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Structural Characterization of an α-Amylase Inhibitor from a Wild Common Bean (*Phaseolus vulgaris*): Insight into the Common Structural Features of Leguminous α-Amylase Inhibitors

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The primary structures of two subunits of an α-amylase inhibitor (αAI-2) from a wild common bean (*Phaseolus vulgaris*) were revealed by a comparison of the amino acid sequence previously deduced from the nucleotide sequence with the amino- and carboxyl-terminal amino acid sequences determined by conventional methods. The polypeptide molecular weight of αAI-2 obtained by the light-scattering technique, considered together with the sequence molecular weights revealed for the subunits, indicated that αAI-2 has the subunit stoichiometry of an $\alpha_2\beta_2$ complex. These structural features were closely similar to those recently elucidated for a white kidney bean (*P. vulgaris*) α-amylase inhibitor, which is quite different in the inhibitory specificity from αAI-2. The post-translational processing of the precursor glycoproteins to form the tetrameric structure appeared to require an Arg residue close to the processing site. Further, the proper associations of the subunits into the tetrameric structures seemed to be strictly controlled by a few amino acids on the subunit interfaces.

Key words: α-amylase inhibitor, kidney bean, post-translational processing, primary structure, subunit structure.

Seeds of the common bean (*Phaseolus vulgaris*) contain a high level of a family of defense proteins that includes α-amylase inhibitor, lectin, α-amylase inhibitor-like protein, and arcelin (*1*). The genes for these proteins are encoded at a single locus in the *P. vulgaris* genome (*2*), and the homologous genes seem to have arisen by duplication of an ancestral gene. The amino acid sequences of these proteins have been deduced from their cDNAs and show high degrees of homology among them (*3*).

Recently, a series of structural studies has been made on a white kidney bean (*P. vulgaris*) α-amylase inhibitor (αAI-1)[2], which is composed of two kinds of glycopolypeptide subunits, α and β (*4-7*). These studies disclosed for the first time that a legume α-amylase inhibitor has a tetrameric structure, $\alpha_2\beta_2$, which is essential for the inhibitory activity. In this study, with a view to gaining an insight into the common structural features of to leguminous α-amylase inhibitors, we undertook to determine the structure of an α-amylase inhibitor (αAI-2) from a wild common bean (*P. vulgaris*) (*8*), which has an inhibitory specificity quite different from αAI-1. The amino acid sequences established for the two kinds of subunits, α and β, of αAI-2 were highly homologous to those of αAI-1. Polypeptide molecular weight of αAI-2 determined by size-exclusion chromatography (SEC)/light-scattering technique, considered together with the sequence molecular weights of the subunits, α and β, demonstrated that αAI-2 is also a tetrameric complex, $\alpha_2\beta_2$, analogous to αAI-1, suggesting that the tetrameric structure is common to leguminous α-amylase inhibitors. Further, a comparison of the amino acid sequences of these inhibitors and other *P. vulgaris* defense proteins suggested that the post-translational processing of the precursors to form an active tetramer needs an Arg residue close to the processing site.

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[2] Referred to as PHA-I in the previous papers (*4-7*).

Abbreviations: αAI-1, α-amylase inhibitor of *Phaseolus vulgaris* white kidney bean; αAI-2, α-amylase inhibitor of a *Phaseolus vulgaris* wild common bean; PHA, *Phaseolus vulgaris* lectin; SEC, size-exclusion chromatography.

MATERIALS AND METHODS

Materials—αAI-2 (*9*) and αAI-1 (*4*) were prepared as described previously. Laboratory colonies of *Callosobruchus chinensis* and *Zabrotes subfasciatus* were also obtained as described previously (*10*). Carboxypeptidase Y was purchased from Wako Pure Chem. Aqueous urea was always treated with ion-exchange resin AG501-X8D (Bio-Rad) just before its use. Other chemicals used were described in the previous paper (*4, 6, 7*).

Isolation of αAI-2 Subunits—αAI-2 (2.0 mg) was dissolved in 50 mM Tris-HCl (pH 8.0, 0.5 ml) containing 8 M urea, 2 mM EDTA, and 20 mM 2-mercaptoethanol, and allowed to stand at 37°C for 4 h. Part (0.4 mg/0.1 ml) of the solution was submitted to HPLC on a Shimadzu model LC-10AS chromatograph. A Cosmosil DEAE column (7.5×

75 mm; Nacalai Tesque) was equilibrated with 50 mM Tris-HCl (pH 8.0) containing 8 M urea and eluted with a linear NaCl gradient from 0 to 0.1 M in the same buffer at a flow rate of 0.3 ml/min. The elution of polypeptides was monitored by tyrosyl fluorescence at 304 nm (excitation at 275 nm). The peak effluents were collected, dialyzed against 1% acetic acid (Spectrapor 3 membrane, Spectrum Medical Industries), then lyophilized.

Amino Acid and Sugar Analysis—Amino acid analysis was performed with a Hitachi 835 amino acid analyzer after hydrolysis with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110°C for 24 h or 72 h *in vacuo*. Values for isoleucine and leucine were those of 72 h hydrolysis. Individual neutral sugars were determined by gas-liquid chromatography as their alditol acetates as described previously (*4*). *N*-Acetylglucosamine was determined on a micro scale by a modification of the Elson-Morgan method (*12*).

Carboxyl-Terminal Sequence Analysis—The carboxyl-terminal sequences of peptides were determined with carboxypeptidase Y. Peptides were digested with carboxypeptidase Y in 50 mM ammonium acetate buffer (pH 4.0). At appropriate intervals, a part of the reaction mixture was withdrawn and heated at 100°C for 5 min to stop the reaction. The reaction mixture was lyophilized and the amount of free amino acids was analyzed with an amino acid analyzer.

Other Methods—αAI-1 assay was done exactly as described previously (*4*). The insect α-amylases were extracted from larvae and used for αAI-2 activity estimation by the method previously described (*11*). The amino-terminal sequence analysis was carried out on a Shimadzu model PSQ-1 gas-phase protein sequencer. Other analytical methods including SDS-PAGE and SEC/light-scattering technique were previously described (*4, 7*). Parental and chimeric inhibitors were constructed from the subunits of αAI-1 and αAI-2 by the method described in a previous paper (*6*). Gel-filtration HPLC of the construction products was performed on a column of TSK-GEL G3000SW (0.75×30 cm) (Tosoh) developed with 40 mM glycerophosphate buffer (pH 6.9) containing 25 mM NaCl at a flow rate of 0.4 ml/min. The elution of polypeptides was monitored by tyrosyl fluorescence at 304 nm as described above.

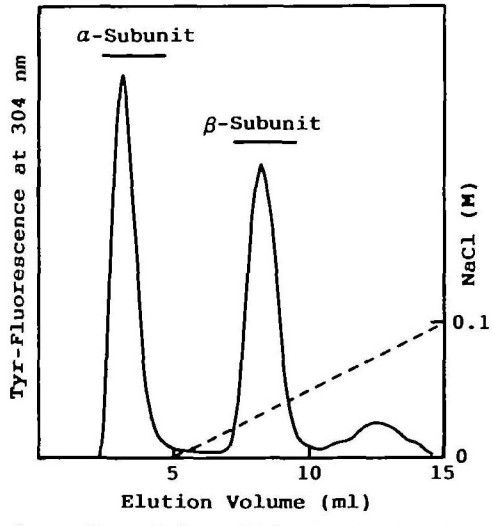

Fig. 1. **Separation of the αAI-2 subunits by DEAE-HPLC under denaturing conditions.** A Cosmosil DEAE column was first eluted with 50 mM Tris-HCl (pH 8.0) containing 8 M urea, then with a linear NaCl gradient from 0 to 0.1 M in the same buffer.

RESULTS

Isolation of αAI-2 Subunits—In a preliminary experiment, SDS-PAGE of αAI-2 after chemical deglycosylation yielded two protein bands, suggesting that αAI-2 may be composed of two different subunits. In this study, an attempt was first made to isolate and purify these subunits easily in sufficient quantity. As shown in Fig. 1, the α- and β-subunits were satisfactorily separated by HPLC on a DEAE column with 33 and 40% weight yields, respectively, based on αAI-2 subjected to subunit dissociation.

SDS-PAGE of αAI-2 Subunits—The subunit preparations obtained by HPLC were submitted to SDS-PAGE. The α-subunit migrated as two bands, a major one and a minor one (Fig. 2, lane b), both of which were positive to the periodic acid-Schiff staining test (data not shown). The β-subunit also gave two bands (Fig. 2, lane c), and the minor one was positive to the same test, whereas the major one was only slightly positive (data not shown). These results indicated that each subunit has a heterogeneous sugar moiety, as in the case of αAI-1 subunits.

αAI-2 subunits were compared with αAI-1 subunits in polypeptide molecular size by SDS-PAGE after chemical deglycosylation treatment (Fig. 2, lanes d, e, and f). The α- and β-subunits showed almost the same mobilities as those of αAI-1, respectively, showing that each subunit of αAI-2 is closely similar in polypeptide size to the corresponding subunit of αAI-1. From this analysis, the polypeptide molecular weights of α- and β-subunits of αAI-2 were estimated to be about 8,000 and 15,000, respectively.

Amino Acid and Sugar Compositions of the Subunits—The chemical compositions of the subunits were determined as summarized in Table I. No tryptophan was detected in the α-subunit and no cysteine in the β-subunit. Valine and lysine contents were much higher in the β-subunit. The α-subunit contained about 27% by weight of carbohydrate, whereas the β-subunit had only 4%. The sugar compositions of these subunits suggested the possibility that the α-subunit has an abundance of high man-

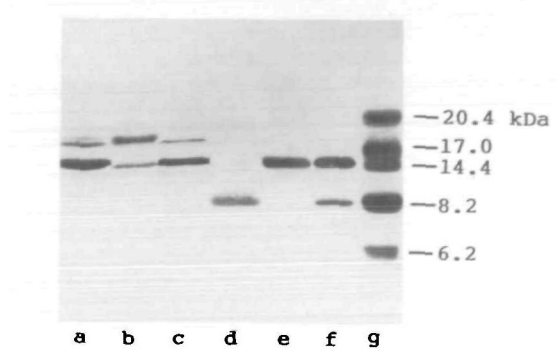

Fig. 2. **SDS-PAGE of αAI-2 and its subunits before and after deglycosylation.** Lane a, αAI-2; lane b, α-subunit; lane c, β-subunit; lane d, deglycosylated α-subunit; lane e, deglycosylated β-subunit; lane f, deglycosylated αAI-1; lane g, molecular weight standards.

nose-type oligosaccharides and the β-subunit contains one short xylomannose-type oligosaccharide chain.

Amino Acid Sequence of α- and β-Subunits—The amino acid sequence of αAI-2 was previously deduced from the nucleotide sequence of the cloned gene (*13*). To establish the complete sequences of the α- and β-subunits, therefore, these subunits were subjected to terminal sequence analyses. As shown in Fig. 3, the N-terminal sequences of the α- and β-subunits were directly determined with the automated sequencer up to the 34th and 24th residues, respectively. The yields of PTH-Ala from the α-subunit and PTH-Ser from the β-subunit in the first cycles were 109 and 13%, respectively. Subsequently, the C-terminal residues of both of the subunits were identified as arginine by hydrazinolysis. The amino acid sequences adjacent to the C-terminal Arg residues of these subunits were in good agreement with the time-courses of the yields of amino

TABLE I. **Amino acid and sugar compositions of the subunits of αAI-2.**

Component	α-subunit (residues/mol of subunit)	β-subunit (residues/mol of subunit)
Asx	9.3 (11)[a]	15.2 (17)
Thr	9.5 (11)	8.1 (9)
Ser	8.3 (10)	19.0 (21)
Glx	4.2 (5)	12.1 (13)
Pro	1.2 (1)	3.8 (4)
Gly	3.4 (3)	5.2 (6)
Ala	3.3 (4)	4.5 (5)
Cys	0.5 (1)	—[b]
Val	3.3 (3)	14.9 (16)
Met	1.5 (2)	0.9 (1)
Ile	3.4 (4)	5.1 (6)
Leu	3.5 (4)	5.2 (6)
Tyr	2.3 (3)	2.6 (3)
Phe	5.1 (6)	6.0 (7)
Lys	0.7 (1)	6.3 (8)
His	0.6 (1)	2.3 (3)
Arg	1.5 (2)	5.9 (7)
Trp	—[b]	2.4 (3)
GlcNAc	3.6	0.8
Man	12.1	2.0
Xyl	1.0	0.4
Fuc	0.5	—[b]

[a]Values in parentheses are taken from the sequence data. [b]Not detected.

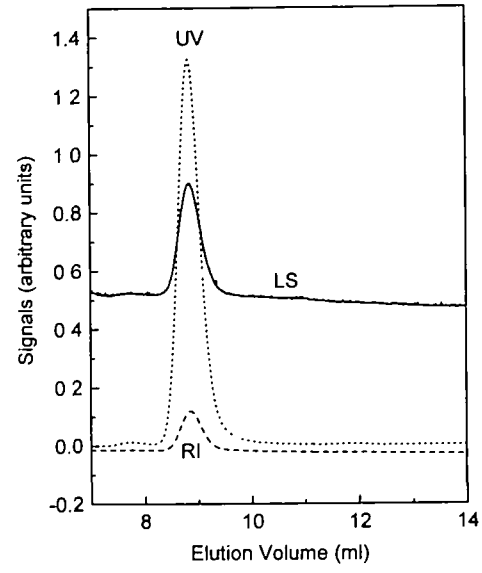

Fig. 4. **SEC elution profile of αAI-2.** αAI-2 (0.1 ml of 0.97 mg/ml) was injected into a Superdex-200 column and the effluent was monitored by absorbance at 280 nm (UV, dotted line), light-scattering (LS, solid line), and refractive index (RI, dashed line).

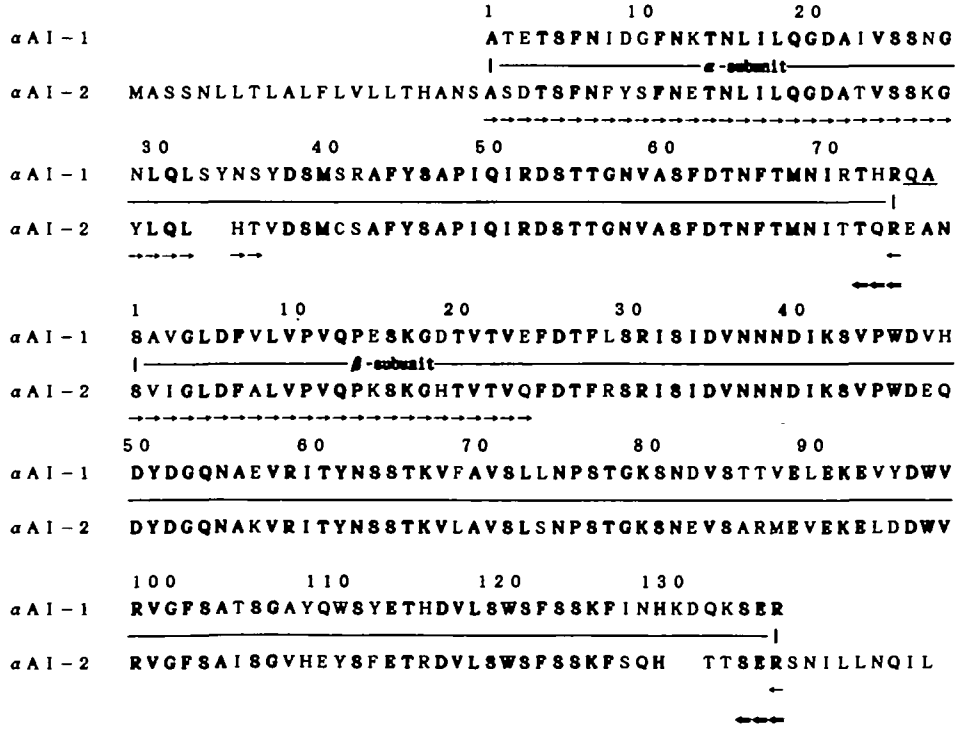

Fig. 3. **Complete amino acid sequences of and sequencing strategies for αAI-2 α- and β-subunits.** The amino acid sequences of the subunits were determined by comparing the amino acid sequence previously deduced from the nucleotide sequence (*13*) with the N- and C-terminal amino acid sequences of the subunits. →, automated Edman degradation; ←, hydrazinolysis; ⇐, carboxypeptidase Y digestion. The sequences obtained are compared with those of the subunits of αAI-1, and residues that are common to the two inhibitors are indicated by boldfaced letters. The underlined sequence is derived from a limited heterogeneity in the C-terminal region of αAI-1 α-subunit (*7*). Residues are numbered according to the sequences of αAI-1 subunits previously determined (*7*).

acids released by carboxypeptidase Y (data not shown). The α- and β-subunits were finally found to be comprised of 72 and 135 amino acids, respectively, and a comparison of αAI-2 with αAI-1 revealed a high degree of identity (78%) in their amino acid sqeuences.

Molecular Weight and Subunit Structure of αAI-2—The polypeptide molecular weight of αAI-2 was estimated by the light-scattering technique. Figure 4 shows the SEC elution patterns on Superdex-200 of αAI-2, *i.e.*, chromatograms for UV absorbance at 280 nm, light-scattering, and refractive index. All three detectors displayed a symmetrical single peak, suggesting that the αAI-2 preparation used is homogeneous. Combining three signals and the extinction coefficients of the α- and β-subunits calculated from their amino acid compositions gave a polypeptide molecular weight of 48,600 for αAI-2 (Table II). This value is nearly twice the sequence molecular weight of the heterodimer α β, *i.e.*, 23,122 (corrected for isotopic composition), demonstrating that αAI-2 is a tetrameric complex $\alpha_2\beta_2$ analogous to the structure recently elucidated for αAI-1 (*7*).

Constructions of Chimeric Inhibitors by Subunit Exchange between αAI-1 and αAI-2—We have previously found that αAI-1 can be reconstituted from the subunits in a 64% yield (*6*). The subunits of αAI-2 were also capable of assembling into an active form in good yield, though each subunit was inactive independently (data not shown). An attempt, therefore, was made to construct chimeric inhibitors by replacing the α- or β-subunits of αAI-2 with the corresponding subunit of αAI-1 with a view to characterizing the subunit assembly further. Despite attempts to control such conditions as protein concentration, pH, and temperature of the reaction solutions, no inhibitory activity against any of the α-amylases was observed with the chimeric products. Nor were oligomeric chimeras detected by gel-filtration HPLC (data not shown).

TABLE II. **Molecular weight of αAI-2 measured by light-scattering.**[a] Molecular weight of αAI-2 determined using the light-scattering data was compared with the sequence molecular weights of the subunits.

Protein	ε[b] (ml/mg·cm at 280 nm)	Theoretical molecular weight from amino acid sequence	Experimental molecular weight from light-scattering
α-subunit	0.48	7,973	
β-subunit	1.38	15,149	
α-subunit+β-subunit	1.07	23,122	
αAI-2			48,600[c]

[a]All molecular weights are given without carbohydrate contributions. [b]Calculated from amino acid compositions as described under "MATERIALS AND METHAODS." [c]Performed using a Superdex-200 column.

DISCUSSION

It is known that αAI-1 inhibits both the activity of the larval α-amylase of *C. chinensis* and porcine pancreatic α-amylase, but not that of *Z. subfascistus*. On the other hand, αAI-2 inhibits only the amylase activity of *Z. subfascistus* (*9*). It is noteworthy that the tetrameric

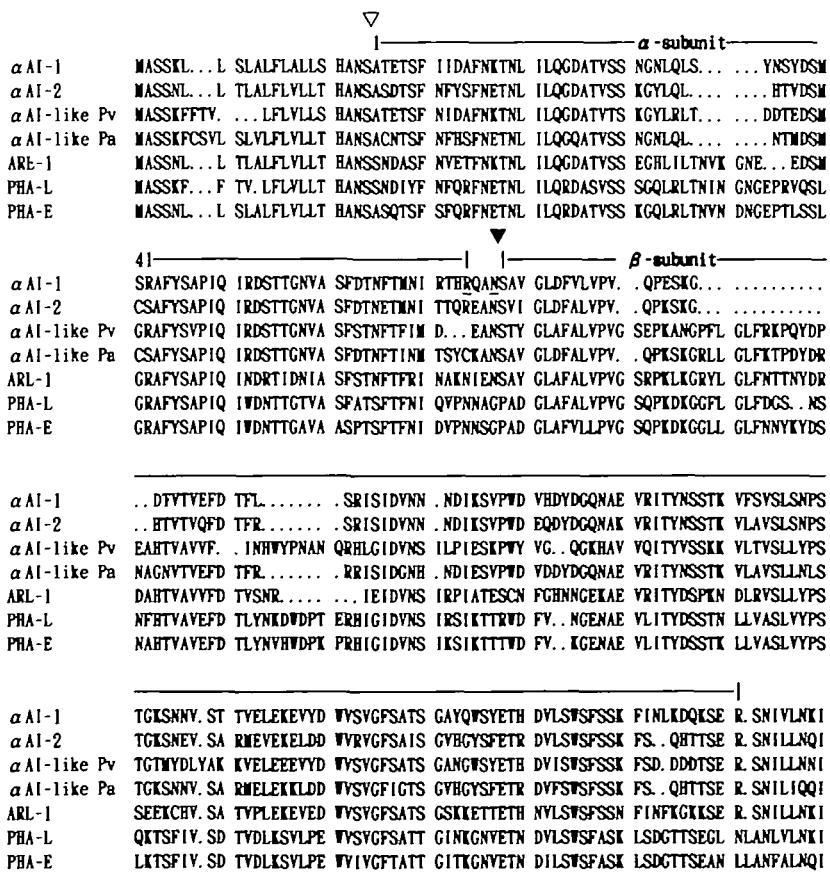

Fig. 5. **Comparison of the deduced amino acid sequences of the genes in the *P. vulgaris* defense proteins, *i.e.*, α-amylase inhibitors (αAI-1 and αAI-2) (*13*), α-amylase inhibitor-like proteins (αAI-like Pv and αAI-like Pa) (*3*), arcelin (ARL-1) (*13*), and lectins (PHA-L and PHA-E) (*13*).** Residues are numbered according to the sequence of αAI-1, starting at the N-terminus of the α-subunit. Arg74 and Asn77 are underlined and gaps are indicated by dots. The open and filled arrows represent the processing sites for removal of the signal peptides and for formation of the oligomeric structures, respectively.

structure $\alpha_2\beta_2$ is common to two leguminous α-amylase inhibitors which are quite different from each other in their inhibitory specificity. This finding, together with the fact that the individual subunits of these inhibitors have no inhibitory activity (*6*), strongly suggests that the post-translational processing of the precursor glycoproteins to form the tetrameric structure is essential for the functions of leguminous α-amylase inhibitors. It may also be noted in connection with the processing that the C-termini of the subunits of αAI-1 and αAI-2 are occupied exclusively by Arg residues (Fig. 3), except for a limited heterogeneity of the C-terminus of the α-subunit of αAI-1 (*7*). Pueyo *et al.* have recently reported that activation of α-amylase inhibitors requires proteolytic processing of the precursors at Asn77 (Fig. 5) and that the defense proteins other than α-amylase inhibitors do not undergo this processing (*14*). Interestingly, it seems likely that the processing at Asn77 needs Arg74 residue for the following reasons: (1) The defense proteins other than α-amylase inhibitors lack Arg74 (Fig. 5). (2) α-Amylase inhibitor-like proteins and arcelin are not processed, in spite of the fact that they have the potential processing site Asn77 (Fig. 5). (3) Although a mutation of Arg74 of αAI-1, which had been viewed as an amino acid residue involved in the active site of αAI-1, abolished the expression of an active inhibitor (*15*), a chemical modification of Arg residues of αAI-1 did not result in its significant inactivation of αAI-1 (Takahashi, T. and Yamaguchi, H., unpublished observations), suggesting that the disappearance of Arg74 seems to have made impossible the processing at Asn77 into an active tetramer. (4) The C-terminus of an α-amylase inhibitor-like protein, *i.e.*, αAI-like Pv (Fig. 5), proved to be arginine by hydrazinolysis, in harmony with the C-terminal residues of the β-subunits of αAI-1 and αAI-2 (data not shown). This finding, when considered with the fact that the C-terminal sequences of *P. vulgaris* lectins, *i.e.*, PHA-L and PHA-E, are isoleucyl-leucine (*16*), supports the idea that an Arg residue is required for the processing. It may well be said, therefore, that the Asn-specific processing protease requires an Arg residue for its hydrolytic action. To confirm this point, it might be fruitful to create mutants having Arg74 from α-amylase inhibitor-like proteins and arcelin.

Assembly of the subunits of αAI-1 and αAI-2 into chimeric oligomers was unsuccessful, in spite of the high degree of sequence homology between these inhibitors. It is probable that most of individual subunit polypeptides fold properly during the renaturation reaction, because the parental inhibitors can be reconstituted in good yields from their denatured subunits. It seems most likely, therefore, that proper association of the folded monomers into a tetrameric complex is strictly controlled by a few amino acid residues on the subunit interfaces and/or by a subtle conformation of each subunit.

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