SEQUENCE HOMOLOGY BETWEEN BARLEY TRYPSIN INHIBITOR AND WHEAT α-AMYLASE INHIBITORS

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1. Introduction

Protein proteinase inhibitors are one of the most studied of all proteins from the viewpoint of evolution. They frequently exist as 'multi-headed' inhibitors capable of inhibiting more than one enzyme simultaneously. Structural analysis of the multi-headed inhibitors revealed the presence of 2–7 repeating sequences, each of which contains a reactive (binding) site for a proteinase [1]. Furthermore, a single species of plants or animals usually contains a set of many inhibitor variants, which, in spite of their closely resembling structures, show a wide variety of inhibition spectra resulted from minor changes of amino acid sequence around the reactive sites. The above findings are accepted as a strong evidence for the divergent evolution of proteinase inhibitors from a limited number of ancestors by fused or separated gene multiplication and mutation [1]. This divergent evolution of the inhibitors so far observed is limited within the inhibitors of proteolytic enzymes. During the course of sequence determination of barley trypsin inhibitor we have noted that the amino-terminal sequence of this inhibitor is homologous to the partially known sequence of α-amylase inhibitors. Here, we present the first evidence for a possible evolutionary relationship between two apparently unrelated inhibitors, a trypsin inhibitor and α-amylase inhibitors.

2. Materials and methods

2.1. *Materials*

Barley trypsin inhibitor was purified from finely ground barley (*Holdeum vulgae*) seeds according to [2]. Pancreatic enzymes were obtained from Worthington Biochem. Corp. (New Jersey). A highly purified fungal α-amylase, Taka-amylase A (*Aspergillus oryzae*) was a generous gift of Drs Hase and Ikenaka, Osaka University.

2.2. *Fragmentation of the inhibitor with CNBr*

Barley trypsin inhibitor (30 mg) was treated with CNBr (100-fold molar excess over Met residues) in 1.0 ml 70% formic acid for 20 h at 25°C. The CNBr digest was reduced and *S*-carboxymethylated as in [3], and fractionated on a DEAE–Sephadex A-25 column (0.9 × 50 cm, 0.02 M NH_4HCO_3) developed with a linear gradient of NH_4HCO_3 (400 ml 1.0 M into 400 ml 0.02 M).

2.3. *Trypsin digestion and separation of the peptides*

Reduced, and *S*-carboxymethylated inhibitor (30 mg) was digested in 0.1 M NH_4HCO_3 with 0.6 mg L-1-chloro-(3-(toluene-sulfonamido)-4-phenyl-2-butanone treated trypsin for 6 h at 25°C, and directly applied to a DEAE–Sephadex A-25 column (0.9 × 50 cm, 0.02 M NH_4HCO_3) and chromatographed as above. Peak fractions were further purified by paper electrophoresis.

2.4. *Amino acid analysis*

Protein and peptide samples were hydrolyzed in 5.7 N HCl under vacuum at 110°C for 20 h. Amino acid composition was analysed on a Hitachi KLA-3B amino acid analyser [4].

2.5. *Sequence analysis*

Sequence of peptides and proteins was analysed by the manual Edman degradation method [5]. Identification of the phenylthiohydantoins was performed by silica gel thin-layer chromatography [6] and by back hydrolysis to parent amino acids [7].

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Table 1
Amino acid compositions of amino-terminal CNBr-fragment CB-1, tryptic peptides (T-1–T-4) and a chymotryptic peptide CB-1C-3, corresponding to CB-1

	CB-1	T-1	T-2	T-3	T-4	CB-1C-3
Asx	4.0 (4)	2.9 (3)			1.0 (1)	
Thr	1.7 (2)			0.9 (1)	0.9 (1)	
Ser	2.6 (3)	0.8 (1)		0.9 (1)	0.8 (1)	0.6 (1)
Glx	1.9 (2)			2.1 (2)		1.7 (2)
Pro	4.2 (4)	3.1 (3)		1.0 (1)		1.0 (1)
Gly	2.6 (3)	2.0 (2)		1.0 (1)		1.0 (1)
Ala	3.0 (3)	1.9 (2)	1.1 (1)			
Cys[a]	3.0 (3)	0.9 (1)	0.8 (1)	0.7 (1)		0.8 (1)
Val	1.6 (2)			1.9 (2)		1.7 (2)
Met					0.8 (1)	
Ile	1.1 (1)			1.0 (1)		1.0 (1)
Leu	4.2 (4)	2.0 (2)			2.1 (2)	2.1 (2)
Tyr	1.2 (1)			0.8 (1)		
Phe	1.0 (1)	0.9 (1)				
Lys					1.0 (1)	
His	1.7 (2)	0.9 (1)		1.0 (1)		0.6 (1)
Arg	3.1 (3)	0.9 (1)	1.0 (1)	1.0 (1)		1.1 (1)
Hse	0.7 (1)					
Total	(39)	(17)	(3)	(13)	(7)	(13)
Yield (%)	31	79	65	50	42	40

[a] Determined as *S*-carboxymethylcysteine

Expressed as molar ratios. Values in parentheses are the nearest integers

2.6. *Enzyme inhibition assay*

Proteinase inhibitor activities were measured as in [8]. For measurement of α-amylase inhibitory activity, the inhibitor was preincubated with the amylase for 1 h at pH 7.0 (0.02 M phosphate buffer, 7 mM NaCl) and 37°C before addition of soluble starch [9]. Amylase activity remaining after preincubation was determined by the dinitrosalicylic acid method [10].

3. Results and discussion

3.1. *Characterization of the purified inhibitor*

Purified inhibitor was homogeneous by SDS–polyacrylamide (12.5%) gel electrophoresis [11] ($M_r \sim 14\,000$), and by amino-terminal sequence analysis (single amino-terminal sequence, Phe–Gly–Asp–, was identified by Edman degradation). The protein

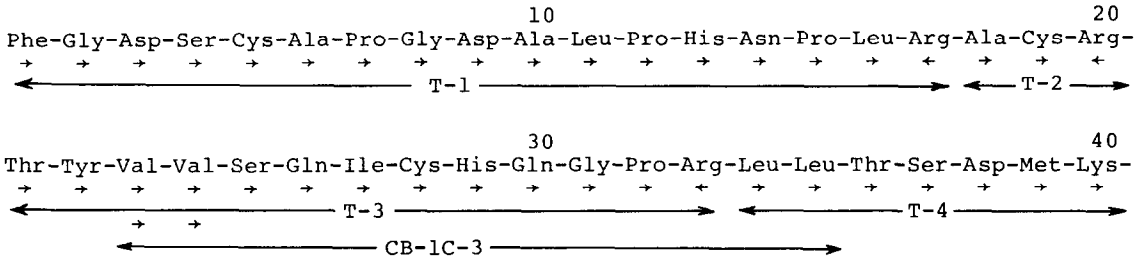

Fig.1. Summary of sequence analyses on the amino-terminal CNBr-fragment, CB-1, of barley trypsin inhibitor: (→) Edman degradation; (←) identified by amino acid analysis of the residue of the Edman reaction. Lysine at position 40 is the amino-terminus of the second CNBr-fragment, CB-2.

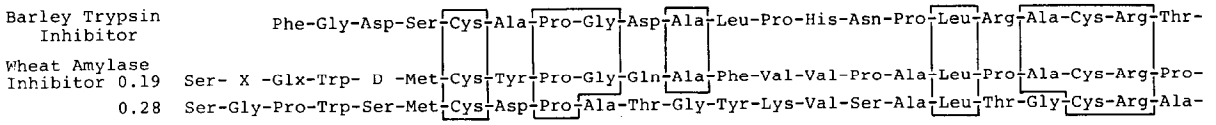

Fig.2. Comparison of the amino-terminal sequence of barley trypsin inhibitor with those of wheat amylase inhibitor 0.28 [13] and amylase inhibitor 0.19 [14]. Residues identical with barley inhibitor are in boxes: (X) unidentified residue; (D) deletion.

stoichiometrically inhibited bovine trypsin, but not bovine α-chymotrypsin and porcine elastase. The chemical composition was very close to those reported in [2,12]. The protein contained 2 Met residues.

3.2. *CNBr fragments*

CNBr treatment of the inhibitor followed by reduction and *S*-carboxymethylation yielded the expected 3 fragments, CB-1, CB-2, and CB-3, on DEAE–Sephadex A-25 chromatography. The sum of the amino acid compositions of these 3 fragments accounted for the composition of the inhibitor (not shown). Amino-terminal sequence of CB-1 (Phe–Gly–Asp–) indicated that this fragment was derived from the amino-terminus of the inhibitor.

3.3. *Tryptic peptides and amino acid sequence of CB-1*

Among 10 peptides obtained from the tryptic digest by DEAE–Sephadex A-25 chromatography, peptides T-1–T-4 were derived from the amino-terminal portion of the protein and the sum of the amino acid compositions of the 4 tryptic peptides comprised the composition of CB-1 plus one lysine residue (table 1). The determination of the amino acid sequence of the 4 tryptic peptides and an overlapping peptide CB-1C-3 obtained by the chymotryptic digestion of CB-1 established the whole sequence of CB-1 as shown in fig.1.

3.4. *Comparison with other plant proteins*

Fig.2 shows the comparison of the amino-terminal sequence of barley trypsin inhibitor and wheat α-amylase inhibitors 0.28 [13] and 0.19 [14]. Although no further sequence data are available for the amylase inhibitors, homology (including the disposition of 2 half-cystine residues) between the trypsin inhibitor and the amylase inhibitors is evident within these rather short segments. This finding, together with a report [15] on the discovery of an unusual plant (*Eleusine coracana*) inhibitor that inhibits porcine pancreatic amylase and trypsin simultaneously led us to investigate α-amylase inhibitory activity of barley trypsin inhibitor. However, no inhibition of porcine pancreatic amylase and Taka-amylase A (*Aspergillus oryzae*) was observed even in the presence of a 10-fold molar excess inhibitor over the enzymes. Human salivary amylase activity was also not affected by the inhibitor.

Two examples for the possible evolution from a common ancestor of inhibitors against proteinases of different classes have been reported; that is, a sequence homology between legume serine proteinase inhibitors and a pineapple thiol proteinase inhibitor [16], and a weak one between potato inhibitors for a serine proteinase and a metalloproteinase [17]. The present result provides the first structural evidence for an evolutionary relationship between inhibitors directed toward two entirely unrelated enzymes, trypsin and α-amylase. Similar molecular properties, such as $M_r \sim 14\,000$ and relatively high cystine contents (7–8%) [9], suggest further sequence homology of the 2 proteins. Detailed comparative study on the structures of these trypsin and amylase inhibitors will contribute to understanding this unusual evolutionary process as well as the inhibition mechanisms for respective target enzymes of the 2 protein inhibitors.

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