well as by maltose seems to go against the hypothesis of an *in vivo* inhibition ability and questions the biological significance of the currently used *in vitro* tests. In no case has a complex of the amylase inhibitor fed to the insect and its target α-amylase ever been isolated subsequent to ingestion.

However, the only group of α-amylase inhibitors in which *in vivo* inhibition of the endogenous α-amylase has been observed are those purified from the *Streptomyces* (see Introduction and Vertey et al., 1984). These inhibitors show irreversible binding with α-amylases as well as much faster complex (EI) formation.

3.13 Suggestion for Future Work

Preliminary inspections of the amino acid sequences of SIa4 and SIa5 for known protein sequence motifs, revealed the existence of a region which has structural similarities with the **Leucine Zipper** motif (repeating heptads of hydrophobic residues, usually leucine), but this region did not have the typical nearby basic region designated **b Zip** (Landschulz et al., 1988; Vinson et al., 1989; O'Shea et al., 1989a, 1989b; Talanian et al., 1990; Turner and Tjian, 1989). The respective SIa4 (indicated by * in Fig.29) and SIa5 (Fig.31 not indicated) regions were between the Leu11 to Val39, and Leu8 to Leu37 respectively. It is not known whether these findings are just coincidence or whether they have some implications in the biological role of these proteins.

It is not known why the members of the cereal superfamily (Trypsin inhibitors, α-amylase inhibitors and the so called CM proteins) which have such high levels of primary structure homology between themselves, appear to have such different activities (or no reported activity for some of the CM proteins). Apart from the reactive sites which can be observed in the trypsin inhibitors and are apparently missing in the other proteins, no other obvious differences in structural motifs have been found in these proteins up to now. It is possible that the different groups of proteins that belong to this family could have "minor" structural differences which would be directly related with their activities (like the single arginine-serine peptide bond reactive sites of the trypsin inhibitors). On the other hand, it is also possible that the different activities observed might be related to different protein foldings due to differences in the disulphide bond patterns of each group of protein within the same family (i.e. trypsin inhibitors, α-amylase inhibitors and CM proteins being considered as isomers of intramolecular disulphide bonds).

Regarding the surprising homology found between the sequences of SIα1, SIα2, SIα3, γ-hordothionin and γ1- and γ2-purothionin (Colilla et al., 1990; Mendez et al., 1990) to a region of a larger trypsin inhibitor precursor from potato (Stekema et al.,

1988), it would be interesting to know whether potato (or other *Solanaceae*) tissues contain the similar thionin like proteins as native products.

BIBLIOGRAPHY

Abdul-Hussain, S. and Paulsen, G.M. (1989) *J. Agric. Food Chem.* 37: 295-299

Aitken, A., Geisow, M.J., Findlay, J.B.C., Holmes, C. and Yarwood, A. (1989) *In* "Protein Sequencing - A Practical Approach" (Findlay, J.B.C. and Geisow, M.J. eds.) pp.43-68. IRL Press, Oxford

Alai, M., Denirev, P., Ienselau, C. and Cotter, R.J. (1980) *Anal. Chem.* **58**: 1303-1308

Algiman, E., Kroviarski, S. and Sing, Y.L.K. (1990) *J. Chromatogr.* **510**: 165-175

Allen, G. (1981) *Sequencing of Proteins and Peptides In* "Laboratory Techniques in Biochemistry and Molecular Biology" (Work, T.S. and Burdon, R.H. eds.). North-Holland Publ. Co., Amsterdam

Amons, R. (1987) *FEBS Lett.* **212**: 68-72

Ampe, C., Van Damme, J., De Castro, L.A.B., Sampaio, M.J.A.M., van Montagu, M. and Vanderkerckhore, J. (1986) *Eur. J. Biochem.*. **159**: 697-604

Ary, M.B., Richardson, M. and Shewry, P.R. (1989) *Biochim. Biophys. Acta* **993**: 260-266

Austen, B.M. and Smith, E.L. (1976) *Biochem. Biophys. Res. Commun.* **72**: 411-417

Ashauer, H., Vertesy, L. and Braunitzer, G. (1981) *Hoppe Seyler's Z. Physiol. Chem.* **362**: 465-467. Sitzungsber. Bayer. Akad. Wiss., Mat. Nat. Klasse, 37-38

Baker, J.E. (1987) *Insect Biochem.*. **17**: 37-44

Baker, J.E. (1988) *Insect Biochem.* **18**: 107-116

Baker, J.E. and Woo, S.M. (1985) *Archives of Insect Biochem. Physiol.* **2**: 415-428

Balls, A.K., Hale, W.S. and Harris, T.H. (1942a) *Cereal Chem.* **19**: 277-288

Balls, A.K., Hale, W.S. and Harris, T.H. (1942b) *Cereal Chem.* **19**: 840-844

Balls, A.K. and Harris, T.H. (1944) *Cereal Chem.* **21**: 74-79

Barber, D., Sanchez-Monge, R., Garcia-Olmedo, F., Salcedo, G. and Mendez, E. (1986) *Biochim. Biophys. Acta* **873**: 147-151

Barber, D., Sanchez-Monge, R., Gomez, L., Carpizo, J., Armentia, A., Lopez-Otin, C., Juan, F. and Salcedo, G. (1989) *FEBS Lett.* **248**: 119-122

Barber, D., Sanchez-Monge, R., Mendez, E., Lazaro, A., Garcia-Olmedo, F. and Salcedo, G. (1986) *Bichim. Biophys. Acta* **869**: 115-118

Barkin, D. and De Walt, B.R. (1988) *In* "Sorghum and the Mexican Food Crisis" *Latin American Research Review* Vol. **23**: 30-59

Battershell, V.G. and Henry, R.J. (1990) *J. Cereal Sci.* **12**: 73-81

Becker, T., Weber, K. and Johnsson (1990) *EMBO J.* **9**(13): 4207-4213

Bedetti, C., Bozzini, A., Silano, V. and Vittozzi, L. (1974) *Biochim. Biophys. Acta* **362**: 299-307

Bekes, F. and Lasztity, R. (1981) *Cereal Chem.* **58**; 360-361

Bernard, W.R. and Somerville, C.R. (1989) *Arch. Biochem. Biophys.* **269**: 695-697

Bernfeld, P. (1955) *Methods Enzymol.* **1**: 149-158

Bieth, J. (1974) in "Proteinase Inhibitors" (M. Fritz et al. eds), Bayer-Symposium V pp.463-469, Springer- Verlag

Billeter, M., Kline, A.D., Braun, W., Huber, R. and Wuthrich, K. (1989) *J. Mol. Biol.* **206**: 677-687

Bird, R. and Hopkins, R.H. (1954) *Biochem. J.* **56**: 86-89

Birk, Y. (1976) *Methods. Enzymol.* **6**: 695-751

Blanco-Labra, A. and Iturbe-Chinas, F.A. (1981) *J. Food Chem.* **5**: 1-17

Bo-Linn, G.W., Santa-Ana, C.A., Morawski, S.G. and Fordtran, J.S. (1982) *New Engl. J. Med.* **307**: 1413-1416

Bohlmann, H. and Apel, K. (1987) *Mol. Gen. Genet.* **207**: 446-454

Bohlmann, H., Clausen, S., Behnke, S., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V. and Apel, K. (1988) *EMBO J.* **7**: 1559-1565

Bowman, D.E. (1945) *Science* **102**: 358-361

Braun, W., Epp, O., Wuthrich, K. and Huber, R. (1989) *J. Mol. Biol.* **26**: 669-676

Breu, V., Guerbette, F., Kader, J-C., Kannangara, C.G., Svensson, B. and von Wettstein-Knowles, P. (1989) *Carlsberg Res. Commun.* **54**: 81-84

Brunger, A.T., Clore, G.M., Gronenborn, A.M. and Karplus, M. (1986) *Proc. Natl. Acad. Sci. USA* **83**: 3801-3805

Brunger, A.T., Campbell, R.L., Clore, G.M., Gronenborn, A.M., Karplus, M. Petsko, G.A., Teeter and M.M. (1987) *Science* **235**: 1049-1053

Brunger, A.T., Clore, G.M., Gronenborn, A.M. and Karplus, M. (1986) *Proc. Natl. Acad. Sci. USA* **83**: 3801-3805

Brunix, E. and Rudstam, G. (1961) *Nucl. Instrum. Methods* **131**: 131-136

Buchanan, B.B., Wolosivk, R.A., Shurmann, P. (1979) *Trends Biochem. Sci.* **4**: 93-96

Buisson, G., Duee, E, Payan, F. and Haser, E. (1987) *Food Hydrocolloids* **1**: 399-406

Buonocore, V., Giardina, P., Parlamenti, R., Poerio, E. and Silano, V. (1984) *J. Sci. Food. Agric.* **35**: 225-232

Buonocore, V., Gramenzi, F., Pace, W., Petrucci, T., Poerio, E. and Silano, V. (1980) *Biochem. J.* **187**: 637-645

Buonocore, V., Petrucci, T. and Silano, V. (1977) *Phytochemistry* **16**: 811-820

Buonocore, V., Poerio, E., Gramenzi, F. and Silano, V. (1975) *J. Chomatogr.* **114**: 109-115

Buonocore, V. and Silano, V. (1986) *In* "Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods" (M.Friedman ed.) pp. 483-507, Plenun Press, New York

Campos, F.A.P. and Richardson, M. (1983) *FEBS Lett.* **152**:300-304

Campos, F.A.P. and Richardson, M. (1984) *FEBS Lett.* **167**: 221-225

Cantagalli, P., Di Giorgio, G., Morisi, G., Pocchiari, F. and Silano, V. (1971) *Sci. Food Agric.* **22**:256-262

Carrasco, J.F. and Xavier-Filho, J. (1981) *Ann. Bot.* **47**: 259-266

Carrasco, L., Vazquez, D., Hernandez-Lucas, C., Carbonero, P. and Garcia-Olmedo, F. (1981) *Eur. J. Biochem.* **116**: 185-189

Cavins, J.F. and Friedman, M. (1970) *Anal. Biochem.* **35**: 489-493

Ceska, M., Birath, K. and Brown, B. (1969) *Clin. Chim. Acta* **26**: 437-440

Cha, S. (1976) *Biochem. Pharm.* **25**: 2695-2702

Cha, S. (1980) *Biochem. Pharm.* **29**: 1779-1789

Chandrasekher, G. and Pattabiraman, T.N. (1983) *Ind. J. Biochem. Biophys* **20**: 241-245

Chandrasekher, G., Raju, D.S. and Pattabiraman, T.N. (1981) *J. Sci. Food Agric.* **32**: 9-16

Chang, J.Y., Brauer, D. and Wittman-Liebold, B. (1978) *FEBS Lett.* **93**: 205-214

Chota, C. and Janin, J. (1981) *Prot. Natl. Acad. Sci. USA* **78**: 4146-4150

Chrazascz, T. and Janicki, J. (1933) *Biochem. Z.* **260**: 354-368

Clore, G.M., Brunger, A.T., Karplus, M. and Gronenborn, A.M. (1986a) *J. Mol. Biol.* **191**: 523-551

Clore, G.M., Nilges, M., Sukumaran, D.K., Brunger, A.T., Karplus, M. and Gronenborn, A.M. (1986b) *EMBO J.* **5**: 2729-2735

Clore, G.M., Sukumaran, D.K. and Gronenborn, A.M. (1987) *J. Mol. Biol.* **193**: 571-578

Colilla, F.J., Rocher, A. and Mendez, E. (1990) *FEBS Lett* **270**: 191-194

Cornelissen, B.J.C., Hooft, Van, Huijsduijnen, R.A.M. and Bol, J.T. (1986) *Nature* **321**: 531-532

Crestfield, A.M., Moore, S. and Stein, W.H. (1963) *J. Biol. Chem.* **238**: 622-626

Croft, L.R. (1980) *In* "Handbook of Protein Sequence Analysis" John Wiley and Sons. Chichester, New York

Corcoran, M.R. (1966) *Plant Physiol.* **41**: 1265-1267

Daley, L.S. and Theriot, L.J. (1987) *Agric. Food Chem.* **35**: 680-687

Davis, A.B. and Hoseney, R.C. (1979) *Cereal Chem.* **56**(4): 314-316

Deponte, R., Parlamenti, R., Petrucci, T., Silano, V. and Tomasi, M. (1976) *Cereal Chem.* **53**: 805-811

Drapeau, G.R. (1977) *Methods. Enzymol.* **47**: 189-191

Edens, L., Heslinga, L., Klok, R., Ledeboer, A.M., Maat, J., Toonen, M.Y., Visser, C. and Verrips, C.T. (1982) *Gene* **18**: 1-12

Edsall, J.T. and Wyman, J. (1958) "Biophysical Chemistry" pp. 610-620

Elliot, B.B. and Leopold, A.C. (1953) *Physiol. Plant* **6**: 65-71

Erlanger, B.F., Kokowski, N. and Cohen, W. (1961) *Arch. Biophys.* **95**: 271-275

Feng, G.-H., Chen, M. Kramer, K.J. and Reeck, G.R. (1991) *Cereal Chem.* **68**: 95-99

Fernandez de Caleya, R. (1973) PhD Thesis Univ. Pol. de Madrid, Spain

Fernandez de Caleya, R., Gonzalez-Pascual, B., Garcia-Olmedo, F. and Carbonero, P. (1972) *Appl. Microbiol.* **23**: 998-1000

Fernandez de Caleya, R., Hernandez-Lucas, C., Carbonero, P. and Garcia-Olmedo, F. (1976) *Genetics* **83**: 687-699

Finardi Filho, F. and Lajolo, F.M. (1982) *Cienc. Tecnol. Aliment.* **2**: 1-20

Fontecilla-Camps, J.C., Almassy, R.J., Suddath, F.L., Watt, D.D. and Bugg, C.E. (1980) *Proc. Natl. Acad. Sci USA* **77**: 6496-6500

Fossum, K. and Whitaker, J.R. (1974) *J. Nutr.* **104**: 930-934

Frels, J.M. and Rupnow, J.H. (1985) *J. Food Sci.* **50**: 72-77

Garcia-Olmedo, F. and Carbonero, P. (1970) *Phytochemistry* **9** 1495-1497

Garcia-Olmedo, F., Carbonero, P., Hernandez-Lucas, C., Paz-Ares, J., Ponz,F., Vicente, O. and Sierra, J.M. (1983) *Biochim. Biophys. Acta* **740**: 52-56

Garcia-Olmedo, F., Rodriguez-Palenzuela, P., Hernandez-Lucas, C., Ponz, F., Marana, C., Carmona, M.J., Lopez-Fando, J., Fernandez, J.A. and Carbonero, P. (1989) *In* "Oxford Surveys of Plant Molecular and Cell Biology" (Miflin, B.J. ed.), Vol. 6 pp.31-60. Oxford Univ. Press, Oxford

Garcia-Olmedo, F. Salcedo, G., Sanchez-Monge, R., Gomez, L., Royo, J. and Carbonero, P. (1987) "Oxford Survey of Plant Molecular and Cell Biology" (Miflin, B.J. ed.), Vol. 4 pp. 275-334. Oxford Univ. Press, Oxford

Garcia-Olmedo, F., Sotelo, I and Garcia-Faure, R. (1968) *Anales Inst. Nac. Invest. Agro.* **17**: 433-443

Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) *J. Mol. Biol.* **120**: 97-120

Gatehouse, A.M.R. (1984) *In* "Developments in Food Proteins-3" (B.J.F. Hudson ed.) pp. 245-294. Elsevier, Amsterdam

Gatehouse, A.M.R., Fenton, K.A., Jepson, I. and Pavey, D.J. (1986) *J. Sci. Food Agric.* **37**: 727-734

Gatehouse, A.M.R., Minney, B.H., Dobie, P. and Hilder, V.A. (1990a) *In* "Bruchids and Legumes: Economics, Ecology and Coevolution" (K.Fujii ed.) pp. 241-256. Kluwer Academic Publisher, Netherlands

Gatehouse, J.A., Hilder, V.A. and Gatehouse, A.M.R. (1990b) *In*"Plant Genetic Engeneering" Plant Biotechnology Series (D. Grierson ed.) pp 83 -135. Blackie, London

Gausing, K. (1987) *Planta* **171**: 241-246

Gayler, K.R., Kolivas, S., Macfarlane, A.j., Lilley, G.G., Baldi, M., Blagrove, R.J. and Johnson, E.D. (1990) *Plant Mol. Biol.* **15**: 879-893

Gomez, L., Martin, E., Hernandez, D., Sanchez-Monge, R., Barber, D., del Pozo, V., de Andres, B., Armentia, A., Lahoz, C., Salcedo, G. and Palomino, P. (1990) *FEBS Lett.* **261**: 85-88

Goto, A., Matsui, Y., Ohyama, K., Arai, M. and Murao, S. (1983) *Agric. Biol. Chem.* **47**: 83-88

Goto, A., Matsui, Y., Ohyama, K., Arai, M. and Murao, S. (1985) *Agric. Biol. Chem.* **49**: 435-439

Granum, P.E. (1978) *J. Food Biochem.* **2**: 103-120

Granum, P.E. (1979) *Food Chem.* **4**: 173-178

Granum, P.E. and Eskeland, B. (1981) *Nutr. Rep. Int.* **23**: 155-162

Granum, P.E., Holm, H., Wilcox, E. and Whitaker, J.R. (1983) *Nutr. Rep. Int.* **28**: 1233-1244

Guitierrez, C., Sanchez-Monge, R., Gomez, L., Ruiz-Lapiador, M., Castalera, P. and Salcedo, G. (1990) *Plant Sci.* **72**: 37-44

Halayko, A.J. Hill, R.D. and Svensson, B. (1986) *Biochim. Biophys. Acta* **873**: 92-101

Gatehouse, J.A., Hilder, V.A. and Gatehouse, A.M.R. (1990b) *In*"Plant Genetic Engeneering" Plant Biotechnology Series (D. Grierson ed.) pp -135. Blackie, London

Gausing, K. (1987) *Planta* **171**: 241-246

Gayler, K.R., Kolivas, S., Macfarlane, A.j., Lilley, G.G., Baldi, M., Blagrove, R.J. and Johnson, E.D. (1990) *Plant Mol. Biol.* **15**: 879-893

Gomez, L., Martin, E., Hernandez, D., Sanchez-Monge, R., Barber, D., del Pozo, V., de Andres, B., Armentia, A., Lahoz, C., Salcedo, G. and Palomino, P. (1990) *FEBS Lett.* **261**: 85-88

Goto, A., Matsui, Y., Ohyama, K., Arai, M. and Murao, S. (1983) *Agric. Biol. Chem.* **47**: 83-88

Goto, A., Matsui, Y., Ohyama, K., Arai, M. and Murao, S. (1985) *Agric. Biol. Chem.* **49**: 435-439

Granum, P.E. (1978) *J. Food Biochem.* **2**: 103-120

Granum, P.E. (1979) *Food Chem.* **4**: 173-178

Granum, P.E. and Eskeland, B. (1981) *Nutr. Rep. Int.* **23**: 155-162

Granum, P.E., Holm, H., Wilcox, E. and Whitaker, J.R. (1983) *Nutr. Rep. Int.* **28**: 1233-1244

Guitierrez, C., Sanchez-Monge, R., Gomez, L., Ruiz-Lapiador, M., Castalera, P. and Salcedo, G. (1990) *Plant Sci.* **72**: 37-44

Halayko, A.J. Hill, R.D. and Svensson, B. (1986) *Biochim. Biophys. Acta* **873**: 92-101

Halford, N.G., Morris, N.A., Urwin, P., Williamson, M.S., Kasarda, D.D., Lew, E.J.L., Kreis, M., Shewry, P.R. (1988) *Biochim Biophys. Acta* **950**: 435-440

Harlan, J.J. and deWet, J.M.J. (1972) *Crop Sci* **12**: 172-173

Hase, T., Matsubara, H. and Yoshizumi, H. (1978) *J. Biochem.* **83**: 1671-1678

Hayashi, K., Imoto, T. and Funatsu, M. (1963) *J. Biochem.* **54**: 381-387

Heinzmann, C.W. and Hunziker, W. (1991) *TIBS* **16**: 98-103

Hendrickson, W.A. and Teeter, M.M. (1981) *Nature* **290**: 107-113

Hernandez,A. and Jaffe, W.G. (1968) *Acta Cient. Venez.* **19**: 183-191

Hernandez-Lucas, C., Fernandez de Caleya, R. and Carbonero, P. (1974) *Appl. Microbiol.* **28**: 165-168

Hernadez-Lucas, C., Carbonero, P. and Garcia-Olmedo, F. (1978) *J. Agric. Food Chem.* **26**: 794-796

Higgins, D.G. and Sharp, P.M. (1988) *Gene* **73**: 237-244

Higgins, D.G. and Sharp, P.M. (1989) *CABIOS* **5**: 151-153

Huang, W.Y. and Tietz, N.W. (1982) *Clin. Chem.* **28**: 1525-1527

Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J. and Steigemann, W. (1974) *J. Mol. Biol.* **89**: 73-101

Inglis, A.S., McMahon, D.T.W., Roxburgh, C.M. and Takayanagi (1976) *Analytical Biochem.* **72**: 86-94

Irshad, M. and Sharma, C.B. (1981) *Biochim. Biophys. Acta* **659**: 326-330

Ishimoto, M. and Kitamura, K. (1988) *Jap. J. Breed.* **38**: 367-370

Ishimoto, M. and Kitamura, K. (1989) *Appl. Ent. Zool.* **24**: 281-286

Jaffe, W.G., Moreno, R. and Wallis, V. (1973) *Nutr. Rep. Int.* **7**: 169-174

Jany, K.D. and Lederer, G. (1985) *Biol. Chem. Hoppe-Seyler* **366**:807-808

Johnson, T.C., Wada, K., Buchaman, B.B. and Holmgren, A. (1987) *Plant Physiol.* **85**; 446-451

Jones, B.L. and Cooper, D.B. (1980) *J. Agric. Food Chem.* **28**: 904-908

Jones, B.L. and Mak, A.S. (1977) *Cereal Chem.* **54**: 511-523

Jones, B.L. and Meredith, P. (1982) *Cereal Chem.* **59**: 321

Jonsson, G., Hedin, A., Hakansson, P., Sundquist, B., Save, G., Nielsen, P.F., Roepstorff, P., Johansson, K.E., Kamensky, I. and Lindberg, M. (1986) *Anal. Chem.* **58**: 1084-1088

Jordan, E.A., Martin, C.R., McNeal, C.J. and Macfarlane, R.D. (1986) Proc. 32nd. Ann. Conf. on Mass Spectrom. and Allied Topics, San Antonio, American Society for Mass Spectrometry pp 26-27

Kashlan, N. and Richardson, M. (1981) *Phytochemistry* **20**: 1781-1784

Kasarda, D.D., Lew, E.J-L., Kreis, M. and Shewry, P. (1988) *Biochim. Biophys. Acta* **950**: 435-440

Kim, C.-H. and Kim, D.-S. (1990) *J. Chromatogr* ***512***: 131-137

Kline, A.D., Braun, W. and Wuthrich, K. (1988) *J. Mol. Biol.* **204**: 675-724

Kneen, E. and Sandstedt, R.M. (1943)*J. Am. Chem. Soc.* **65**: 1247-1252

Kneen, E. and Sandstedt, R.M. (1946) *Arch. Biochem.* **9**: 235-238

Konarev, A.V. (1982) *In* "Peculiarities of Biology and Utilization of Species and Varieties of Cultivated Plants in Breeding" Bull. n.1 of the Vavilov Institute of Plant Industry Fasc. 118 ISSN 0202-5361

Konopa, J., Woynarowsky, J.M. and Lewandowska-Gumieniak, M. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**: 1525-1533

Kortt, A.A., Caldwell, J.B., Lilley, G.G. and Higgins, T.J.V. (1991) *Eur. J. Biochem.* **195**: 329-334

Kotaru, M., Saito, K., Yoshikawa, H., Ikeuchi, T. and Ibuki, F. (1987a) *Agric. Biol. Chem.* **51**: 577-578

Kotaru, M., Yoshikawa, H., Ykeuchi, T. Saito, K. Iwami, K., and Ibuki, F. (1987b) *J. Nutr. Sci. Vitaminol.* **39**: 359-367

Kreis, M., Shewry, P.R., Forde, B.G., Forde, J. and Miflin, B.J. (1985a) *In* "Oxford Surveys of Plant Molecular Biology" (Miflin, B.J. ed.), Vol. 2, pp. 253-317. Oxford Press, Oxford

Kreis, M. Shewry, P.R., Forde, B.G., Forde, J. and Miflin, B.J. (1985b) *J. Mol. Biol.* **183**: 499-502

Krishna Sharma, K. and Pattabiraman, T.N. (1980) *J. Sci. Food Agric.* **31**: 981-986

Krishna Sharma, K. and Pattabiraman, T.N. (1982a) *Ind. J. Biochem. Biophys.* **19**: 54-56

Krishna Sharma, K. and Pattabiraman, T.N. (1982b) *J. Sci.* Food *Agric.* **33**: 255-258

Kumar, P.M.H., Virupaksha, T.K. and Vithayathil, P.J. (1978) *Int. J. Peptide Protein Res.* **12**: 185-196

Kunitz, M. (1945) *Science* **101**: 668-669

Kutty, A.V.M. and Pattabiraman, T.N. (1986a) *J. Agric. Food Chem.* **34**: 552-557

Kutty, A.V.M. and Pattabiraman, T.N. (1986b) *J. Food Biochem.* **10**: 117-124

Kutty, A.V.M. and Pattabiraman, T.N. (1986c) *Biochem. Arch.* **2**(3): 203-208

Kutty, A.V.M. (1987) PhD Thesis, Bangalore University, India

Kutty, A.V.M. and Pattabiraman, T.N. (1987) *Biochem. Arch.* **3**: 231-240

Kyte, J. and Doolittle, R. (1982) *J. Mol. Biol.* **157**: 105-132

Laemmli, U.K. (1970) *Nature* **227**: 680-685

Lajolo, F.M. and Finardi Filho, F. (1985) *J. Agric. Food Chem.* **33**: 132-138

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1988) *Science* **240**: 1759-1764

Laskowski, M.Jr. and Kato, I. (1980) *Ann. Rev. Biochem.* **49**: 593-626

Lauriere, C., Mayer, C., Renard, H., MacGregor, A.W. and Daussant, J. (1985) *In* "Proceedings of the European Biochemical Congress", Helsinki, pp. 675-682

Layer, P., Zinsmeister, A.R. and Dimagno, E.P. (1986) *Gastroenterology* **91**: 41-48

Lazaro, A., Barber, D., Salcedo, G. and Garcia-Olmedo, F. (1985) *Eur. J. Biochem.* **149**: 617-623

Lecomandeur, D., Lauriere, C. and Daussant, J. *Plant Physiol. Biochem.* **25**: 711-715

Lecomte, J.T.J., Jones, B.L. and Llinas, M. (1982) *Biochemistry* **21**: 4843-4849

Liener, I.E. and Kakade, M.L. (1980) *In* "Toxic Constituents of Plant Footstuffs", (Liener, I.E. ed.)pp. 7-71, Academic Press, London

Longstaff, C. and Gaffney, P.J. (1991) *Biochemistry* **30**: 979-986

Lowe, C.R. and Pearson, J.C. (1984) *Methods Enzymol.* **104**: 97-113

Lyons, A., Richardson, M., Tatham, A.S. and Shewry, P.R. (1987) *Bichim. Biophys. Acta* **915**: 305-313

Macfarlane, R.D., McNeal, C.J. and Martin, C.R. (1986) *Anal. Chem.* **58**: 1091-1095

Macri, A., Parlamenti, R., Silano, V. and Valfre, F. (1977) *Poultry Sci.* **56**: 434-441

Maeda, K. (1986) *Bichim. Biophys. Acta* **871**: 250-256

Maeda, K., Hase, T. and Matsubara, H. (1983a) *Bioch. Biophys. Acta* **743**: 52-57

Maeda, K., Wakabayashi, S. and Matsubara, H. (1983b) *J. Biochem.* **94**: 865-870

Maeda, K., Wakabayashi, S. and Matsubara, H. (1985a) *Biochim. Biophys. Acta* **828**: 213-221

Maeda, K., Tamakoshi, K., Yamashita, A. and Fukumoto, T. (1985b) *Biochim. Biophys. Acta* **828**: 222-228

Mahoney, W.C., Hermodson, M.A., Jones, B., Powers, D.D., Corfman, R.S. and Reeck, G.R. (1984) *J. Biol. Chem.* **259**: 8412-8416

Manjunath, N.H., Keerabhadrappa, P.S. and Virupaksha, T.K. (1983) *Phytochemistry* **22**: 2349-2357

Mantsala, P. and Zalkin, H. (1979) *J. Biol. Chem.* **254**: 8540-8547

Marshall, J.J. and Lauda, C.M. (1975a) *Die Starker* **27**: 274-278

Marshall, J.J and Lauda, C.M. (1975b) *J. Biol. Chem.* **250**: 8030-8037

Martin, F.W. (1984) "Handbook of Tropical Food Crops" (Martin, F.W. ed.), pp. 17-22. CRC Press, Boca Raton, Florida

Matsubara, H., Sasaki, R.K., Tsuchiya, D.K. and Evans, M.C.W. (1970) *J. Biol. Chem.* **245**: 2121-2131

Matsuura, Y., Kusunoki, M., Harada, W. and Kakudo, M. (1984) *J. Biochem.* **95**: 697-702

Mattoo, A.K. and Modi, V.V. (1970) *Enzymologia* **39**: 237-247

McMullen, B.A., Fujikawa, K. and Davie, E.W. (1991) *Biochemistry* **30**: 2050-2056

Mellstrand, S.T. (1974) *Acta Pharm. Suec.* **11**: 410-412

Mellstrand, S.T. and Samuelsson, G. (1973) *Eur. J. Biochem.* **32**: 143-147

Mellstrand, S.T. and Samuelsson, G. (1974) *Acta Pharm. Suec.* **11**: 347-360

Mendez, E., Moreno, A., Colilla, F., Pelaez, F., Limas, G.G., Mendez, R., Soriano, F., Salinas, M. and deHaro, C. (1990) *Eur. J. Biochem.* **194**: 533-539

Miflin, B.J. (1989) "Oxford Suerveys of Plant Molecular and Cell Biology" (Miflin, B.J. ed.) Vol.**6**: 31-60. Oxford Univ. Press, Oxford

Militzer, W., Ikeda, C. and Kneen, E. (1946a) *Arch. Biochem.* **9**: 309-320

Militzer, W., Ikeda, C. and Kneen, E. (1946b) *Arch. Biochem.* **9**: 321-329

Miller, S. (1989) *Protein Engineering* **3**(2): 77-83

Moreno, J. and Chrispeels, M.J. (1989) *Proc. Natl. Acad. Sci. USA* **86**: 7885-7889

Morrison, J.F. (1982) *TIBS* **7**: 102-105

Mundy, J. Hejgaard, J. and Svendsen, I. (1984) *FEBS Lett.* **167**: 210-214

Mundy, J. and Rogers, J.C. (1986) *Planta* **169**: 51-63

Mundy, J., Svendsen, I. and Hejgaard, J. (1983) *Carlsberg Res. Commun.* **48**: 81-90

Murai, H., Hara, S., Ikenaka, T., Goto, A., Arai, M. and Murao, S. (1985) *J. Biochem.* **97**: 1129-1133

Murao, S., Goto, A., Matsui, Y. and Ohyama, K. (1980a) *Agric. Biol. Chem.* **44**: 1679-1681

Murao, S., Goto, A. and Arai, M. (1980b) *Agric. Biol. Chem.* **44**: 1683-1684

Murao, S., Goto, A., Matsui, Y., Ohyama, K. and Arai, M. (1981) *Agric. Biol. Chem.* **45**: 2599-2604

Murao, S. and Ohyama, K. (1975) *Agric. Biol. Chem.* **39**: 2271-2274

Murao, S. and Ohyama, K. (1979) *Agric. Biol. Chem.* **43**: 679-681

Murao, S., Oouchi, N., Goto, A. and Arai, M. (1983) *Agric. Biol. Chem.* **47**: 453-454

Nagaraj, R.H. and Pattabiraman, T.N. (1985a) *J. Agric. Food Chem.* **33**: 646-650

Nagaraj, R.H. and Pattabiraman, T.N. (1985b) *J. Biosci.* **7**: 257-268

Nagaraj, R.H. and Pattabiraman, T.N. (1986) *Ind. J. Med. Res.* **84**: 89-92

Nakajima, R., Imanaka, T. and Aiba, S. (1986) *Appl. Microbiol. Biotechnol.* **23**: 355-360

Nakanishi, T., Yoshizumi, H., Tahara, S., Hakura, A. and Toyoshima, K. (1979) *Gann* **70**: 323-326

Nelson, N. (1944) *J. Biol. Chem.* **153**: 375-379

Nose, Y. and Ichikawa, M. (1968) *J. Ferment. Technol.* **46**: 915-925

O'Connor, C.M., M.D. O'Donnell and McGeeney, K.F. (1981a) *Biochim. Biophys. Acta* **658**: 387-396

O'Connor, C.M. and McGeeney, K.F. (1981b) *Biochim. Biophys. Acta* **658**: 397-405

Odani, S., Koide, T. and Ono, T. (1983) *J. Biochem.* **93**: 1701-1704

O'Donnell, M.D., Fitzgerald, O. and Mcgeeney, K.F. (1977) *Clin. Chem.* **23**: 560-566

O'Donnell, M.D. and McGeeney, K.F. (1976) *Biochim. Biophys Acta* **422**: 159-169

O'Donnell, M.D. and McGeeney, K.F. (1983) *Clin. Chem.* **29**: 510-512

Ohtani, S., Okada, T., Kagamiyama, H. and Yoshizumi, H. (1975) *Agric. Biol. Chem.* **39**: 2269-2270

Ohtani, S., Okada, T., Yoshizumi, H. and Kagamiyama, H. (1977) *J. Biochem.* **87**: 753-767

Ohyama, K. and Murao, S. (1977) *Agric. Biol. Chem.* **41**: 2221-2228

Okabe, H., Uji, Y, Netsu, K. and Noma, A. (1984) *Clin. Chem.* **30**: 1219-1224

Okada, T. and Yoshizumi, H. (1970) *Agric. Biol. Chem.* **34**: 1089-1094

Okada, T. and Yoshizumi, H. (1973) *Agric. Biol. Chem.* (1973) **37**: 2289-2294

Okon, E.U. and Uwaifo, A.O. (1984) *J. Agric. Food Chem.* **32**:11-14

Onesti, S., Brick, P. and Blow, D. (1990) *Acta Crystalogr.* A46, PS-03.11.03

Osborn, T.C., Alexander, D.C., Sun, S.S.M., Cardona, C. and Bliss, F.A. (1988) *Science* **240**: 1505-1509

O'Shea, E.K., Rutkowski, R. and Kim, P.S. (1989a) *Science* **243**: 538-542

O'Shea, E.K., Rutkowski, R., Stafford, W.F. and Kim, P.S. (1989b) *Science* **245**: 646-648

Ozaki, Y., Wada, K., Hase, T., Matsubara, H., Nakanishi, T. and Yoshizumi, H. (1980) *J. Biochem.* **87**: 549-555

Ozals, J. and Gerard, C. (1977) *J. Biol. Chem.* **252**: 5986-5989

Pace, W., Parlamenti, R., Ur Rab, A., Silano, V. and Vittozzi, L. (1978) *Cereal Chem.* **55**: 244-254

Pal, G.P., Betzel, C. Jany, K.D. and Saenger, W. (1986) *FEBS Lett.* **197**; 111-114.

Parviainen, M.T., Koivula, T. and Jokela, M. (1984) *J. Clin. Chem. Cli. Biochem.* **22**: 41-48

Payan, F., Haser, R., Pierrot, M., Frey, M. and Astier, J.P. (1980) *Acta Cryst* **B36**: 416-421

Pearson, W.R. and Lipmann, D.J. (1988) *Proc. Natl. Acad. Sci. USA* **85**: 2444-2448

Peruanskii, Y.V. and Gabsattarova, B.S. (1979) *Khim. Prir. Soedin.* **5**: 742-744

Petrucci, T., Sannia, G., Parlamenti, R. and Silano, V. (1978) *Biochem. J.* **173**: 229-235

Petrucci, T., Tomasi, M., Cantagalli, P. and Silano, V. (1974) *Phytochemistry* **13**: 2487-2495

Petrucci, T., Ur Rab, A., Tomasi, M. and Silano, V. (1976) *Biochim. Biophys. Acta* **420**: 288-297

Pflugrath, J.W., Wiegand, G. and Huber, R. (1986) *J. Mol. Biol.* **189**: 383-386

Pick, K.H. and Wober, G. (1978a) *Hoppe-Seyler's Z. Physiol. Chem.* **359**: 1371-1377

Pick, K.H. and Wober, G. (1978b) *Hoppe-Seyler's Z. Physiol. Chem.* **359**: 1379-1384

Pick, K.H. and Wober, G. (1979) *Prep. Biochem.* **9**: 293-301

Powell, J.M. (1985) *Trop. Agric.* 62: 77-81

Powers, J.P. and Whitaker, J.R. (1977a) *J. Food Biochem* **1**: 217-238

Powers, J.P. and Whitaker, J.R. (1977b) *J. Food Biochem.* **1**: 239-260

Puls, W. and Keup, U. (1973) *Diabetologia* **9**: 97-101

Rackis, J.J., Wolf, W.J. and Baker, E.C. (1986) *In* "Nutritional and Toxicological Significance of Enzyme Inhibitors in Food". (M.Friedman ed.) pp. 299-347. Plenum Press, New York

Rausher, E., Neumann, U., Wahlefeld, A.W., Hagen, A., Gruber, W. and Ziegenhorn, J. (1979) Ger.Offen. 2.741,192 (Cl.G01N31/14)

Redman, D.G. (1975) *Biochem. J.* **149**: 725-734

Redman, D.G. (1976) *Biochem. J.* **155**: 193-201

Richardson, M. (1977) *Phytochemistry* **16**: 159-169

Richardson, M. (1981a) *Food Chem.* **6**: 235-253

Richardson, M. (1981b) *J. Biol. Educ.* **15**: 178-182

Richardson, M. (1991) *In* "Methods in Plant Biochemistry" (Rogers, L.J.eds.) Vol.5, pp.259-305. Academic Press, New York

Richardson, M. and Kashlan, N. (1981) *In* "Regulators of Intestinal Absorption in Obesity, Diabetes and Nutrition" (Berchtold, P., Cairella, M., Jacobelli, A. and Silano, V. eds.), pp. 103-125. Societa Editrice Universo, Roma

Richardson, M. Valdes-Rodriguez, S. and Blanco-Labra, A. (1987) *Nature* **327**: 437-434

Robertson, M. and Hill, R. (1989) *J. Cereal Sci* **9**: 237-246

Rodriguez-Loperena, M.A., Aragoncillo, C., Carbonero, P. and Garcia-Olmedo, F. (1975) *Phytochemistry* **14**: 1219-1223

Ryan, C.A. (1973) *Ann. Rev. Pl. Physiol.* **24**; 173-196

Ryan, C.A. (1979) *In* "Herbivores: Their Interaction with Secondary Plant Metabolities" (G.A.Rosenthal and D.H.Janzen eds.) pp. 599-618. Academic Press, New York

Ryan, C.A. (1981) *In* "The Biochemistry of Plants" (A.Marcus ed.), Vol.6, pp. 351-370. Academic Press, New York

Ryan, C.A. and Walker-Simmons, M. (1981) *In* "Biochemistry of Plants" (E.Marcus ed.), Vol.6, pp. 321-347. Academic Press, New York

Sadowski, J., MacGreggor, A.W. and Daussant, J. (1986) *Electrophoresis* **7**: 176-179

Saito, N. (1982) *J. Biol. Chem.* **257**(6): 3120-3125

Samuelsson, G. (1974) *Syst. Zool.* **22**: 566-569

Samuelsson, G. and Pettersson, B. (1977) *Acta Pharm. Suec.* **14**: 245-254

Sanchez-Monge, R. Fernandez, J.A. and Salcedo, G. (1987) *Theor. Appl. Genet.* **74**: 811-816

Sanchez-Monge, R., Gomez, L., Garcia-Olmedo, F. and Salcedo, G. (1986) *FEBS Lett.* **207**: 105-109

Sanchez-Monge, R., Barber,D., Mendez, E., Garcia-Olmedo, F. and Salcedo, G. (1986b) *Theor. App. Genet.* **72**: 108-113

Sandstedt, R.M. and Beckford, O.C. (1946) *Cereal Chem.* **23**: 548-555

Saunders, R.M. (1975) *Cereal Foods World* **20**: 282-285

Saunders, R.M. and Lang, J.A. (1973) *Phytochemistry* **12**: 1237-1243

Shainkin, R. and Birk, Y. (1970) *Biochim. Biophys. Acta* **221**: 502-508

Shewry, P.R., Lafiandra, D., Salcedo, G., Aragoncillo, C., Garcia-Olmedo, F., Lew, E.J.-L., Dietler, M.D. and Kasarda, D.D. (1984) *FEBS Lett.* **175**: 359-363

Shewry, P.R., Parmar, S. and Field, J.M. (1988) *Electrophoresis* **9**: 727-737

Shewry, P.R. and Tatham, A.S. (1990) *Biochem. J.* **267**: 1-12

Shivaraj, B. and Pattabiraman, T.N. (1980) *Ind. J. Biochem. Biophys.* **17**: 181-185

Shivaraj, B. and Pattabiraman, T.N. (1981) *Biochem* J. **193**: 29-36

Shivaraj, B., Rao, H.N. and Pattabiraman, T.N. (1982) *J. Sci. Food Agric.* **33**: 1080-1091

Shivaraj, B., Sharma,K.S. and Pattabiraman, T.N. (1979) *Ind. J. Biochem. Biophys.* **16**, April: 52-55

Silano, V. (1986) *In* "Enzymes and Their Role in Cereal Technology", (J.E. Kruger et al., eds.) pp. 141-199, Amer. Assn. Cereal Chem.

Silano, V., Furia, M., Gianfreda, L., Macri, A., Palescandolo, R. Rab, A., Scardi, V. Stella, E. and Valfre, F. (1975) *Biochim. Biophys. Acta* **391**: 170-178

Silano, V., Pocchiari, F. and Kasarda, D.D. (1973) *Biochim. Biophys. Acta* **317**: 139-148

Silano, V., Poerio, E. and Buonocore, V. (1977) *Mol. Cell. Biochem.* **18**: 87-91

da Silva, A.C. R. and Reinach, F.C. (1991) *Trends in Biochem. Sci.* **16**: 53-57

Singh, N.K., Bracker, C.A., Hasegawa, P.M., Handa, A.K., Buckel, S., Hermodson, M.A., Pfankoch, E., Regnier, F.E. and Bressan, R.A. (1987) *Plant Physiol.* **85**: 529-536

Singh, U., Kherdekar, M.S. and Jambunathan, R. (1982) *J. Food Sci.* **47**: 510-512

Singh, N.K., Nelson, D.E., Kuhn, D. Hasengawa, P.M. and Bressan, R.A. (1989) *Plant Physiol.* **90**: 1096-1101

Smith, B.W. and Roe, J.M. (1957) *J. Biol. Chem.* **227**: 357-362

Sodini, G., Silano, V., De Agazio, M., Pocchiari, F., Tentori, L. and Vivaldi, G. (1970) *Phytochemistry* **9**: 1167-1172

Somogyi, M. (1938) *J. Biol.Chem.* **125**: 399-403

Soyama, K. and Ono, E. (1983) *Clin. Chim. Acta* **131**: 149-153

Stankovic, C.S. and Marcovic, N.D. (1960) "A Study of Amylase Inhibitors in the Acorn" *Glas. Hem. Drus. Beograd* **25-26**: 519 (Cited in *Chem. Abstr.* (1963) **59**: 3084d)

Steers, E., Craven, G.R. and Anfinsen, C.R. (1965) *J. Biol. Chem.* **204**: 2478-2485

Sternberg, M.J.E. and Islam, S.A. (1990) *Protein Engineering* **4**: 125-131

Stiekema, W.J., Heidekamp, F., Dirkse, W.G., van Beckum, J., de Haan, P., ten Bosch, C.and Louwerse, J.D. (1988) *Plant Mol. Biol.* **11**: 255-269

Strumeyer, D.H. (1972) *Nutr. Rep. Int.* **6**: 45-51

Strumeyer, D.H. and Fisher, B.R. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**: 624-630

Strumeyer, D.H. and Malin, M.J. (1969) *Biochim. Biophys. Acta* **184**: 643-649

Stuat, L.S. and Harris, T.H. (1942) *Cereal Chem.* **19**: 288-300

Suehiro, I., Otsuki, M., Yamasaki, T., Ohki, A., Sakamoto, C., Yuu, H., Maeda, M. and Baba, S. (1981) *Clin. Chim. Acta* **117**: 145-151

Svensen, I., Helgaard, J. and Mundy, J. (1986) *Carlsberg Res. Commun.* **51**: 43-50

Svensson, B., Asano, K., Jonassen, I., Poulsen, F.M., Mundy, J. and Svendsen, I. (1986) *Carlsberg Res. Commun.* ***51***: 493-500

Talanian, R.V., McKnight, C.J. and Kim, P.S. (1990) *Science* **249**: 769-771

Tashiro, M. and Maki, Z. (1986) *Agric. Biol. Chem.* **50**: 2955-2957

Teeter, M.M. and Hendrickson, W.A. (1979) *J. Mol. Biol.* **127**: 219-223

Teeter, M.M., Mazer, J.A. and L'Italien, J.J. (1981) *Biochemistry* **20**: 5437-5443

Thurnberg, E. (1983) *Acta Pharm. Suec.* **20**: 115-122

Thurnberg, E. and Samuelsson, G. (1982) *Acta Pharm. Suec.* **19**: 447-456

Tietz, N.W. and Shuey, D.F. (1984) *Clin. Chem.* **30**: 1227-1230

Turner, A.J. (1981) *Trends in Biochem. Sci.* **6**: 171-173

Turner, R. and Tjian, R. (1989) *Science* **243**: 1689-1694

Tuzhilin, S.A., Gonda, M., Carbonell, G. and Dreiling, D.A. (1982) *Am. J. Gastroenterol.* **77**: 18-26

Uehara, Y., Tonomura, B. and Hiromi, K. (1980) *Arch. Biochem. Biophys* **202**: 250-258

Van Etten, C.H., Nielsen, H.C. and Peters, J.E. (1965) *Phytochemistry* **4**: 467-473

Vermeulen, J.A.W.H., Lamerichs, R.M.J.N., Berliner, L.J., De Marco, A., Llinas, M., Boelens, R., Alleman, J. and Kaptein, R. (1987) *FEBS Lett.* **219**: 426-430

Vernon, L.P., Evett, G.E., Zeikus, R.D. and Gray, W.R. (1985) *Arch. Biochem. Biophys.* **238**: 18-29

Vertesy, L., Oeding, V., Bender, R., Nesemann, G., Sukatsch, D.A. and Zepf, K. (1981) Proc. 3rd Int. Congr. Obesity, Siena: "Regularotrs of Intestinal Absorption in Obesity, Diabetes and Nutrition", Vol. 2, pp. 269-274 Societa Editrice Universo, Roma

Vertesy, L., Oeding, V., Bender, R., Zepf, K. and Nesemann, G. (1984) *Eur. J. Biochem.* **141**: 505-512

Vertesy, L. and Tripier, D. (1985) *FEBS Lett.* **185**: 187-190

Vinson, C.R., Sigler, P.B. and McKnight, S.L. (1989) *Science* **246**: 911-916

Vittozzi, L. and Silano, V. (1976) *Theor. Appl. Genet.* **48**: 279-286

Wada, K. and Buchanan, B.B. (1981) *FEBS Lett.* **124**: 237-240

Wall, J.S. and Ross, W.M. (1970) "Sorghum Production and Utilization". AVI Publishing, Westport, Conn.

Walton, E.V. and Holt, O.M. (1959) *In* "Profitable Southern Crops", pp. 211-261. Prentice-Hall, Inc., Englewood Cliffs, N.J. USA

Warchalewski, J.R. (1976) *Pol. Sci. Ser. Sci. Biol.* **24**: 559-563

Warchalewski, J.R. (1978) *Rocz. Acad. Roln. Poznaniv* **82**: 1-6

Warchalewski, J.R. (1983) *Nahrung* **27**: 103-117

Warchalewski, J. R. (1987) *Nahrung* **31**: 1015-1031

Webb, J.L. (1963) *In* "Enzyme and Metabolic Inhibitors" (Webb, J.L. ed.), Vol I, pp. 66-82. Academic Press, New York

Weder, J.K.P. (1986) *In* "Nutritional and Toxicological Significance of Enzyme Inhibitors in Plant Foods" (M.Friedman ed.), pp. 299-347. Plenum Press, New York

Weselake, R.J, MacGregor, A.W. and Hill, R.D. (1983a) *Plant Physiol.* **72**: 809-812

Weselake, R.J., MacGregor, A.W., Hill, R.D. and Duckworth, H.H. (1983b) *Plant Physiol.* **73**: 1008-1012

Weselake, R.J., MacGregor, A.W. and Hill, R.D. (1985a) *Cereal Chem.* **62**: 120-123

Weselake, R.J., MacGregor, A.W. and Hill, R.D. (1985b) *Cereal Sci* **3**: 249-259

Whitaker, J.R. (1981) *In* "Impact of Toxicology on Food Processing" (J.C.Ayres and J.C.Kirschman eds.) pp.57-104. Avi Publishing Co. Inc., Westport, USA

Whitaker, J.R. (1983) *In* "Xenobiotics in Food and Feeds", Am. Chem. Soc. Symposium Series **234**, pp. 15-46

Wilcox, E.R. and Whitaker, J.R. (1984a) *J. Food Biochem.* **8**: 189-213

Wilcox, E.R. and Whitaker, J.R. (1984b) *Biochemistry* **23**(8): 1783-1791

Wilding, P. (1963) *Int. J. Vitam. Nutr. Res.* **51**: 161-168

Williams, J.W. and Morrison, J.F. (1979a) *Methods in Enzymol.* **63**, Part A: 437-467

Williams, J.W., Morrison, J.F. and Duggleby, R.G. (1979b) *Biochem.* **18**: 2567-2573

Williams, R.W. and Teeter, M.M. (1984) *Biochemistry* **23**: 6796-6802

Winterfeld, V.K. and Bijl, L.H. (1949) *Justus Liebigs Annalen der Chemie* **561**: 107-115

Xavier-Filho, J. and Campos, F.A.P. (1984) *Arq. Biol. Tecnol.* **27**: 407-418

Xavier-Filho, J. and Campos, F.A.P. (1989) *In* "Toxicants of Plant Origin" (P.R.Cheeke ed.) Vol. 3: Proteins and Amino Acids, pp. 1-27. CRC Press Inc., Boca Raton, FL.

Xavier-Filho, J. and Ventura, M.M. (1988) *Comments Agric. Food Chem.* **1**: 239-264

Yarwood, A., (1989) *In* "Protein Sequencing - A Practical Approach" (Findlay, J.B.C. and Geisow, M.J. eds.), pp. 119-145. IRL Press, Oxford

Yetter, M. A., Saunders, R.M. and Boles, H.P. (1979) *Cereal Bioch.* **56**: 243-244

Yoshida, M., Nakai, T., Fukuhara, K., Saitoh, S., Yoshikawa, W., Kobaiashi, Y. and Nakamura, H. (1990) *J. Biochem.* **108**: 158-165

Yu, Y.G., Chung, C.H., Fowler, A. and Suh, S.W. (1988) *Arch. Biochem Biophys.* **265**: 466-475

Zacharius, R.M., Zell, T.E., Morrison, J.H. and Woodlock, J.J. (1969) *Anal. Biochem.* **30**: 148-152

Zawistowska, U., Longstaff, J. and Bushuk, W. (1988) *J. Cereal Sci.* **8**: 207-209

Zemke, K.J., Muller-Fahrnow, A., Jany, K.-D., Pal, G.P. and Saenger, W. (1991) *FEBS Lett.* **279**: 240-242

Zhou, J.-M., Liu, C. and Tsou, C.-L., (1989) *Biochemistry* **28**: 1070-1076