Phytochemistry, 1977, Vol. 16. pp. 159–169, Pergamon Press, Printed in England.

REVIEW

THE PROTEINASE INHIBITORS OF PLANTS AND MICRO-ORGANISMS

M. RICHARDSON

Department of Botany, University of Durham, Science Laboratories, South Road, Durham City DH1 3LE

(*Received* 27 *September* 1976)

Key Word Index—Plants; micro-organisms; proteinase inhibitors; specificity; occurrence and distribution; assay; isolation and purification; structure; reactive sites; homologies; physiology; functions; nutritional significance.

Abstract—Recent (post-1972) advances in our knowledge of the proteinase inhibitors of plants and micro-organisms are reviewed. Details of the specificity, occurrence and distribution of these proteins are summarized, and modern methods for their isolation, purification and assay are discussed. Certain homologies revealed by comparison of the amino acid sequences of several inhibitors are noted. Details of their reactive (inhibitory) sites are tabulated and discussed in relation to the proposed mechanisms of action of these proteins. Recent experiments on the intracellular localization of the inhibitors, their physiology and possible functional roles are described. The nutritional significance, possible therapeutic use and value of the proteinase inhibitors as laboratory tools are also discussed.

INTRODUCTION

Plants and micro-organisms as well as animals contain a number of proteins which have the peculiar property of forming reversible stoichiometric protein–protein complexes with various proteolytic enzymes, thus bringing about competitive inhibition of their catalytic functions. For many years these so-called proteinase inhibitors have been the subject of much investigation for a wide variety of different reasons.

Much of the initial work in this field came from those interested in animal nutrition who were concerned about the possibly deleterious dietary effects exerted by the proteinase inhibitors found in important food plants and their products. At the same time biochemists were attracted to their study because of the possible opportunities they afford to explore the basic protein–protein interactions which underly the mechanism of proteolytic digestion. In the pharmacological and medical fields, investigations have been made into the potential of the proteinase inhibitors as therapeutic agents in the treatment of a wide range of disorders. Biologists on the other hand are still trying to determine what physiological functions these proteins might have within the living tissues of plants and animals under normal conditions. Research in this latter area has been greatly stimulated by some intriguing but as yet merely speculative suggestions.

All this interest has culminated recently in two International Conferences on Proteinase Inhibitors [1, 2] and a number of reviews, some of which have dealt with proteinase inhibitors in general [3–5] and others which have concentrated in particular on specific aspects of the proteinase inhibitors found in plants [6–10]. This article attempts to give a broad survey of the proteinase inhibitors found in plants and micro-organisms and to summarize the recent (post-1972) progress in this rapidly expanding field.

Specificity

The proteolytic enzymes found in nature can be conveniently subdivided into four main groups which are characterized by the nature of their active sites and the reaction mechanism involved. These are the serine proteinases (e.g. trypsin, chymotrypsin, thrombin, plasmin and elastase); the sulphydryl proteinases (papain, bromelain and ficin); the metalloproteinases (carboxypeptidases A and B, and the aminopeptidases); and the acidic proteinases (pepsin and rennin). Nearly all of these types of enzymes have been shown to be inhibited by proteins or peptides isolated from the cells of plants and micro-organisms. In some cases the proteinase inhibitors exhibit a very narrow range of specificity, being capable of inhibiting only one or two closely related proteinases, whilst others of broad specificity are active against a wide range of different enzymes. However one difficulty frequently encountered in determining the specificity of a particular inhibitor stems from the fact that some of the early investigations were made with heterogeneous preparations containing several inhibitory proteins, and/or the effects of these proteins were sometimes tested on unresolved mixtures of proteinases such as pronase.

Much of the early work on the proteinase inhibitors of plant origin concentrated almost exclusively on the inhibitors of trypsin [8] an important serine protease of the digestive tract of animals. It soon became apparent however that many of the so-called trypsin inhibitors were also inhibitory to the related enzyme chymotrypsin [3, 11]. In some cases the reactive site of the

inhibitor was the same for both enzymes [12], but several of the other inhibitors were demonstrated to be 'double-headed' or 'polyvalent', i.e. containing different reactive sites for the independent inhibition of the two proteolytic enzymes [13, 14]. It should also be noted that some potent inhibitors of trypsin are inactive or only weakly active against chymotrypsin [15, 16] and vice versa [17, 18]. Sometimes the trypsin or chymotrypsin inhibitors are strictly specific for these two enzymes [19] but other examples have subsequently been shown to inhibit a range of other serine proteases such as elastase [20], thrombin [21], plasmin and kallikrein [22].

The possible therapeutic application of the thrombin, plasmin and kallikrein inhibitors in the control of pathophysiological processes such as blood clotting and fibrinolysis related to the kinogen–kinin system in mammals has led to numerous studies on this type of inhibitor in plants [22, 23]. Japanese workers have recently [24] shown that potato tubers are a rich source of a family of proteins which are strongly inhibitory to the human plasma kallikreins and at the same time show a weaker activity against the glandular kallikreins and several other serime proteases such as trypsin and chymotrypsin.

There are a few reports that inhibitors of the serine proteases are also active against enzymes in the other groups. For example the trypsin and chymotrypsin inhibitors purified from broad beans [21] also inhibited the sulphydryl enzyme papain. Similarly the proteinase inhibitors isolated from cultivated cells of *Scopolia japonica* included the acidic proteinase pepsin in their broad spectrum of specificity [22]. The soybean trypsin inhibitor (Kunitz) has been shown to inhibit clostripain, a microbial enzyme similar to trypsin, but having the catalytic site of an —SH protease [25].

Apart from the inhibitors from *Scopolia* [22], none of the other proteinase inhibitors which have been isolated from higher plants appears to be active against the acidic proteinases such as pepsin and rennin. There has, however, been much recent work on the inactivation of the acid proteinase A found in yeast by macromolecular inhibitors from the same organism [26], and on pepstatin, the pepsin inhibitor produced by various Actinomycetes [27].

Details of plant inhibitors of the sulphydryl enzymes such as papain, bromelain and ficin are also relatively scanty. Those which are known appear to be highly specific. For example the cubical proteinaceous crystals isolated from potato tubers by Rodis [28] were strongly inhibitory to papain, chymopapain and ficin, but failed to act against the related bromelain or any of the other proteolytic enzymes tested. The iso-inhibitors of bromelain found in pineapple stems [29] were also active against papain and ficin. Several of the proteinase inhibitors such as leupeptin and antipain which have been isolated from culture filtrates of *Streptomyces* spp. [30] and other Actinomycetes [31] are known to inhibit papain, but like pepstatin, are very different from the macromolecular plant inhibitors in that they are small acylated peptides containing arginial. The only records of macromolecular inhibitors of the microbial proteinase subtilisin are the proteins produced by *Streptomyces albogriseolus* [32] and barley [33].

Whilst most attention has been focussed on the plant inhibitors of the endopeptidases, similar proteins affecting the metal-containing exopeptidases have not been completely neglected. The purification and characterization of a potent inhibitor of the pancreatic carboxypeptidases A and B has recently been reported from potato tubers [34]. In yeast there is a highly specific inhibitor of the endogenous carboxypeptidases [35].

Investigations on the spectrum of enzymes affected by the proteinase inhibitors from plants have been widened to include the digestive enzymes found in the guts of insects and their larvae which often feed on plants and their products [19, 36]. This follows the suggestion by a number of workers that the accumulation of proteinase inhibitors which are potentially harmful to insects might play an important role in plant protection [36, 37].

Finally in considering the specificity of action of plant proteinase inhibitors it would be particularly relevant to know whether they are capable of inhibiting the endogenous proteinases and could thus function in regulating proteolysis *in vivo*. This possibility is considered in detail in a later section of this review.

Occurrence and distribution

The occurrence of proteinase inhibitors in plants has been known since 1938 and they are extremely widespread in their distribution throughout the plant kingdom [3, 6, 7, 9]. They appear to have been most extensively studied in the Leguminosae, Gramineae and Solanaceae, probably because of the large number of species in these families which form important sources of food.

The seeds of the Leguminosae have long been recognized as excellent sources of protein. It is perhaps not surprising therefore that a great deal of the early work on protease inhibitors was on the now well-characterized inhibitors of trypsin and chymotrypsin from the widely-grown soya (*Glycine max*) and lima beans (*Phaseolus lunatus*) [6, 8]. There has been a growing realization in the last few years that many other peas and beans might be attractive as alternative sources of protein, particularly in view of the expanding commercial interest in textured protein products. This has led to studies on the proteinase inhibitors of an even wider range of these species [16, 19, 21, 38–48].

Tubers of the potato (*Solanum tuberosum* L.) are a notable source of a wide diversity of proteinase inhibitors. In this tissue, there are at least seven different types of proteinase inhibitor which can be distinguished on the basis of their varying molecular weights, amino acid compositions, *N*- and *C*-terminal amino acids, stability during heating and specificity [23, 34, 49–55]. Frequently the picture is further complicated by the fact that several of the inhibitor types may exist in the form of a number of closely related iso-inhibitors. For example chymotryptic inhibitor I has recently been resolved into at least ten iso-inhibitor species of different iso-electric points [56]. Other fleshy tubers, such as those of the taro (*Colocasia antiquorum*) and the banana (*Musa sapientum*) are also known to contain considerable inhibitory activity towards chymotrypsin [57].

The proteinase inhibitors also represent a quantitatively important fraction of the protein found in most cereal grains. Mikola and Kirsi [58] have demonstrated that several different inhibitors are present in high concentrations (5–10% of the water soluble protein) both in the embryos and endosperms of grains of barley, wheat, oats and rye. They are also well known in the grains of rice [59], maize [60] and sorghum [61].

A recent survey [62] for trypsin inhibitors in the plant tissues which are commonly found in human diets revealed that the potato and sweet corn (maize) contained the highest levels, but they also were found in moderate amounts in spinach, broccoli, brussels spouts, radish and cucumber. Negligible levels of this type of inhibitor were found in fruits of peach, plum and avocado.

Whilst several macromolecular inhibitors have been isolated from yeast [26, 35], *Neurospora crassa* [4] and *Aspergillus sojae* [3] and various smaller peptide inhibitors from the Actinomycetes [27, 30, 31], there are as yet no reports of proteinase inhibitors from other fungi. Similarly there are no details of any well characterized inhibitors from the algae. One suspects that this apparent shortage of information results not from their absence in these groups but from a lack of enquiry.

Assay

The standard methods for the assay of proteinase inhibitors generally involve spectrophotometry or pH-titration [11] to determine the decrease (inhibition) of the enzymatic hydrolysis of natural (e.g. casein, haemoglobin) or synthetic (e.g. α-*N*-benzoyl-L-arginine-*p*-nitroanilide, *N*-benzoyl-L-tyrosine ethyl ester) substrates caused by the inhibitor, but a wide variety of other methods such as vasodilation assays (for kallikrein inhibitors) and milk clotting have also been used.

Rapid screening methods have been devised for use in extensive surveys of plants and other biological materials. Perhaps one of the most notable of these is the quantitative immunological method developed by Ryan [63] for the estimation of chymotryptic inhibitor I in the various tissues of potato and other members of the Solanaceae [64, 65]. This method involves the radial diffusion of plant extracts in agar gels containing antibodies to the inhibitor obtained from rabbit serum after purified chymotryptic inhibitor I was used as the antigen. The radial diffusion of inhibitor I from the extract into the agar results in the formation of a precipitin ring, the diameter of which is a function of the concentration of inhibitor in the extract.

A micro-complement fixation technique has been used for making immunological comparisons of chymotryptic inhibitor I among several genera of the Solanaceae [66] and qualitative immunological methods have also been used by Kirsi [67] for comparing the trypsin inhibitor in developing barley grains with that in mature seeds and with the inhibitors from other cereals [58].

Isolation and purification

The isolation and purification of proteinase inhibitors from plants has involved the full range of general techniques for the separation of proteins, but in addition certain novel and special methods have been employed.

Many of the plant proteinase inhibitors are extremely resistant to denaturation by heat and this property has frequently been exploited. For example, several isolation procedures [15, 18, 22, 34, 40, 49, 68] include a preliminary step in which the extract or homogenized suspension is heated at 80–100° for approximately 10 min during which period various contaminating proteins become precipitated and can be removed by filtration or centrifugation. The inhibitors themselves are left essentially unaltered by this process. Alternatively, the inhibitors are sometimes isolated in the form of the complex with their proteinase, and following further purification the complex is dissociated, and then the proteinase removed as an irreversibly inactivated precipitate by heating.

Another increasingly popular method of purification for these inhibitors involves affinity chromatography [19, 22, 39, 43, 69–71] on columns containing the proteinase enzymes bound to insoluble resins or polymerized dextrans such as Sepharose. One of the attractions of this method is that crude or only partially purified extracts of plant material may be applied to the columns at near neutral pH values. As the extract passes through the column the inhibitors form stable complexes with the immobilized proteinases and are retained on the column during subsequent washing which removes most of the other impurities. The inhibitors can then be released from their complexes by lowering the pH and increasing the ionic concentration of the eluting buffer. Whilst this method of purification has the attractive features of relative speed and specificity, it does however suffer from the major drawback that partial proteolysis of the reactive site of the inhibitor may occur [72] (see later section). Such cleavage of the reactive site results in the formation of modified forms of the inhibitor which can interfere with the further purification, particularly if a multiplicity of iso-inhibitors are involved [73].

As might be expected ion-exchange chromatography has been widely employed for the separation of different proteinase inhibitors found in plant extracts. The technique has also been adapted for use in the presence of dissociating solvents such as 4 M guanidine or 8 M urea and this has permitted the separation of the dissociated protomers (sub-units) of the many inhibitors which are normally polymeric in their native form [49]. The recently introduced technique of iso-electric focusing has proved to be particularly useful in separating the proteins of families of iso-inhibitors [19, 39, 50, 51, 56] where the individual components sometimes only differ from one another by slight variations in their iso-electric (pI) points.

Structure

The well characterized macromolecular proteinase inhibitors from plants are all proteins with little or no additional carbohydrate moieties. Generally they are relatively small with molecular weights falling in the lower part of the range 4000–80000. The best studied of the smaller inhibitors is the carboxypeptidase inhibitor from potatoes [34] which has a molecular weight of 4300 [74, 75], but the same tissue also contains inhibitors of chymotrypsin with molecular weights in the region of 5400 [54, 55]. Other small inhibitors in the range 4000–6000 have been reported in *Scopolia japonica* [22], the egg-plant (*Solanum melogena*) [68], mung beans [76] and pineapple [77].

The majority of the inhibitors from plants are proteins containing between 70 and 90 amino acids (corresponding to a molecular weight of 8000–10000) [14, 18–20, 40, 42, 78–80] and moreover many of the apparently larger (> 10000) inhibitors are actually polymeric (usually di-, tri-, or tetrametric) proteins whose protomers have minimum molecular weights of under 10000 [26, 49, 51, 60, 80, 81]. The few notable exceptions to this rule are the trypsin inhibitors from sweet potatoes (*Ipomoea batatas*) (23000–24000) [15], oats (43500) [58], soybean (Kunitz

inhibitor, 19900) [13] and the kallikrein inhibitors from potatoes (25200–27100) [50]. The largest plant inhibitor known so far is the papain inhibitor from potato tubers [28] which is a glycoprotein with a molecular weight of approximately 80000.

The first details of the primary structures of the plant proteinase inhibitors started to appear in 1971 with the sequences of the lima bean inhibitor [82, 83], and the soybean (Kunitz) inhibitor [84]. Further work in the last five years has now revealed the complete amino acid sequences of a number of other inhibitors including the soybean (Bowman–Birk) inhibitor [78], the chymotryptic inhibitor I from potatoes [85–87], the bromelain inhibitor from pineapples [77], the garden bean (*Phaseolus vulgaris*) inhibitor [20], and the carboxypeptidase inhibitor from potatoes [74, 75]. The determination of the latter sequence provides an interesting example of the use of combined gas chromatography–computerized mass spectrometry for the analysis of primary structure [88]. In addition details have been published of an active fragment of the potato proteinase inhibitor IIa [89] and partial sequences, of a chick pea trypsin inhibitor [90] and a small chymotrypsin inhibitor in potatoes [54].

These sequences have revealed a striking degree of structural homology between certain of the inhibitors. For example, Fig. 1. shows the comparison of the primary structures of a number of the trypsin inhibitors and clearly indicates their close structural similarities. These details obviously permit comparisons to be made at several different taxonomic and phylogenetic levels. In this figure alone we have illustrated intergeneric (*Phaseolus/Glycine*), interspecific (*Phaseolus vulgaris/Ph. lunatus*) and intraspecific (the iso-inhibitors of *Ph. lunatus*) homologies.

Very recent work by Ryan and co-workers [54] has shown that in the amino acid sequences of the potato carboxypeptidase inhibitor and a small chymotrypsin inhibitor from the same source there is evidence that an extraordinary divergent evolution has generated from a single ancestral gene two homologous inhibitors directed against non-homologous classes of enzymes, the serine endopeptidases and the metallo-carboxypeptidases. In the same way the bromelain inhibitor from pineapple [77] has been shown to exhibit homology with numerous trypsin inhibitors from a variety of animal sources, but surprisingly it seems to show little or no similarity to the other known plant inhibitors.

In several of the plant proteinase inhibitors it has been noticed that the primary structures contain repetitive sequences or regions of internal homology. This phenomenon was first reported by Tan and Stevens [83] in the lima bean inhibitor, but has also been recorded in the soya bean (Bowman–Birk) inhibitor [78] and the garden bean inhibitor [20] (see Fig. 1). These and similar repetitive sequences in other proteins are thought to have resulted from the extension of shorter polypeptide chains by a process of gene duplication.

These regions of internal homology frequently contain the reactive (inhibitory) sites of the inhibitor which interact with the active site of the relevant proteinase. Ozawa and Laskowski [91] first proposed the reactive

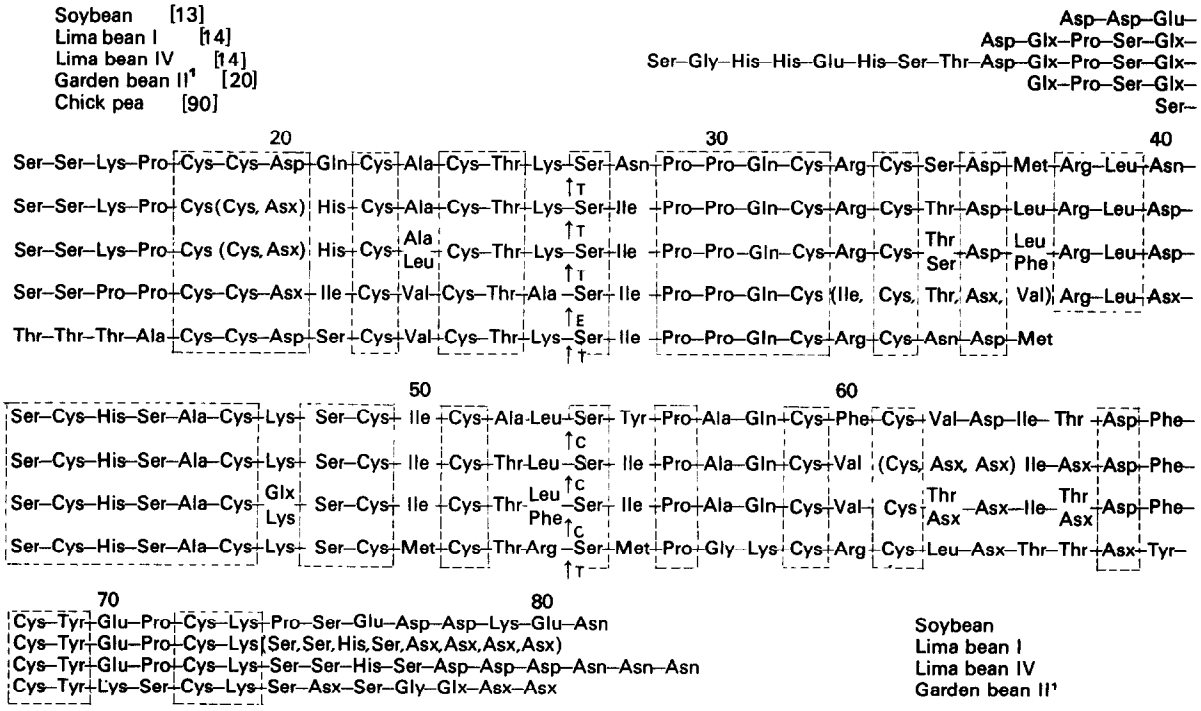

Fig. 1. Comparison of the amino acid sequences of the proteinase inhibitors from soybean (Bowman–Birk), lima bean and garden bean and a fragment of the chick pea inhibitor. Segments of identical (homologous) sequences in the proteins are enclosed in the dashed boxes. The reactive (inhibitory) sites of each protein are indicated by the arrows; T, site reacting with trypsin; C, chymotrypsin, E, elastase. The numbering of the residues shown refers to that proposed for the lima bean IV sequence by Stevens *et al.* [14]. The sequences are also arranged to facilitate comparison of the regions of internal homology (repetitive sequences) around the reactive sites (e.g. residues 14–36 and 41–63).

site model whereby the interaction of naturally occurring trypsin inhibitors with trypsin involved the cleavage of a single LYS-X or ARG-X peptide bond in the inhibitor. In the case of chymotryptic inhibitors the bond cleaved was expected to be LEU-X, TYR-X or PHE-X. Most frequently these sites have been identified by carrying out a limited hydrolysis of the inhibitor with catalytic amounts of the enzyme at low pH (2.5–4.0) and then separating the cleaved (modified) fragments after any necessary reduction and S-alkylation [71, 91]. Removal of the newly formed carboxy-terminal residue (e.g. LYS or ARG for trypsin inhibitors) by the action of carboxypeptidase B usually abolishes the inhibitory activity of the protein.

Studies on the properties of the reactive sites of many naturally occurring proteinase inhibitors have enabled Laskowski and Sealock [5] to draw up certain tentative rules for these reactive sites. Invariably the reactive sites are found to be located within a loop of the protein closed by a disulphide bridge. Table 1 shows the different reactive sites which have been identified in the plant inhibitors.

In the case of the lima bean inhibitor the reactive sites for chymotrypsin and trypsin are in separate but homologous regions of the molecule. This has led workers to describe this inhibitor as 'double-headed'. Similar inhibitors with separate sites are those from soybean (Bowman–Birk) [13, 78], chickpeas [90] and *Phaseolus vulgaris* var *nanus* [94]. One of the 'double-headed' isoinhibitors from black-eyed peas is anomalous in that both sites inhibit trypsin and have no effect on chymotrypsin [95]. On the other hand the potato inhibitors IIa and IIb have a single peptide bond (LYS^{63}–SER^{64}) which serves as the main reactive site not only for trypsin but also for chymotrypsin and the bacterial proteinase Nagarse [96, 97]. This and similar examples [12] are referred to as 'single-headed' inhibitors.

Table 1. The reactive (inhibitory) sites of proteinase inhibitors [from plants]

Enzyme inhibited	Reactive site residues	Species/Inhibitor	Reference
Trypsin	ARG–ALA	Wheat, rye,	[72]
	ARG–ILE	Soybean (Kunitz)	[91]
	ARG–LEU	Maize	[60]
	ARG–SER	Garden bean	[20]
	LYS–SER	Soybean (Bowman–Birk)	[78]
		Chick pea	[90]
		Lima bean	[93]
		Potato (IIa)	[96]
		Phaseolus vulgaris var *nanus*	[94]
Chymotrypsin	ARG–ILE	Soybean (Kunitz)	[91]
	LEU–SER	Lima bean (Variant 1)	[14]
		Soybean (Bowman–Birk)	[13]
		Phaseolus vulgaris var *nanus*	[94]
	PHE–SER	Lima bean (Variant IV)	[14]
	LYS–SER	Potato IIa/IIb	[96, 97]
	LEU–ASP	Potato (Inhibitor I)	[92]
	MET–ASP	Potato (Inhibitor I)	[86, 87]
Elastase	ALA–SER	Garden bean	[20]
Nagarse	LYS–SER	Potato IIa/IIb	[96, 97]
Subtilisin	MET–VAL	*Streptomyces albogriseolus*	[32]

Odani and Ikenaka [98] have shown that it is possible by chemical and enzymatic means to cleave a 'double-headed' inhibitor into two smaller 'single-headed' fragments. In the case of the Bowman–Birk soybean inhibitor which contains 71 amino acid residues in its native form, treatment with cyanogen bromide and pepsin yielded two separate fragments of 38 and 29 residues. The larger of these, which contained the LYS–SER peptide bond, retained 84% of the anti-trypsin activity of the intact protein, and the smaller one containing the LEU–SER bond had 16% of the anti-chymotrypsin activity. Cleavage of the 'double-headed' inhibitor from chick peas with cyanogen bromide and pepsin also resulted in the formation of two active fragments. However in this case the fragment with activity against trypsin was shown to be twice as active as the native protein suggesting that unmasking of another trypsin inhibitory site had probably occurred during the cleavage [99].

In a similar manner smaller active fragments have also been produced from 'single-headed' inhibitors. For example Iwasaki *et al.* [73,100] have cleaved an active fragment of 45 amino acid residues from the native potato inhibitor IIa (97 residues) by prolonged tryptic digestion of an equimolar complex of the inhibitor with trypsin. The active fragment contained the LYS–SER reactive peptide bond and was still a potent inhibitor of trypsin, but had lost most of its activity against chymotrypsin and Nagarse. A similar low molecular weight (4300) active fragment of potato proteinase inhibitor IIb obtained by incubating the inhibitor with an equimolar amount of trypsin at pH8 retained very strong activity against chymotrypsin and subtilisin [101]. Thus the specificities of the two fragments are quite different despite the fact that they both contain the same LYS–SER reactive site peptide bond.

An interesting development in this area is the recent finding by Laskowski and co-workers that the ARG^{63}–ILE^{64} reactive peptide bond in the soybean (Kunitz) trypsin inhibitor can be modified in various ways by chemical and enzymatic means without the inhibitor losing its activity. For example the ARG^{63} can be replaced by LYS^{63}, in which case the modified inhibitor behaves like the native form, or by TRP^{63} where the protein becomes a good inhibitor of chymotrypsin [102]. Alternatively the inhibitor will tolerate the replacement of ILE^{64} by either ALA^{64}, LEU^{64} or GLY^{64} whilst still retaining activity [103]. If, however, an additional amino acid residue (such as Ile, Ala or Glu) is inserted between the ARG^{63} and ILE^{64} residues (i.e. ARG^{63}–GLU^{63a}–ILE^{64}) this abolishes inhibitory activity and converts the trypsin inhibitor into a trypsin substrate [104].

Two contrasting theories have been put forward to explain the mechanism and the great strength of the protein–protein binding which occurs between proteinase inhibitors and the serine proteinase enzymes. Laskowski and Sealock [5] believe that a covalent acyl intermediate is formed between the inhibitor molecule and the hydroxyl group of the serine at the active site of the enzyme. Other workers [105] think that the strength and specificity of the binding is due to the summation of many weak non-covalent forces between the active surfaces similar to those which operator in antigen–antibody reactions. For

example, various workers [106, 107] have shown that catalytically inactive enzymes such as anhydrotrypsin and anhydrochymotrypsin, in which the elements of water have been removed from the serine residue at the active sites, cannot be acylated by substrates but nevertheless can still bind strongly and stoichiometrically to the proteinase inhibitors.

Tschesche [4] has summarized the available evidence which suggests that in certain cases the complex formed between inhibitor and protease is an adduct with a tetrahedral intermediate state approaching a covalent bond. In particular our understanding of the mechanism has been greatly assisted by the recent crystal structure analysis carried out on the complex of the soybean (Kunitz) inhibitor with trypsin by Sweet *et al.* [108]. The high resolution (2.6 Å) three dimensional map prepared from X-ray crystallographic data shows that only 12 out of the 181 amino acids in the inhibitor actually make contact with the trypsin molecule. As might be expected, these 12 particularly include the sequence SER^{61}–PHE^{66} which contains the reactive peptide bond ARG^{63}–ILE^{64}.

Similar studies have recently begun on the mechanism of enzyme-inhibitor interaction for the potato carboxypeptidase inhibitor [109]. The carboxyl terminus of the inhibitor was shown to be the region of contact with the enzyme in the complex and inhibitor residues Tyr^{37} and Gly^{39} were particularly important in the binding region. Both carboxypeptidases A and B were inhibited by this same reactive site. Studies of the circular dichroic spectra of the chromophoric derivative arsanilazocarboxypeptidase A have confirmed that binding of the inhibitor occurs at the active site of the protease [110]. However catalytic activity was not required for inhibitor binding since carboxypeptidase A which was inactivated with *N*-bromoacetyl-*N*-methyl-L-phenylalanine still bound the inhibitor nearly as strongly as the native enzyme [110]. In the proposed model of action the inhibitor was thought to fit like a cap over the shallow depression at the active site of the enzymes, but not to penetrate into the binding pocket [109, 110].

As yet little is known about the reactive sites of the plant inhibitors of other protease enzymes such as papain [28] and bromelain [29, 77]. The large (80000) inhibitor of papain which is found as cubical crystals in potato tubers was found to be a multisite and polyvalent inhibitor [28] in which there appeared to be eight enzyme binding sites, four of which were capable of binding either papain or chymopapain while the remaining four sites could accommodate chymopapain exclusively. There is also some evidence that the latter sites of the inhibitor undergo conformational changes when the former sites are occupied by papain.

Physiological functions

The physiology of the proteinase inhibitors in plants is poorly understood despite the fact that they often form an important part of the protein found in the tissues. In the grains of the Gramineae, for example, it is reported that they comprise some 5–10% of the water-soluble protein [58], whereas in seeds of the Leguminosae their content has been variously recorded as 0.25 g/kg for mung beans [76], 1.5 g/kg for chick peas [19], and as much as 3.6 g/kg for the kidney bean [39]. It must be remembered however that the reported yields often vary significantly depending on the harvest time, the freshness of sample and the cultivar or species under investigation [67]. This is particularly so for the inhibitors found in potato tubers where for example the yields of the inhibitors IIa and IIb from different batches ranged from 10 to 20 mg/kg [18], and the kallikrein inhibitors found in 38 different varieties of potatoes were present in concentrations ranging from 59–189 kallikrein inhibitory units/g tuber (average content 15 mg/kg) [50]. These kallikrein inhibitors were distributed throughout the tuber and appeared to be stable during storage [18], whereas chymotryptic inhibitor I which represents some 1–5% of the total soluble protein of the tuber was more concentrated in the cortical regions and tended to decrease during storage [57].

The technique of polyacrylamide gel electrophoresis has been used to demonstrate the significant levels of genetic and environmental variation in the trypsin inhibitor content and activity of various soybean lines. Within the genus *Glycine* there was very little electrophoretic difference between the inhibitors of the species *G. soja*, *G. gracilis* and *G. max*, but by contrast there was an unusual amount of variation in the banding patterns of the trypsin inhibitors in examples of the species *G. wightii* [111]. The limitations of the technique have been illustrated by Clark and Hymowitz [112] who showed that a difference in electrophoretic mobility may not correspond to a difference in specific activity and also that uniformity of electrophoretic mobility may not indicate uniformity in specific activity. Nevertheless the technique has been useful in demonstrating that the two major electrophoretic forms of the Kunitz inhibitor found in soybean seeds from experimental backcrosses represented the expression of the two co-dominant alleles (T_1^1 and T_1^2) at a single locus [113]. The geographical origin and distribution of these two forms in different populations has also been studied [114]. Significant differences in the concentrations of the trypsin inhibitors have also been reported in 115 cultivars and lines of Faba beans (*Vicia faba* var *minor*) obtained from the World Collection [115]. The transitory nature of some of these inhibitors and the fluctuations in their concentrations due to redistribution during development, maturation and germination have been well summarized by Ryan [9].

There is still some uncertainty as to the exact intracellular localization of many of the plant proteinase inhibitors. Shumway and Ryan believe that Inhibitor I forms a significant part of the vacuolar protein bodies which are conspicuous in the very young cells found in the vegetative and floral apices of members of the Solanaceae [116] and in particular in the detached leaves of tomato [117]. This belief was initially based on the direct and consistent relationship found between the presence of inhibitor I as demonstrated by using an immunological assay and the occurrence of dense protein bodies visible in the vacuoles by electron microscopy [116, 117]. Recently however the presence of inhibitor I as one of the components of the intravacuolar protein bodies has been confirmed by the use of specific antibodies labelled with ferritin, fluorescein, or cytochrome *c* [118]. The perfectly shaped cubical crystals visible in the cells of the outer cortex of potato tubers have been isolated in their original morphological form [119] and subsequently demonstrated to be composed largely (90%) of a proteinase inhibitor of papain, chymopapain and ficin [28].

Studies on the compartmentalization of various proteins in the organelles purified from cotyledons of seeds of *Phaseolus vulgaris* by differential centrifugation have shown that an appreciable part of the trypsin inhibitory activity was definitely associated with the protein bodies [120]. Miège and co-workers have recently analysed the protein bodies isolated from cotyledonary cells of *Lablab purpureus* and *Ph. vulgaris* by iso-pycnic centrifugation. The trypsin inhibitors were found to be associated with the globulin proteases in fragments of the protein bodies which could be further dissociated by dilution [121, 122]. Other workers have however suggested the possibility that some or all of the inhibitors could be cytoplasmic in origin and merely adsorbed onto organelle surfaces during extraction. For example some 50% of the inhibitory activity found to be associated with unwashed protein bodies extracted from pea seeds could be removed by washing and then subsequently rebound [123].

Confirmation of the exact sub-cellular location of the proteinase inhibitors is clearly important for our understanding of their *in vivo* physiological functions. Several possible roles have been suggested. They may merely act as storage or reserve proteins, or they may, because of their peculiar properties actually regulate the endogenous protease enzymes and hence control protein turnover and metabolism. Alternatively they may be part of the plants' defence mechanisms against the attacks of insects and micro-organisms. In some tissues at least they may simultaneously fulfil several or all of these roles or other as yet unsuspected functions.

The presence of inhibitors in various tissues may be correlated with different physiological stages of development. For example the accumulation of Inhibitor I in solanaceous plants coincides with the establishment or maintenance of meristematic regions [116]. In other plants the inhibitors are associated particularly with the resting stages such as the seeds and other storage organs, and tend to decrease during germination [59, 124]. It is difficult to generalize however since the content of some inhibitors increases during germination [39, 124].

The relative levels of chymotryptic inhibitor I at various growth stages in nearly all tissues of the potato are strongly indicative of its function as a storage protein [9]. The initially high concentration of inhibitor in the tuber decreases rapidly as the sprouts appear and growth of the young plant occurs. Thereafter the inhibitor accumulates steadily in the new leaves and aerial tissues. This continues until the plants begin to set tubers at which time the inhibitor disappears from the vegetative tissues and starts to build up in the new tubers [57]. This inhibitor and other inhibitors of trypsin also accumulate as protein bodies in the vacuoles of detached leaves of potato and tomato [125]. Similarly the inhibitor accumulated in significant amounts during the growth and aging of tobacco callus cultures, but decreased rapidly when 26 day old callus tissues were transferred to a new medium, suggesting that it was degraded and utilized for establishing new callus growth [64].

The tobacco callus tissue cultures probably provide a good model system for studies on the regulation of accumulation and utilization of such inhibitors, since during the growth of this tissue Inhibitor I accumulates in extraordinarily large quantities until it represents over 10% of the soluble proteins [64].

The fact that the application of glutamine and asparagine to detached leaves of potato and tomato stimulated the production of the inhibitor [126] also argues for its possible reserve nature, since both amides are known to be intimately involved in nitrogen pools and the deposition and mobilization of proteins in plants.

Pusztai [39] has drawn attention to another interesting structural feature of many of the protease inhibitors, namely their high cystine content which might make them attractive as sulphur depot proteins.

There is still some controversy over the possible role of the inhibitors in the regulation of proteolysis. Many seeds (particularly the legumes) which have proteins as their major food reserves are characterized by high levels of proteinase inhibitors. Some workers believe that the proteolytic enzymes in the dormant seeds are kept inactive by the presence of the inhibitors and that the food stores are only mobilized during germination because the endogenous proteases become activated as the inhibitor content declines. This could be the case in seeds of lettuce [127, 128], barley [129], rice [59], rye [130], black-eyed peas [95] and *Vigna* seeds [43–45] where the inhibitors have actually been shown to inhibit certain of the seed proteases but the picture is far from complete, as in other species [39] there appears to be little or no correlation between the relative alterations in the protease and inhibitor concentrations. It is unlikely that protease/inhibitor interactions are the only controlling factor in proteolysis since we know that during germination some of the proteases are formed *de novo*, and some control might also be achieved if the proteases and their substrates were suitably compartmentalized.

The remarkable specificity of the inhibitors in pineapple stem [29] towards the protease found in the same tissue is strongly suggestive of their being involved in some regulatory role. The same is probably true for the potato carboxypeptidase inhibitor [34] which was found to strongly inhibit a bradykinin inactivating carboxypeptidase isolated from potatoes [9], but Baumgartner and Chrispeels [131] came to the conclusion that the specific inhibitor of the major endopeptidase in mung bean cotyledons had little or no role in regulating normal protein turnover, but was more likely to protect the cytoplasm from the effects of an accidental rupture of the protease-containing protein bodies in this species.

The yeast *Saccharomyces cerevisiae* probably provides one of the best understood examples of the control of intracellular proteolysis by proteinase inhibitors. In this organism the specific inhibitors (I^A, I^B and I^C) of the proteinases A and B and carboxypeptidase Y (previously called proteinase C) were all shown to be located in the soluble cytoplasm whilst their respective proteinases appeared to be compartmentalized in the vacuoles [132]. The complicated interplay of the three yeast proteinases and their respective specific inhibitors is illustrated in Fig. 2. Proteinase A inactivates the proteinase B inhibitor, proteinase B inactivates the proteinase A inhibitor and both proteinases A and B inactive inhibitor C. Carboxypeptidase Y on the other hand does not inactivate any of the inhibitors [26, 35]. This 'overcrossing' phenomenon can lead to an amplification of the activation of proteinases A and B and carboxypeptidase Y in an explosion-type time course when traces of free proteinase A or B appear in, or are added to, a mixture of inhibited proteinases (A–I^A, B–I^B and Y–I^C). The trigger for this overall cyclic process of activation might not only be the addition

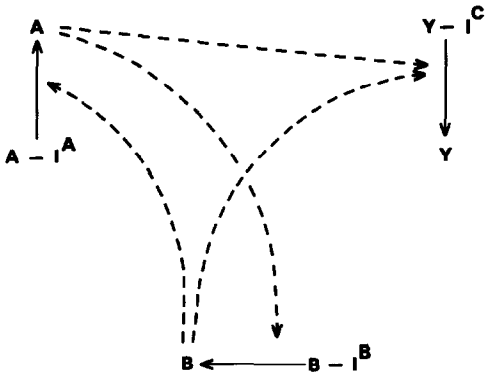

Fig. 2. Autocatalytic cycle of activation of the inhibited proteinases A and B and carboxypeptidase Y in yeast by traces of active proteinases A or B. A, B and Y represent the respective yeast proteinases. I^A, I^B and I^C represent the specific inhibitors. ----→ stands for activation of inhibited proteinase by proteolytic inactivation of the respective inhibitor (modified after Saheki *et al.* [26] and Matern *et al.* [35]).

of a surplus of free proteinases A or B, but also any physiological changes which lead to a dissociation of the inhibitor-proteinase complexes. One such change might be a reduction in pH brought about by lysosomal action, since the greatest inhibition of proteinase activity occurs at pH 6.5, the overall intracellular pH value of yeast cells, but is much reduced at pH 3 [26]. Studies are currently in progress on the activity of these proteases and their inhibitors throughout the period of enhanced protein degradation which occurs during yeast sporulation [133].

Recent developments have once more focussed attention on the possibility that the proteinase inhibitors might have an important function in protecting plants against the attacks of insects, or invasion by micro-organisms. Ryan [9] has summarized the considerable body of evidence that the proteinase inhibitors found in plants are capable of inhibiting the digestive enzymes of a range of insect pests which commonly use plants and their products as food. The best studied interactions are those between the plant inhibitors and the gut proteinases of members of the genera *Tribolium* and *Tenebrio* [134, 135]. The presence of inhibitors might be expected to make the plant less palatable, perhaps lethal to the insects and thus confer some selective advantage to the plant.

In 1972 Green and Ryan [37] made a significant advance in this field when they demonstrated that leaves of young tomato and potato plants infected with adult Colorado potato beetles or their larvae accumulated much greater amounts of Inhibitor I than did uninfested control plants. This effect of insect damage could be simulated by wounding or crushing the leaves with a paper punch or wooden dowl and file. Damage induced mechanically, or by the insects on a single leaf also caused an increase in the inhibitor levels in undamaged leaves elsewhere on the same plant. The latter observation suggested that a chemical signal or hormone, now known as Proteinase inhibitor-inducing factor (PIIF), which initiates Inhibitor I accumulation was produced or released near the site of wounding and was capable of being translocated out of the leaf to the rest of the plant. Production of PIIF and subsequent accumulation of the inhibitor were shown to be entirely light dependent requiring about 6000 lux at an optimum temperature of 36° [65].

Ryan [136] has described an assay method for PIIF and has also clearly shown that this factor induces the accumulation of other proteinase inhibitors besides Inhibitor I. The exact chemical nature of PIIF is as yet unknown, although it appeared to have a molecular weight in excess of 2000, was insoluble in lipid solvents, was unaffected by several hydrolytic enzymes (e.g. RNase, cellulase, amylase, chymotrypsin, phosphodiesterase), contained a large percentage of carbohydrate and had no detectable phosphate or ninhydrin reactive material [135, 137]. PIIF-like activity was found to be widely distributed, its presence being detected in the tissue juices of 37 out of 39 species representing 20 families of plants [137, 138]. Perhaps surprisingly juice from the fruiting body of the fungus *Agaricus campestris* was particularly effective, exhibiting about 20 times more PIIF-like activity than tomato juice. It remains to be seen whether the PIIF-like activities throughout the plant kingdom have the same or similar chemical composition and whether they are all capable of inducing proteinase inhibitor accumulation in their own leaves. Nevertheless Ryan has described this system as an excellent example of a primitive immune response contributing to the overall defence of a plant, and has suggested that it may hold promise for designing new approaches to biological pest control [9]. The discovery of mutant varieties with greatly enhanced or reduced levels of proteinase inhibitor production in response to wounding should prove particularly useful in such studies.

Not much is known about the possible role of the proteinase inhibitors in the defence of plants against parasitic micro-organisms, but in view of the almost certain participation of the pathogens' extracellular proteinases in the infection process a connection might reasonably be expected. Mosolov *et al.* [139] have recently shown that proteinase inhibitors from a number of higher plants and in particular the trypsin inhibitor from potatoes were very effective against the exogenous proteases found in culture filtrates of the phytopathogenic fungus *Fusarium solani*, whereas ovomucoid, a trypsin inhibitor of animal origin had no effect.

Recent work [140] has shown that Inhibitor I was induced in crown gall tumours of tobacco caused by the bacterium *Agrobacterium tumefaciens*, whereas uninfected tissues do not normally contain detectable quantities of the inhibitor. Surprisingly the tumours induced on potato and tomato plants by the same bacterium did not accumulate the inhibitor despite their family relatedness and ability to accumulate Inhibitor I under the influence of other stimuli. It is probably premature to ascribe a defensive role to this particular response. Another possible protective function has been suggested by Vogel *et al.* [3] who believe there is a correlation between the levels of proteinase inhibitors in some species of plants and the presence of symbiotic bacteria such as *Rhizobium*, and that the physiological role of the inhibitors is to prevent the plant tissues from being over-run by the bacteria.

It is rather obvious, but sometimes forgotten that the proteinase inhibitors probably have an important role in the endozoic dispersal of seeds. Birds and other animals which eat fruits or whole plants are known to subsequently excrete some seeds in a viable condition. The presence of proteinase inhibitors in relatively high concentrations in

so many seeds must increase the chances of their passing through the animals' alimentary canal without being damaged by the digestive system. Inhibitors of other digestive enzymes besides the proteinases will of course add to the protective effect if they are also present in the seeds; it is interesting that proteinaceous inhibitors of the starch degrading enzyme α-amylase have recently been isolated in large amounts (4–5 g/kg) from seeds of kidney bean [141] and wheat [142]. The specificity of these inhibitors for animal β-amylases [141, 143] also suggests that they are more likely to serve as protective agents against predators rather than as regulators of the endogenous amylases of the plant.

The exact nutritional significance of the plant proteinase inhibitors in the diets of animals and man is difficult to assess [8]. The observation of growth inhibition when animals were fed with plant products known to contain high levels of proteinase inhibitors led to these proteins being implicated as the cause of certain nutritional disorders. Experiments in which rats and chickens were fed with raw soybean products, or the partially purified crystalline trypsin inhibitors resulted in pancreatic hypertrophy and excessive enzyme secretion [3, 36]. There are also suggestions that the inhibitors may cause metabolic disturbance of the utilization of methionine and cystine [3].

The nutritive value of many leguminous seeds was reported to improve during germination or following controlled heat treatment [6], both of which were presumed to inactivate or reduce the levels of the inhibitors. However it is by no means certain that these presumptions are always correct. Pusztai [39] has shown that the content of proteinase inhibitors in kidney bean seeds remains constant or actually increases during germination. The proteinase inhibitors purified from different seeds vary considerably in their thermal stability and the extent to which they are destroyed by heat *in vivo* is a function of several variables such as particle size and moisture content. In some cases the excessive amount of heating required to destroy the inhibitors can lead to a decline in the overall biological value of the food. For example the inhibitors in chick peas retained all of their activity after being heated at 80° for 5 min and were only 50% inactivated by boiling at 100° for 5 min or roasting at 130° for 8 min [19]. Similarly the trypsin inhibitors in Faba beans (*Vicia faba* var *minor*) still contained 20% of their original activity after heating in a boiling water bath for 60 min [144].

The improvement in nutritive value produced in some cases by heating or germination may be due to the elimination of growth inhibitors other than the proteinase inhibitors since there is now good evidence [145, 146] that the trypsin inhibitors are not the only causes of growth inhibition to be found associated with the soybean and other legume proteins. Amongst the other antinutritional factors the most important appear to be the phytohaemagglutinins, goitrogens, cyanogenic glycosides, anti-vitamin factors, metal-binding constituents, lathyrogens and the substances repsonsible for causing favism. Several of these toxic substances are also eliminated together with some proteinase inhibitors by heating [147].

Heat treatment have been used most successfully to reduce the levels of proteinase inhibitors and other growth retardants during the commercial preparation of soybean products such as flour [148] and textured meat analogues. The inhibitors are still present on the surface of insolubilized soy protein [149] and although they are further inactivated during the process of converting this material into fibre it has been necessary to have a semi-automated method of determining the residual levels of trypsin inhibitors as a production control parameter in the manufacture of textured soy proteins [150].

Another complication is the fact that many comments on the significance of the plant inhibitors in human nutrition have been drawn from experiments in which bovine proteinases have been used instead of human proteinases because of their ready commercial availability. Yet the enzymes from these two sources differ in their susceptibility to inhibition [151]. The likelihood of a particular proteinase inhibitor causing adverse physiological effects in man depends to a considerable extent on its stability under acid conditions (pH 2–3) and its resistance to digestion by pepsin. This is the environment that the proteins have to survive in the stomach before they can exert their inhibitory activities on the trypsin and chymotrypsin in the intestine. Many inhibitors are known to be very stable under these conditions [11, 19, 21, 24, 38] but a few are quickly destroyed [11, 18].

During the 1950's there were many investigations of the possible therapeutic use of proteinase inhibitors from plants and other sources in the treatment of pancreatitis, shock, allergic reactions and various inflammatory disorders [3]. As a result of these studies the basic bovine pancreatic kallikrein trypsin inhibitor is now marketed under the pharmacological names of Trasylol, Iniprol and Contrykol [152] and has proved to be most promising [153]. In general the clinical experiments with the plant inhibitors have been less satisfactory [153] but the search continues for potentially useful proteins from plant tissues [154].

Finally looking to the future one may reasonably predict that the use of the proteinase inhibitors from plants as valuable laboratory tools will increase. Already in the field of affinity chromatography the immobilized inhibitors, covalently attached to inert supports have been widely used for the purification of specific proteases [155, 156] and for the removal of contaminating protease activity from other enzyme preparations [157, 158].

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Note added in proof: Peng and Black [159] have recently demonstrated a correlation between an increase of proteinase inhibitor activity in tomato plants following infection with *Phytophthora infestans* and the resistance of this species to the fungal pathogen.