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A Chimera-Like α-Amylase Inhibitor Suggesting the Evolution of *Phaseolus vulgaris* α-Amylase Inhibitor[1]

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White kidney bean (*Phaseolus vulgaris*) contains two kinds of α-amylase inhibitors, one heat-stable (αAI-s) and one heat-labile (αAI-u). αAI-s has recently been revealed to be a tetrameric complex, $\alpha_2\beta_2$, with two active sites [Kasahara *et al.* (1996) *J. Biochem.* 120, 177–183]. The present study was undertaken to reveal the molecular features of αAI-u, which is composed of three kinds of subunits, α, β, and γ. The γ-subunit, in contrast to the α- and β-subunits that are indistinguishable from the α- and β-subunits of αAI-s, was found to correspond to a subunit of an α-amylase inhibitor-like protein, which has been identified as an inactive, evolutionary intermediate between arcelin and the α-amylase inhibitor in a *P. vulgaris* defense protein family. The polypeptide molecular weight of αAI-u determined by the light-scattering technique, together with the polypeptide molecular weights of the subunits, suggests that αAI-u is a trimeric complex, αβγ. The inhibition of αAI-u by increasing amounts of porcine pancreatic α-amylase (PPA) indicates that an inactive 1:1 complex is formed between αAI-u and PPA. Molecular weight estimation of the complex by the light-scattering technique confirmed that it is a complex of αAI-u with one PPA molecule. Thus it seems probable that αAI-u is an evolutionary intermediate of the *P. vulgaris* α-amylase inhibitor.

Key words: α-amylase inhibitor, α-amylase inhibitor-like protein, evolution, *Phaseolus vulgaris*, subunit structure.

Seeds of the kidney bean (*Phaseolus vulgaris*) contain high levels of a family of defense proteins that include lectin (PHA), arcelin (ARL), and α-amylase inhibitor (αAI) (*1–4*). The genes for these proteins are encoded at a single locus in the *P. vulgaris* genome (*2–5*), 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, showing that they are all highly homologous and fall into three groups representing the three proteins (*2–5*). ARL differs from PHA by having a deletion of one short loop segment (G3) in the sequence, and αAI differs from ARL primarily by the deletion of two additional loop segments (G1 and G2) (Fig. 1). It has been revealed that the activation of αAI requires proteolytic processing of the precursor at Asn77 (in the sequence where the processing occurs) to form a tetrameric structure, $\alpha_2\beta_2$ (*7, 8*), and that defense proteins other than αAI do not undergo this processing (*7*). Finardi-Filho *et al.* (*6*) previously reported the existence of a protein referred to as α-amylase inhibitor–like protein (AIL) with a sequence differing from αAI primarily by a 15–amino acid insert around position 115 (G2) (Fig. 1). On a dendrogram of defense protein sequences, the AIL sequence is most closely related to αAI, suggesting that the AIL gene may be an evolutionary intermediate between ARL and αAI (*6, 9*). Interestingly, ARL and AIL are not proteolytically processed (*5, 6*) and exist in a homodimeric form, despite the fact that they have a potential processing site, Asn77. Information from mutation (*10*) and chemical modification (*11*) studies of the amino acid residues, the X-ray crystal structure (*12*), and comparison of the amino acid sequences of *P. vulgaris* defense proteins (*13*), has suggested that the processing at Asn77 requires an Arg74 residue; *i.e.*, the lack of Arg74 seems to make the processing of ARL and AIL to an active tetramer impossible.

White kidney bean (*P. vulgaris*) contains two kinds of α-amylase inhibitors, one heat-stable (αAI-s)[3] and one heat-labile (αAI-u)[4], in a weight ratio of 7:3 (*14, 15*). Recently, a series of structural studies have been carried out on αAI-s,

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Abbreviations: αAI, *Phaseolus vulgaris* α-amylase inhibitor; αAI-s, heat-stable α-amylase inhibitor of *Phaseolus vulgaris* white kidney bean; αAI-u, heat-labile α-amylase inhibitor of *Phaseolus vulgaris* white kidney bean; AIL, *Phaseolus vulgaris* α-amylase inhibitor–like protein; ARL, *Phaseolus vulgaris* arcelin; Endo F, *Flavobacterium* sp. endo-β-*N*-acetylglucosaminidase F, PHA, *Phaseolus vulgaris* lectin; PPA, porcine pancreatic α-amylase; SEC, size-exclusion chromatography.

[3] Referred to as PHA-I in the previous papers (*8, 11, 14, 23*).

[4] Referred to as PHA-II in the previous paper (*15*).

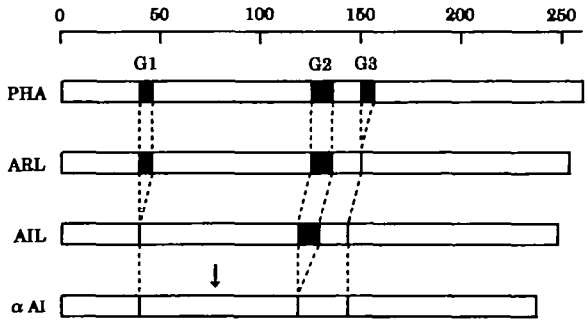

Fig. 1. **Schematic representation of the 4 types of genes at the PHA/ARL/AIL/αAI locus including gaps (G1, G2, and G3) in the amino acid sequence alignments of ARL, AIL, and αAI (*6*).** The scale at the top indicates amino acid position The arrow shows that only αAI is proteolytically processed to form the α- and β-subunits.

which is composed of two kinds of glycopolypeptide subunits, α and β (*8*, *14*). These studies disclosed for the first time that αAI has a tetrameric structure, $\alpha_2\beta_2$, with two independent active sites for α-amylase inhibition (*8*). On the other hand, αAI-u is composed of three kinds of glycopolypeptide subunits, α, β, and γ (*15*). The α- and β-subunits of αAI-u resemble the α- and β-subunits of αAI-s closely in every molecular feature examined, suggesting that these subunits are common to the two inhibitors (*15*). However, the γ-subunit, which seems to be associated with the heat-lability of αAI-u (*15*), has remained unidentified. It would be interesting, therefore, in connection with the evolution as well as the proteolytic processing of αAI, to characterize αAI-u fully. This study shows that the γ-subunit of αAI-u is similar to the subunit of AIL, and that αAI-u is a chimera-like trimer, αβγ, that forms an inactive 1:1 complex with PPA.

MATERIALS AND METHODS

Materials—αAI-u (*15*) and αAI-s (*14*) were purified from white kidney beans (*P. vulgaris*), and AIL (*16*) from Ofuku-5 beans (*P. vulgaris*) as described previously. Their concentrations were calculated using A_{280}[1%/cm] = 11.8 obtained with dried materials. The subunits of αAI-u (*15*) and αAI-s (*14*) were also prepared by the methods described in previous papers. PPA (Type I-A) was purchased from Sigma and its concentration was calculated using A_{280}[1%/cm] = 24.0 and M_r = 52,000 (*17*). Trypsin treated with *N*-tosyl-L-phenylalanyl chloromethyl ketone was also obtained from Sigma, and *Staphylococcus aureus* V8 protease and 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole were from Pierce. Endo-β-*N*-acetylglucosaminidase F (*Flavobacterium* sp.) (Endo F) was a product of Seikagaku Kogyo. Aqueous urea was always treated with ion-exchange resin AG501-X8D (Bio-Rad). Other chemicals used are described in previous papers (*8*, *13*).

Amino Acid and Sequence Analyses—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 *in vacuo*. Amino acid sequence analysis was carried out on a Shimadzu PPSQ-10 gas-phase protein sequencer.

Enzymatic Digestion of the γ-Subunit of αAI-u and Separation of Peptides—The γ-subunit was digested with trypsin at 30°C for 4 h at an enzyme/substrate molar ratio of 1:100 in 50 mM ammonium bicarbonate containing 2 M urea. *S. aureus* V8 protease digestion of a tryptic peptide was performed similarly. The peptides obtained were purified by HPLC on a C_{18} reversed-phase column (Inertsil ODS-3, 4.6 × 250 mm; GL Sciences) eluted with a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid (1%/min), then with 100% acetonitrile in 0.1% trifluoroacetic acid. The elution of the peptides was monitored at 220 nm and the distinct peptide peaks obtained were numbered in the order of their elution.

SEC/Light-Scattering—Size-exclusion chromatography (SEC)/light-scattering experiments were carried out according to the procedures described previously (*18–20*). A SEC column (1 × 30 cm), Superdex-200, was eluted with Dulbecco's phosphate-buffered saline (pH 7.1) at a flow rate of 0.5 ml/min. Stock solutions of PPA (0.86 mg/ml) and αAI-u (0.80 mg/ml) in the same buffer were used. Polypeptide molecular weights (without carbohydrate contributions) were calculated using the signals from the detectors and the extinction coefficients of the protein or complex at 280 nm. Protein concentrations were spectrophotometrically determined using extinction coefficients calculated from the amino acid compositions of the proteins (*21*). The extinction coefficient, ε, of the αAI-u/PPA complex was calculated from the molecular weight and the extinction coefficients ε of each protein in the complex as described previously (*8*).

Isolation of αAI-u/PPA Complexes—A reaction mixture containing 5.1 μM αAI-u and 16 μM PPA, or 15 μM αAI-u and 5.3 μM PPA was incubated in 40 mM glycerophosphate (pH 6.9) (2 ml) containing 25 mM NaCl and 10 mM $CaCl_2$ at 37°C for 1.5 h. The preparation of these complexes was also attempted at protein concentrations 1/20 those of the above solutions. The complexes were separated by gel filtration on a HW-55F column (1.2 × 95 cm) equilibrated and eluted with the same buffer. The effluent fractions (1 ml) were monitored by measuring the absorbance at 280 nm, and the peak fractions were combined for characterization.

Other Methods—The αAI inhibitory activity was assayed exactly as described previously (*14*). SDS-PAGE was done according to the method of Laemmli (*22*) using 12% acrylamide gels. All samples for SDS-PAGE were heated at 100°C for 5 min in 1.7% SDS. The bands were visualized with Coomassie Brilliant Blue R-250. The stained slab gels were scanned on a Shimadzu Chromatoscanner CS-9000 at 595 nm. Parental and chimeric inhibitors were constructed from the α- and β-subunits of αAI-u and αAI-s by the method described in a previous paper (*23*). Chemical deglycosylation of the γ-subunit and AIL was carried out with trifluoromethanesulfonic acid as described previously (*15*). Endo F digestion procedures have also been previously described (*24*).

RESULTS

Characterization of the Subunits of αAI-u—The α- and β-subunits of αAI-u, although their complete amino acid sequences remain to be determined, were previously found to be indistinguishable from the α- and β-subunits of αAI-s, respectively, in every molecular feature examined (*15*). In addition, an attempt to reconstitute αAI-u from its sub-

units showed the combination of the α- and β-subunits to be essential for the acquisition of inhibitory activity, suggesting that the α- and β- subunits are common to the αAI-u and αAI-s inhibitors (*15*). In this respect, it is interesting to see whether the activities of the α-amylase inhibitors are reconstituted from the α- and β-subunits of αAI-u and αAI-s in different subunit combinations. First, a mixture of equimolecular amounts of α- and β-subunits of αAI-u resulted in 56% (mean of three independent measurements) of the specific activity of the native αAI-s upon reconstitution. Similarly two "chimeric" inhibitors reconstituted from α from αAI-u and β from AI-s, and from α from αAI-s and β from αAI-u, showed 58 and 62% of the activity of the native αAI-s, respectively (means of at least three independent measurements). These results, together with the previous finding that the reconstitution of αAI-s from its α- and β-subunits gives about 60% of the activity of the native inhibitor (*23*), strongly support the above suggestion that α- and β-subunits are common to αAI-u and αAI-s.

On the other hand, amino acid analysis showed that the γ-subunit has an amino acid composition more closely similar to the AIL subunit than to the αAI precursor (Table I). To obtain more accurate information about the relationship between the γ-subunit and AIL subunit, the amino acid sequence of the γ-subunit was partially determined. The γ-subunit was digested with trypsin, and thirteen peaks, T1-T13, were well separated on reverse-phase HPLC. The T6 peptide, a long fragment, was further digested with V8 protease, and two major peaks, T6V1 and T6V2, were observed. All of the peptides were subjected to amino acid and N-terminal sequence analyses (HPLC and amino acid analysis data not shown). The amino acid sequences determined for the peptides were aligned by comparing with those previously deduced for the AIL subunit and αAI precursor from their nucleotide sequences. Except for four amino acid residues, the sequence determined for the 240 amino acid residues of the γ-subunit coincided with that of AIL, although two amino acid residues remain to be sequenced (Fig. 2). This result, considered together with the common deletion pattern of their sequences, confirms that the γ-subunit belongs to AIL.

Furthermore, the γ-subunit was compared to the AIL subunit by SDS-PAGE (Fig. 3). The behaviors of the γ-subunit on SDS-PAGE were closely similar to those of the AIL subunit before and after deglycosylation, showing that the carbohydrate content as well as the polypeptide molecular

TABLE I. **Amino acid compositions of the γ-subunit, AIL subunit, and αAI precursor.**

Component	γ-Subunit	AIL subunit[a] (residues/molecule)	αAI precursor[b]
Asx	29 9	31	37
Thr	19.8	20	18
Ser	28.2	26	31
Glx	18 1	18	17
Pro	9.1	10	5
Gly	20 0	17	10
Ala	15 2	16	11
Cys	—[c]	0	0
Val	18.7	20	21
Met	2.7	3	2
Ile	10.7	12	13
Leu	18.3	18	12
Tyr	10.3	11	8
Phe	16 3	14	12
Lys	12.0	11	10
His	4 6	4	4
Arg	6 1	6	7
Trp	4.1	5	4

[a]Values are taken from the sequence data (*25*). [b]Values are taken from the sequence data (*26*). [c]Not detected.

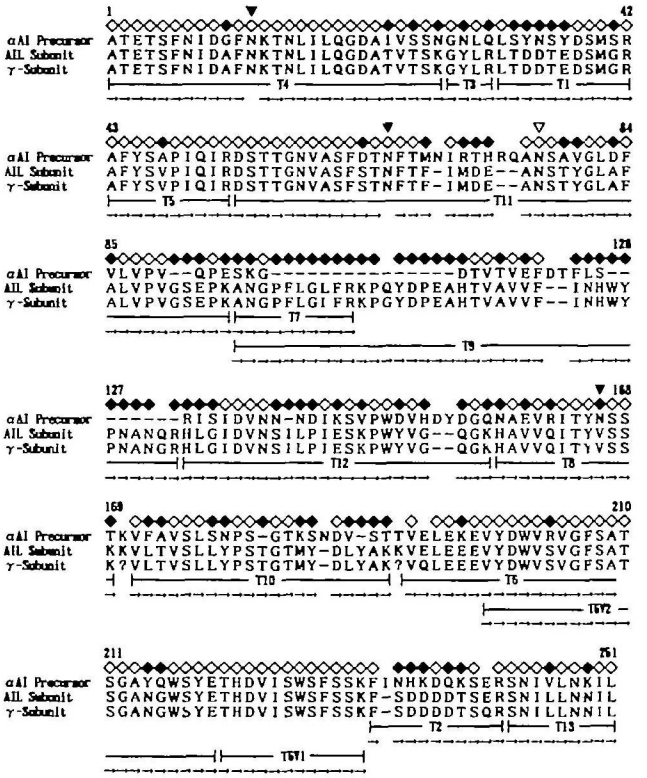

Fig. 2 **Comparison of the amino acid sequences of the peptides from the γ-subunit with the deduced amino acid sequences of the AIL (αAI-3) subunit (*5*) and αAI precursor (*26*) genes.** Residues are numbered according to the sequence starting at the N-terminus after removal of the signal peptide. T1-T13, tryptic peptides, T6V1 and T6V2, V8 protease peptides from T6; →, automated Edman degradation, ?, amino acid residues not determined, ◇, residues common to the three sequences; ◆, residues common only to the γ-subunit and AIL subunit, ▽, processing site Asn77; ▼, *N*-glycosylation site

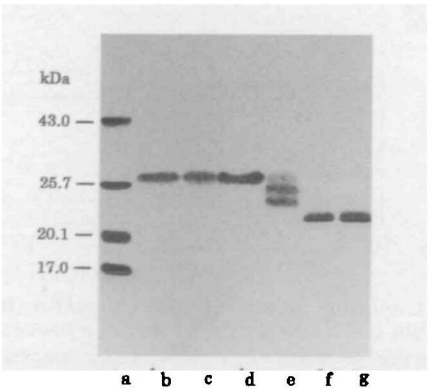

Fig. 3. **Comparison of the γ-subunit and Ofuku-5 bean AIL by SDS-PAGE.** Lane a, molecular weight standards; lane b, AIL; lane c, γ-subunit; lane d, Endo F-treated AIL, lane e, Endo F-treated γ-subunit; lane f, trifluoromethanesulfonic acid-treated AIL; lane g, trifluoromethane-sulfonic acid–treated γ-subunit.

weight resemble each other (lanes b, c, f, and g). However, there was a significant difference between their susceptibilities to Endo F digestion; *i.e.*, the glycans of AIL were completely resistant to Endo F digestion, whereas those of the γ-subunit were partially hydrolyzed under the same conditions (lanes d and e). It is unclear whether the resistance of these glycans to Endo F digestion is due to their structures or to steric hindrance of the polypeptide moiety to enzymatic digestion.

Molecular Weight and Subunit Structure of αAI-u—The polypeptide molecular weight of αAI-u was estimated by the light-scattering technique. Figure 4, panel B, shows the SEC elution patterns on Superdex-200 of αAI-u, *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-u preparation used is homogeneous. The extinction coefficients of the α-, β-, and γ-subunits were calculated from their amino acid compositions (Table II). Since αAI-u is composed of equimolecular amounts of these subunits (*15*), the extinction coefficient of αAI-u was considered to be a weight average of the values of the three polypeptides, *i.e.*, 1.54. Combining the signals and the extinction coefficient of αAI-u gave a polypeptide molecular weight of 44,900 for αAI-u. This value is similar to the sum of the polypeptide molecular weights of each subunit as previously estimated by SDS-PAGE (*15*), suggesting that αAI-u is a trimeric complex, αβγ. However, the extinction coefficient of γ is close to the value of the heterodimer, αβ, and, besides, the polypeptide molecular weights of γ and α plus β are also similar. The possibility, therefore, exists that the observed peak in Fig. 4B might be a mixture of $\alpha_2\beta_2$, αβγ, and γ_2.

Stoichiometry of PPA Inhibition by αAI-u—The inhibitory equivalent of αAI-u to PPA was estimated by measuring the extent of inhibition, using a PPA molecular weight of 52,000 (*17*) and an αAI-u molecular weight of 52,200, obtained by correcting the polypeptide molecular weight of 44,900 for its carbohydrate content (*15*). Figure 5 indicates that an inactive 1:1 complex could be formed between αAI-u and PPA, suggesting that αAI-u is a monovalent inhibitor.

Isolation and Characterization of αAI-u/PPA Complexes—An attempt to isolate αAI-u/PPA complexes by gel-filtration was made in order to gain more insight into the interaction between αAI-u and PPA. Reaction mixtures involving a large excess of PPA (Fig. 6A, top) or αAI-u (Fig. 6A, bottom) were submitted to gel filtration after incubation. The second peaks in Fig. 6A, top and bottom, were found to correspond to free PPA and αAI-u, respectively, by SDS-PAGE (data not shown). The fractions of the first peaks having the same elution volume (designated CI and CII as shown in Fig. 6A, top and bottom, respectively) were combined separately. Both of these complexes, CI and CII,

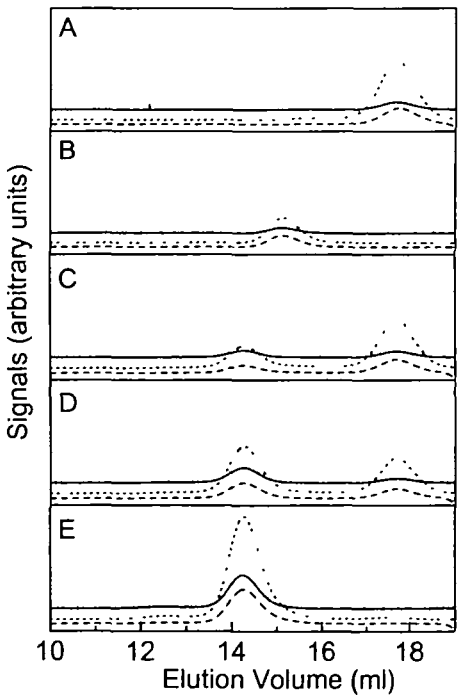

Fig. 4. **SEC elution profiles of PPA (A), αAI-u (B), and mixtures of PPA and αAI-u (C, D, and E).** Stock solutions of PPA and αAI-u were used to prepare individual protein samples and the mixtures PPA and αAI-u were mixed at a molar ratio of 4:1 (C), 2:1 (D), or 1:1 (E) and allowed to complex with each other at 37°C for 90 min. These samples were injected into a Superdex-200 column and the effluent was monitored by absorbance at 280 nm (UV, dotted lines), light-scattering (solid lines), and refractive index (dashed lines). See the text for more details.

TABLE II **Molecular weights of αAI-u and PPA as measured by light-scattering.**[a] The molecular weight of αAI-u determined using the light-scattering data was compared with the molecular weights of the deglycosylated subunits.

Protein	ε^{b} (ml/mg-cm at 280 nm)	Molecular weight from SDS-PAGE[c]	Molecular weight from light-scattering[d]
α-Subunit	0.46	7,800	
β-Subunit	1.89	14,000	
γ-Subunit	1.41	22,000	
α + β + γ (trimer)	1.54	43,800	
αAI-u			44,900
PPA	2.52		53,000

[a]All molecular weights are given without carbohydrate contributions. [b]Calculated from the amino acid compositions as described under "MATERIALS AND METHODS." [c]Quoted from a previous paper (*15*). [d]Performed using a Superdex-200 column.

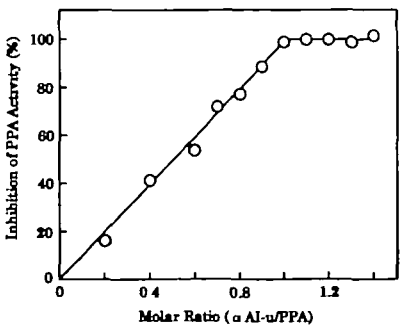

Fig. 5 **Inhibition of PPA by αAI-u.** PPA (0.25 μM) was incubated with increasing amounts of αAI-u in 40 mM glycerophosphate buffer (pH 6.9) (0.1 ml) containing 25 mM NaCl and 10 mM $CaCl_2$ at 37°C for 90 min. After diluting 10-fold with the same buffer, the remaining activity was determined as described previously (*14*). The protein concentrations were calculated using the adsorptibilities and molecular weights of αAI-u and PPA. The αAI-u molecular weight of 52,200 used here was obtained by correcting the polypeptide molecular weight of 44,900 for carbohydrate content (*15*).

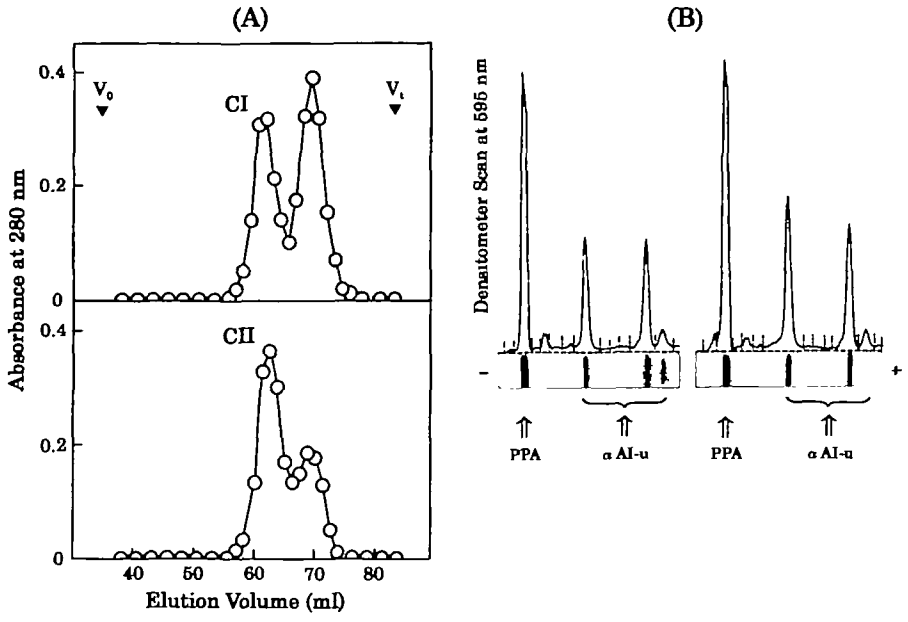

Fig. 6. **Separation and characterization of αAI-u/PPA complexes.** (A) Reaction mixtures containing 5 1 μM αAI-u and 16 μM PPA (top), and 15μM αAI-u and 5.3 μM PPA (bottom) were developed on a TOYO PEARL HW55F column. (B) Complexes, CI (left) and CII (right), separated by gel-filtration were analyzed as described in the text.

exhibited no detectable inhibitory activity and appeared to be homogeneous on native polyacrylamide gel disc electrophoresis at acidic pH (data not shown); but native gel electrophoresis at a higher pH was unsuccessful, due to the unavoidable dissociation of the complexes into their components under alkaline conditions. Lowering the αAI-u and PPA concentrations of the reaction mixtures to 1/20 produced no detectable variations in the gel filtration patterns obtained above, showing that αAI-u binds PPA without any change of its oligomeric structure even in such a dilute solution. To compare the compositions of CI and CII, they were subjected to SDS-PAGE and the band intensities of αAI-u and PPA were estimated by densitometry (Fig. 6B). αAI-u was quantitated by summing the intensities of the three bands derived from the three subunits. The relative band intensities PPA/αAI-u from CI and CII were similar to each other, in agreement with the result from the inhibition test of PPA by αAI-u (Fig. 5).

Molecular Weight of the αAI-u/PPA Complex—In order to obtain corroborating evidence for the monovalent inhibition by αAI-u, an attempt was made to determine the molecular weight of the αAI-u/PPA complex by the SEC/light-scattering technique. Figure 4, panels A and B, show the elution profiles of PPA and αAI-u, respectively, and the molecular weight obtained for PPA was essentially identical to the theoretical value of 52,000 (*17*), indicating that PPA is a monomer (Table II).

Next, the binding of PPA to αAI-u was examined at different molar ratios by the SEC/light-scattering technique (Fig. 4, panels C, D, and E). When PPA was mixed with αAI-u in a molar ratio of 1:1, a single symmetrical peak eluting earlier than either component was observed, and neither PPA nor αAI-u was detected as a free form, suggesting that the αβγ complex (or other combinations, as described above) binds only one PPA molecule. As the ratio of PPA to αAI-u was increased, a peak corresponding to free PPA was observed. At the ratios tested, however, the peak corresponding to the complex of αAI-u with PPA eluted at an identical position. The molecular weight, 98,000, calculated for this complex agrees closely with a total mass of one molecule each of PPA and $\alpha\beta\gamma$, although the complex could contain one PPA molecule per $\alpha_2\beta_2$ or γ_2.

DISCUSSION

Seed defense proteins that accumulate in the protein storage vacuoles are synthesized in the endoplasmic reticulum as proproteins. After removal of the signal peptide and glycosylation at one or more Asn sites, they are transported to the protein storage vacuoles. The arrival of the *P. vulgaris* α-amylase inhibitor at the vacuoles is followed by two rounds of proteolytic processing at the linkages, including the carboxyl groups of Asn77 and Arg214 (*7*, *13*), to form an active tetrameric $\alpha_2\beta_2$. In this study, amino acid sequencing revealed that the γ-subunit of αAI-u corresponds to the subunit of AIL that can not be processed at Asn77. Since αAI-u is known to be composed of equimolecular amounts of the α-, β-, and γ-subunits (*15*), and the polypeptide molecular weights of these subunits are also known (*15*), the estimation of the polypeptide molecular weight of αAI-u must reveal its oligomeric structure. Light-scattering photometry, which is applicable to the estimation of the polypeptide molecular weights of glycoproteins (*27*), clearly showed αAI-u to have a molecular weight very close to the sum of the polypeptide molecular weights of each subunit as estimated by SDS-PAGE. This result, however, can not rule out the possibility that αAI-u is a mixture of $\alpha_2\beta_2$, $\alpha\beta\gamma$, and γ_2, because the subunits all resemble each other in molecular weight. In the previous paper, we showed that αAI-s, $\alpha_2\beta_2$, can bind two PPA molecules simultaneously and that the αβ heterodimer has an independent PPA binding site (*8*). In addition, the α- and β-subunits seem to be common to both αAI-u and αAI-s, and, furthermore, γ_2 is considered unable to bind PPA (*15*). The molecular weight determination of the αAI-u/PPA complex by the SEC/light-scattering technique, therefore, excludes the possibility that the complex is a mixture of $\alpha_2\beta_2$, $\alpha\beta\gamma$, and γ_2, because $\alpha_2\beta_2$ complexed with two PPA molecules should be eluted ear-

lier, and γ_2 later, than the complex comprised of one molecule each of PPA and αAI-u. These results, therefore, are most consistent with the interpretation that αAI-u has the subunit stoichiometry αβγ, and that only the αβ heterodimer is capable of binding PPA. To our knowledge, this is the first demonstration of a chimera-like trimer of an α-amylase inhibitor.

αAI-u does not seem characteristic of white kidney bean for the following reasons. Frels and Rupnow previously purified two kinds of α-amylase inhibitor from *P. vulgaris* black beans and found significant differences in their complexation with PPA, as well as in their thermostabilities (*28*). In addition, it should be noted that the purification of αAI from different varieties of kidney bean has generally been performed by a series of procedures including heat treatment. As pointed out by Frels and Rupnow (*28*), the heat treatment used to coagulate heat-labile proteins is validly thought to destroy the αAI-u in beans. It is possible, therefore, that αAI-u, along with αAI-s, may be widespread among varieties of kidney bean. In view of the results and arguments presented here, it may feasibly be concluded that αAI-u is an evolutionary intermediate between ARL and αAI.

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