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AMINO ACID SEQUENCE OF SERINE PROTEASE INHIBITOR CI-1 FROM BARLEY. HOMOLOGY WITH BARLEY INHIBITOR CI-2, POTATO INHIBITOR I, AND LEECH EGLIN

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Three molecular forms of a protein inhibitor of chymotrypsin and microbial alkaline proteases have been isolated from Hiproly high-lysine barley.

Automated Edman degradation of one of these inhibitor preparations (CI-1C) resulted in the following amino acid sequence (77 residues in total): Tyr-Pro-Glu-Pro-Thr-Glu-Gly-Ser-Ile-Gly-Ala-Ser-Gly-Ala-Lys-Thr-Ser-Trp-Pro-Glu-Val-Val-Gly-Met-Ser-Ala-Glu-Lys-Ala-Lys-Glu-Ile-Ile-Leu-Arg-Asp-Lys-Pro-Asn-Ala-Gln-Ile-Glu-Val-Ile-Pro-Val-Asp-Ala-Met-Val-Pro-Leu-Asn-Phe-Asn-Pro-Asn-Arg-Val-Phe-Val-Leu-Val (His, Lys, Ala, Thr, Thr, Val, Ala, Glx, Val, Ser, Arg) Val-Gly.

The inhibitor (CI-1) is homologous with another barley inhibitor (CI-2), with potato inhibitor I and with the elastase-cathepsin G inhibitor eglin from the leech Hirudo medicinalis (30–50% of the amino acid residues in identical positions). This established »family of cystine-independent inhibitors« also showed some sequence similarities with the cystine-free yeast proteinase B inhibitors 1 and 2. In the reactive site region homologies with the cystine-rich inhibitors of the »Kazal pancreas secretory inhibitor« and the »Streptomyces subtilisin inhibitor« families were observed.

Abbreviations: APNE = N-acetyl-D,L-phenylalanine-2-naphtylester; CI-1 and CI-2 = chymotrypsin inhibitor 1 and 2 from barley (3); LIE = Leech inhibitor eglin (17); PI-I = potato inhibitor I (15); Polybrene = hexadimethrine bromide; PTH = phenylthiohydantoin; SDS = sodium dodecyl sulfate; THEED = N,N,N',N',-tetrakis (2-hydroxyethyl)ethylenediamine; YIB = yeast inhibitor of protease B (11).

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1. INTRODUCTION

Barley grains contain two immunochemically distinct inhibitors of chymotrypsin and microbial subtilisin-like alkaline proteases (3, 5, 13). The inhibitor content is about twenty fold increased in Hiproly high-lysine barley (3, 4) and the two inhibitors, called CI-1 and CI-2, have been obtained in a highly pure form from this source (3, 6). Recently, the amino acid sequence of a lysine-rich protein from barley was determined (19). This protein was identified as inhibitor CI-2 (20) and a high degree of homology with the well characterized potato inhibitor I (15) was demonstrated.

The purpose of the present paper is: 1) to describe further separation of barley inhibitor CI-1 into a number of molecular forms with different charge properties, 2) to present the amino acid sequence of one of these inhibitors, and 3) to discuss sequence homologies between the two barley inhibitors CI-1 and CI-2 and other serine protease inhibitors.

2. MATERIALS AND METHODS

2.1. Materials

Protease inhibitor CI-1, previous purified from Hiproly barley by successive use of ammonium sulfate fractionation, gel filtration and cation exchange chromatography (3) was used as starting material. Immunoelectrophoresis and isoelectric focusing showed that the CI-1 inhibitor was effectively separated from the CI-2 inhibitor, and non-active proteins could not be detected in the inhibitor preparation. The purified inhibitor was composed of at least 4 molecular forms with isoelectric points in the pH-range 4.5–5.5

All reagents used with the sequencer were from Pierce Eurochemie B.V., Rotterdam, Holland, except THEED which was obtained from ICN-K&K Laboratories, Plainview, USA, ethylacetate from Merck, Darmstadt, West Germany, and heptane from Fluka, Buchs, Switzerland. APNE was purchased from Bachem, Bubendorf, Switzerland.

2.2. Methods

2.2.1. Chromatography

Anion exchange chromatography was performed on a column of DEAE-Sephadex A-50 (300 × 15 mm) equilibrated with 25 mM-Tris HCl, pH 7.5. About 170 mg of barley CI-1 inhibitor from previous purification steps (3) was applied in the same buffer. The column was washed with 100 ml buffer before elution with a buffer gradient. Absorbance at 280 nm was measured continuously, 8 ml fractions collected and chymotrypsin inhibitor activity determined. Relevant fractions were pooled, dialyzed and rechromatographed under the same experimental conditions in the presence of 6 M-urea.

2.2.2. Electrophoresis

Isoelectric focusing was made with 2% Ampholine, pH 3–10, at 25 °C in 74 × 2.7 mm polyacrylamide gel rods, and staining for inhibitors of subtilisin was based on the chromogenic substrate APNE (5). SDS-polyacrylamide gel electrophoresis was made in 180 × 180 × 1 mm gel slabs as described previously (3, 20).

2.2.3. Amino acid analysis

The amino acid composition was determined after acid hydrolysis in 5.7 M-HCl in an evacuated and sealed tube at 110 °C for 24 hours. The analysis was performed with a Durrum model 500 amino acid analyzer.

2.2.4. Amino acid sequence determination

Amino acid sequence determinations were made in a Beckman liquid phase sequencer, model 830 C, using the Beckman programme 122974. Instead of Quadrol, THEED was used in a 0.1 M-concentration (1). The PTH-amino acids were identified by HPLC using a linear gradient of methanol from 10% to 50%. Additional information was obtained by thin-layer chromatography on polyamide sheets (9). When the CNBr-peptides were sequenced, Polybrene was added to the cup in order to minimize loss of material in the extraction steps.

Digestion with carboxypeptidase Y was done as described by MARTIN et al. (12) with and without 0.5% SDS in the reaction mixture.

2.2.5. Acetylation and cyanogen bromide cleavage

Acetylation was performed in 0.1 M-NH_4HCO_3, pH 8.0, by addition of acetic anhydride (4 × 25 µl with 20 min intervals). Reaction mixture containing 1 mg of inhibitor was freeze-dried and redissolved in 1 ml 70% formic acid. 30 mg CNBr was added, and the reaction took place overnight in the dark at room temperature. The mixture was purged with a stream of nitrogen to remove excess of CNBr and placed directly in the sequencer cup.

3. RESULTS

3.1. Preparation of inhibitors

In order to separate the inhibitor forms for sequence studies, pooled material from SP-Sephadex cation exchange chromatography (3) was subjected to anion exchange chromatography on DEAE-Sephadex (Figure 1). One minor and three major inhibitor peaks were obtained after elution with a buffer gradient. Pooled material from each of the three major peaks (indicated by bars A, B, and C in Figure 1) was rechromatographed under the same experimental conditions, but in the presence of 6 M-urea. Each of the three experiments resulted in elution of a symmetrical protein peak completely overlapping with the chymotrypsin inhibitor activity. Inhibitor preparations CI-1 A, B, and C eluted at the same salt concentration as in the first anion exchange (cf. Figure 1), and apparently 6 M-urea did not affect the charge properties of the different inhibitors. Central peak fractions corresponding to 40–50% of the total activity were pooled, desalted and freeze dried.

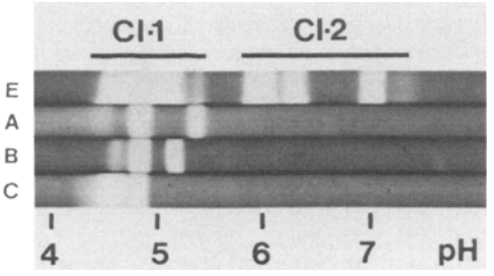

Figure 2. Isoelectric focusing of barley CI–1 inhibitors.

About 0.7 µg of each inhibitor preparation (A, B and C) or Hiproly barley extract (E) corresponding to 0.5 mg grain were applied. After isoelectric focusing the gel was stained for subtilisin inhibitor activity (5). A pH-scale and the isoelectric range of CI-1 and CI-2 inhibitors (3, 5) are indicated on the figure.

The three inhibitor preparations were characterized by isoelectric focusing followed by specific staining for subtilisin inhibitor activity (Figure 2) and by SDS-gel electrophoresis (Figure 3). In contrast to the apparently well-

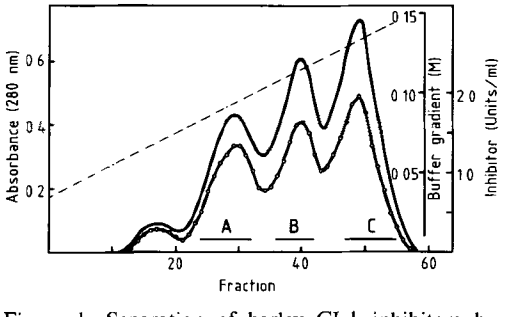

Figure 1. Separation of barley CI-1 inhibitors by anion exchange chromatography on DEAE-Sephadex A-50 at pH 7.5.

– · – · – buffer gradient; ——— absorbance at 180 nm; –O–O–O–O– chymotrypsin inhibitor activity. Fractions 24–32 (CI-1A), 36–42 (CI–1B) and 47–55 (CI-1C) were pooled as indicated by the bars.

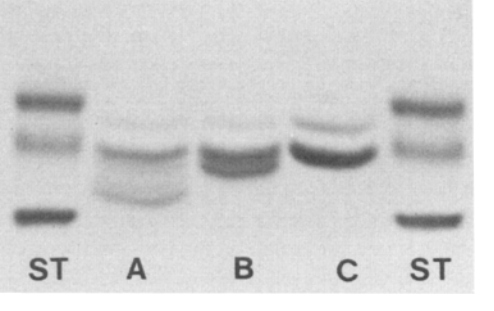

Figure 3. SDS-gel electrophoresis of barley CI-1 inhibitors.

About 5 µg of each inhibitor preparation (A, B and C) were applied, and after electrophoresis the gel was stained for protein with Coomassie blue R-250. ST indicates application of the following standard proteins: cytochrome C (MW 13,000), aprotinin (MW 6,500) and (bottom) glucagon (MW 3,500).

defined fractions isolated by anion exchange (Figure 1), these analytical experiments showed that all inhibitor preparations were composed of two or more forms with different charge properties and molecular sizes. As discussed previously (3, 20), genetic variants, partially degraded N-termini and molecular aggregation may explain these complex patterns. Rocket immunoelectrophoresis experiments (20) with monospecific antibodies (3) towards the two barley inhibitors CI-1 and CI-2 confirmed that the CI-1 inhibitor preparations were not contaminated with inhibitor CI-2. No immunoelectrophoretic relationships with inhibitors in crude potato inhibitor preparations (20) were observed.

Inhibitor preparation CI-1C was selected for the sequence studies. This fraction represented the most acidic inhibitor zones in isoelectric focusing (Figure 2) and appeared to be the most homogeneous with respect to molecular size (Figure 3).

3.2. Sequence determination of inhibitor CI-1C

The intact protein (0.5 mg) was sequenced from the N-terminus through 57 cycles with a repetitive yield of 96%. Two chains were actually sequenced, one starting at Tyr (7) and another starting at Glu (9) (so numbered for comparison with other inhibitors, see Figure 4). The two chains were present in approximately equal amounts, thus a double check on the

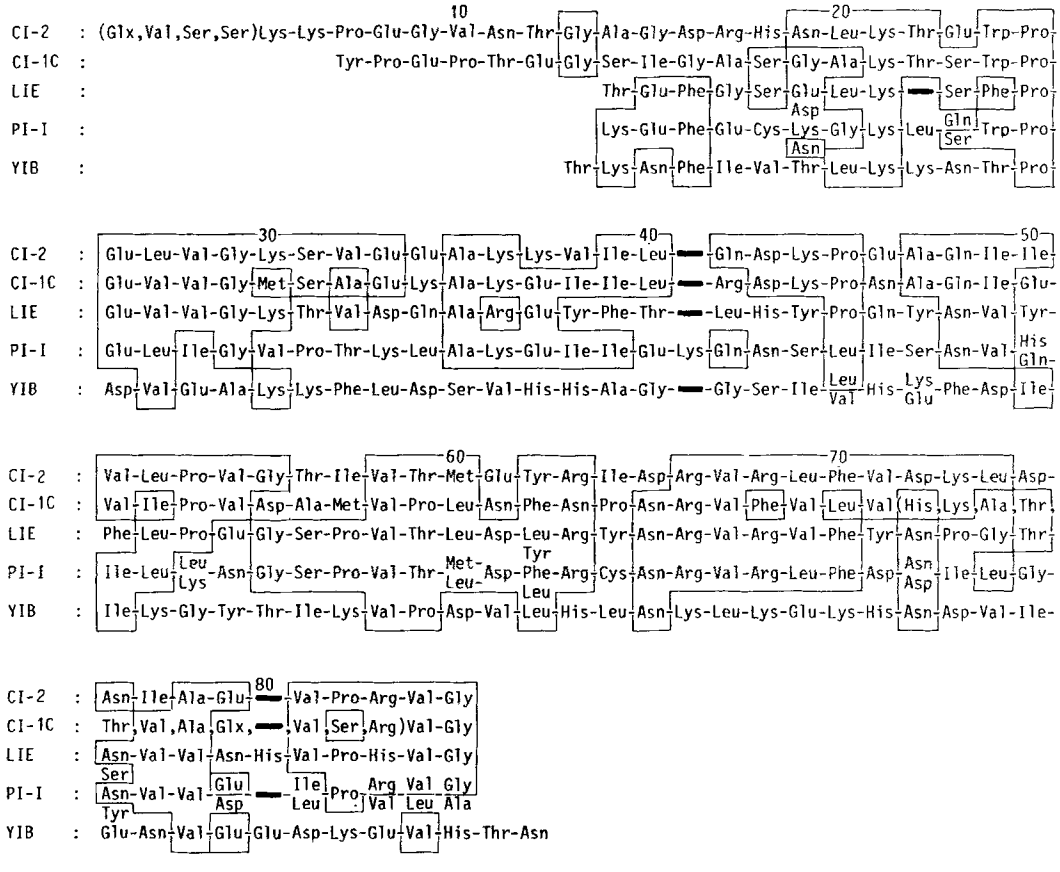

Figure 4. Amino acid sequence of barley inhibitor CI-1C.

Comparison with barley inhibitor CI-2 (19, 20), potato inhibitor I (PI-I, polymorphous sequence) (15), leech eglin (LIE) (17), and yeast protease B inhibitor (YIB, dimorphous sequence) (11). Homologous sequences are boxed. ▬ indicate »gaps« introduced to obtain maximal homology among the inhibitors. The numbering refers to the sequence of the barley CI-2 inhibitor as used previously (20).

individual steps in the sequencing was obtained.

Another 0.5 mg sample was acetylated at the N-terminus with acetic anhydride and then subjected to cleavage with CNBr. After removal of the reagents in a stream of nitrogen the peptide mixture was placed directly in the sequencer cup together with Polybrene. Two sequences could be followed, one starting at Ser (31) and one at Val (58) as expected. Since the first sequence was known throughout, an unknown part of the second sequence was easily determined, and the total sequence was extended to include Val (71) (Figure 4). In several additional experiments with peptides obtained after tryptic or CNBr cleavage it was not possible to extend the sequence beyond Val (71).

Attempts to determine the C-terminal sequence by digestion with carboxypeptidase Y in the absence of SDS were not successful. This method also failed when inhibitor CI-2 (19) and leech eglin (17) were sequenced. If 0.5% SDS was present (12), however, inhibitor CI-1C as well as leech eglin (17) could be digested. Two residues were liberated from the barley CI-1C inhibitor; Gly followed by Val. Thus, the four homologous inhibitors belonging to the »potato inhibitor I family« (see section 3.3) have the same C-terminal sequence Val-Gly and, apparently, this terminal is buried and not directly accessible to degradation by carboxypeptidase. It therefore seems reasonable to presume that inhibitor CI-1 ends in the same position (residue 85, Figure 4) as the three other inhibitors with a C-terminal glycine. If the results from amino acid analysis of the inhibitor CI-1C preparation were calculated on basis of this assumption, an amino acid composition of the C-terminal undecapeptide not sequenced could be obtained by difference (Table I). Tentatively, these amino acids could be lined up in a sequence showing a high degree of homology with the three other inhibitors (Figure 4). With the suggested amino acid composition (Table I) the CI-1C inhibitor should contain 2 methionine residues. This result is in agreement with the presence of three peptides after CNBr-cleavage.

Molecular weights of 8,000 and 8,300 were calculated for the two molecular forms present in the CI-1C preparation. Comparison with the SDS-gel electrophoresis results (Figure 3) suggests that both forms are present in the same major protein band detected after electrophoresis of the CI-1C inhibitor preparation. Another protein corresponding to a slightly higher molecular weight was present in relatively high amounts in the inhibitor CI-1C and apparently also in the two other inhibitor preparations CI-1A and CI-1B (Figure 3). Previous experiments did not show the presence of non-active proteins in the inhibitor mixture used as starting material for the present study (3), and no »background« sequence from a contaminant protein was detected before or after cleavage with CNBr. The most likely explanation seems to be that this protein zone represents an inhibitor form with a blocked N-terminus, in accordance with results obtained during sequencing of barley inhibitor CI-2 (19).

Table I

Amino acid composition of inhibitor preparation CI-1C.

Amino acid	residues[a] per molecule	residues[b] from sequence	difference
Asx	6.2 (6)	6	
Thr	3.7 (4)[c]	2	2
Ser	4.9 (5)[c]	4	1
Glx	7.7 (8)	7	1
Pro	6.7 (7)	7	
Gly	4.7 (5)	5	
Ala	8.4 (8)	6	2
Val	10.8 (11)[d]	9	2
Met	2.3 (2)	2	
Ile	5.2 (5)[d]	5	
Leu	3.4 (3)	3	
Tyr	0.7 (1)	1	
Phe	2.0 (2)	2	
His	0.5 (1)	0	1
Lys	4.5 (5)	4	1
Arg	3.4 (3)	2	1
Trp	1 (1)[e]	1	
Cys	0 (0)[e]	0	
Total residues	77	66	11

[a] Hydrolysis time 24 hours. Calculations based on a Phe content of 2 residues per molecule. [b] Data from Figure 4 residues 7–71 and 84–85. [c] Extrapolated to zero-time based on previous analyses (13). [d] Calculated on previous results from 72 hours hydrolysis (13). [e] Result from previous analyses (3).

In further analogy with the »ragged« N-terminus found in the CI-2 inhibitor and other inhibitors (15, 19, 20), the proteins with lower molecular weights found in the CI-1A and CI-1B inhibitor preparations (Figure 3) may correspond to molecular forms with more degraded N-termini.

3.3. Comparison of sequences

Homology between barley inhibitor CI-2 and potato inhibitor I has been demonstrated previously (20) and, recently, an elastase-cathepsin G inhibitor (called eglin) from the leech Hirudo medicinalis was also found to be homologous with the potato inhibitor (18). Obviously the barley inhibitor CI-1 belongs to the same »inhibitor family« (Figure 4). In addition to the identical C-terminal -Val(84)-Gly(85) discussed above, a high degree of homology between the four inhibitors was found in the stretch of 57 residues (residue 14–71, Figure 4), where the sequence of all four inhibitors has been determined. To obtain maximal homology, one deletion in the leech inhibitor sequence (residue 22) and one insertion in the potato inhibitor sequence Lys (41) had to be introduced. Comparison of amino acids in identical positions in these four inhibitors (Table II) showed between 30% and 46% homology in the sequence of residues 14–71. However, it is impossible from such a comparison to suggest which of the two inhibitors originates from the same monocotyledonous plant species, which one is from a dicotyledonous plant, and which is the inhibitor originating from a primitive invertebrate animal. Apparently, these inhibitors diverged from one another very early during evolution, and thus a wide distribution in plants and animals of homologous inhibitors belonging to the same family may be expected.

Table II

Homology between different inhibitors. Amino acids in identical positions in the sequence of residues 14 to 71 (see Figure 4).

Inhibitors compared	% Homology			
	YIB	LIE	PI-I	CI-2
CI-1	11	32	30	46
CI-2	11	35	40	
PI-I	16	46		
LIE	16			

All four inhibitors inactivate chymotrypsin and subtilisins. The reactive site (P_1-P'_1) of the potato inhibitor and the leech inhibitor have been identified as Met/Leu(60)-Asp(61), using the numbering of the present paper. The presence of Leu(60)-Asn(61) and Met(60)-Glu(61) bonds in the two barley inhititors is in accordance herewith.

In contrast to the three other inhibitors, the potato inhibitor also inactivates trypsin. Treatment of the potato inhibitor with catalytic amounts of trypsin suggested two major and two minor cleavage sites (all different from the chymotrypsin inhibitory site) as potential trypsin binding sites (16). One of the sites suggested was between Lys(41)-Gln(42) and, if this is the true binding site, the insertion of Lys(41) resulting in an extention of the peptide chain with one residue may explain that the potato inhibitor I, but not the barley inhibitor CI-1 with Arg(41/42) in the same position, inhibits trypsin.

The conservation of all intra-chain disulfide bridges has been observed within different inhibitor families and, especially, the localization of the reactive site in a disulfide loop has been considered essential (10). A characteristic property of the two barley inhibitors and the leech inhibitor is the absence of cystine residues. Recently, it has been demonstrated that carboxymethylation of the two cysteines forming the only disulfide bridge present in the potato inhibitor had no effect on the inhibitory properties (14). Obviously, the inhibitor family here established should be classified among families where stabilization of the reactive site tertiary structure is only dependent on non-covalent forces.

Among the few other cysteine-free protease inhibitors hitherto known only yeast inhibitors of the two endogenous proteases A and B have similar peptide lengths of about 70 residues (2, 11). Of these inhibitors the two proteinase B isoinhibitors 1 and 2 show a clear similarity with the four members of the »potato inhibitor I family«. Recent studies suggested that yeast protease B has a »mixed type« specificity

corresponding to that of porcine chymotrypsin C as well as trypsin (8), but the reactive site(s) of the endogenous inhibitor has not been identified. In the sequence of 57 residues discussed above, an 11–16% homology of the yeast inhibitor with the four other inhibitors was observed (Figure 4, Table II). Generally, this degree of homology is not sufficient to suggest a divergent evolution. It should, however, be noted that the two genetic variants of the yeast protease B inhibitor could be aligned with the four other inhibitors without introduction of new insertions or deletions, and the homology is significantly connected with the conservative positions of the four inhibitors, but not especially confined to the active site region. Thus, Lys(21), Pro(25) and Val(58) are found in the inhibitors from all five species, and in the remaining 5 invariant positions in the four highly homologous inhibitors: Glu(26), Gly(29), Ala(35), Arg(66) and Val(67), the yeast inhibitor contains the following residues, respectively: Asp(26), Ala(29), Ser(35), Lys(66) and Leu(67). These observations may be of interest for future studies of the structure, mechanism and specificity of all the inhibitors.

The high degree of homology often found in the active site region of otherwise unrelated inhibitors belonging to different families has, generally, been explained as the result of a convergent evolution (10). When sequences around the reactive site of the four inhibitors from the »potato inhibitor I family« are compared with those of other inhibitors, many similarities are found, especially with animal inhibitors of the »Kazal pancreatic secretory trypsin inhibitor family« and microbial inhibitors of the related »Streptomyces subtilisin inhibitor family« (Table III). Almost all inhibitors sequenced within these families have Thr or Pro in the P_2 position and Pro in the P_4' position. Asx or Glx appear with high frequencies in the P_1', aromatic/hydrophobic amino acids in the P_2' and Arg or Asx in the P_3' position (7, 10). Barley inhibitor CI-2 and potato inhibitor I have, for example, a pentapeptide in the active site (P_2–P_3') identical with domain 3 of pheasant ovomucoid and a domain of canine submandibular gland inhibitor, respectively, (Table III). In contrast, barley inhibitor CI-1 has Pro in both positions P_2 and P_4' as the Streptomyces subtilisin inhibitor and many of the »Kazal family« inhibitors.

Table III

Comparison of reactive site sequences from different »families« of inhibitors.

Inhibitor	Amino acid sequence around reactive sites P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'
Barley inhibitor CI-1	Met-Val-Pro-Leu-Asn-Phe-Asn-Pro
Streptomyces inhibitor S-SI (10)	Met-Cys-Pro-Met-Val-Tyr-Asp-Pro
Canine submandibular gland inhibitor 2 (10)	Met-Cys-Thr-Met-Asp-Tyr-Arg-Pro
Pheasant ovomucoid 3 (7)	Ala-Cys-Thr-Met-Glu-Tyr-Arg-Pro
Barley inhibitor CI-2 (19)	Ile-Val-Thr-Met-Glu-Tyr-Arg-Ile
Potato inhibitor I (16)	Pro-Val-Thr-Met/Leu-Asp-Tyr/Phe/Leu-Arg-Cys
Leech Eglin (17)	Pro-Val-Thr-Leu-Asp-Leu-Arg-Tyr

It should be noted, that the four inhibitors from the »potato inhibitor I family«, as well as the yeast protease B inhibitor, all have a Val in the P_3 position. In contrast, this position is always occupied by a Cys in inhibitors from the »Kazal inhibitor«, the »Streptomyces inhibitor« and also the »Bowman-Birk Plant inhibitor« families (10).

Physiological functions of inhibitor I from potatoes and other Solanaceae plants have been intensively studied and, apparently, this inhibitor is an essential part of a defense system under hormone-like control in the vegetative plant (for references, see 3, 10, 20). We have now shown that the two major inhibitors of microbial proteinases and chymotrypsins present in the barley grain both are highly homologous with the Solanaceae inhibitor I. Hitherto it has not been possible to demonstrate the presence of these inhibitors, or mechanisms inducing their synthesis, in vegetative tissues of the barley plant, and further studies are necessary to elucidate their possible protective role in storage as well as in vegetative parts of barley and other cereal plants.

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