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Complete Sequence, Subunit Structure, and Complexes with Pancreatic α-Amylase of an α-Amylase Inhibitor from *Phaseolus vulgaris* White Kidney Beans

Keiji Kasahara,[*] Kaeko Hayashi,[†] Tsutomu Arakawa,[‡] John S. Philo,[‡] Jie Wen,[‡] Saburo Hara,[†] and Haruki Yamaguchi[*,1]

[*]Department of Applied Biological Chemistry, College of Agriculture, Osaka Prefecture University, Gakuen-cho 1-1, Sakai, Osaka 593; [†]Department of Chemistry and Materials Technology, Faculty of Engineering and Design, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606; and [‡]Amgen Inc., Thousand Oaks, California 91320-1789, USA

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The complete amino acid sequence of a white kidney bean (*Phaseolus vulgaris*) α-amylase inhibitor (PHA-I), which is composed of two kinds of glycopolypeptide subunits, α and β, was established by conventional methods. The polypeptide molecular weight of PHA-I determined by the light-scattering technique, considered together with the sequence molecular weights revealed for the subunits, indicated that PHA-I has the subunit stoichiometry of $(\alpha\beta)_2$ complex. Inhibition test of PHA-I with increasing amounts of porcine pancreatic α-amylase (PPA) suggested that an inactive 2 : 1 complex is formed between PPA and PHA-I. In fact, two complexes differing from each other in the molar ratio of PPA to PHA-I were separated by gel filtration, and molecular weight estimation by the light-scattering technique confirmed that they are complexes of PHA-I with one or two PPA molecules. The binding of PPA to PHA-I appeared to follow simple binomial statistics, suggesting that two binding sites on PHA-I are independent and of high affinity for PPA.

Key words: α-amylase inhibitor, complete structure, inhibition stoichiometry, kidney bean, subunit structure.

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α-Amylase inhibitors from different varieties of the kidney bean (*Phaseolus vulgaris*) have received attention, mainly because of their unique specificity: they are inhibitory only toward insect and animal α-amylases (*1, 2*), suggesting their role in the defense of the starch-rich seeds from attack by insect larvae by inhibiting their α-amylases. Although the biochemical properties of legume α-amylase inhibitors have been studied for over half a century, only a little is known about their structural features, and their inhibition mechanism remains unknown. The amino acid sequence of an α-amylase inhibitor from common bean (*P. vulgaris*) was previously deduced from the nucleotide sequence of the cloned gene (*3, 4*), and the involvement of tryptophan, arginine, and tyrosine residues in its active site was recently suggested based mainly on a three-dimensional model of legume lectins (*5*), which have high homology to legume α-amylase inhibitors. The complete amino acid sequence, however, has not been determined for any legume α-amylase inhibitor. Nor has convincing information been presented on the α-amylase inhibition stoichiometry.

The white kidney bean (*P. vulgaris*) is known to produce two α-amylase inhibitors of different thermostability, and these have been partially characterized (*6-9*). The major inhibitor (PHA-I) of the white kidney bean is composed of two kinds of glycopolypeptide subunits, α and β, which are noncovalently bonded with each other (*6*). In this study, with a view to providing a basis essential for understanding the inhibition mechanism of PHA-I, we first undertook to determine the complete amino acid sequence of PHA-I. Molecular weight estimation of PHA-I by size-exclusion chromatography (SEC)/light-scattering technique, considered together with the sequence molecular weights of α- and β-subunits, demonstrated that PHA-I is a tetrameric complex $(\alpha\beta)_2$. Inhibition test of PHA-I with porcine pancreatic α-amylase (PPA) suggested that an inactive 2 : 1 complex is formed between PPA and PHA-I; and complexes of PHA-I with one or two molecules of PPA were actually prepared and characterized.

MATERIALS AND METHODS

Materials—PHA-I was purified from white kidney beans (*P. vulgaris*) as described previously (*6*), and its concentration was calculated using A_{280} [1%/cm] = 11.8 obtained with dried material. The α- and β-subunits of PHA-I were also prepared by the method described in a previous paper (*9*). 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 (*10*). Trypsin and chymotrypin, treated with *N*-tosyl-L-phenylalanyl chloromethyl ketone and N^{α}-tosyl-L-lysyl chloromethyl ketone, respectively, were also from Sigma, and lysyl endopeptidase from *Achromobacter*

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[1] To whom correspondence should be addressed.

Abbreviations: PHA-I, α-amylase inhibitor of *Phaseolus vulgaris* white kidney bean; PPA, porcine pancreatic α-amylase; SEC, size-exclusion chromatography.

lyticus M497-1 was a product of Wako Pure Chem. *Staphylococcus aureus* V8 protease and 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole were from Pierce.

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. The carboxyl-terminal amino acid residues were determined by hydrazinolysis according to the method of Fraenkel-Conrad and Tsung (*11*).

Enzymatic Digestions of Polypeptides—The α-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. Chymotrypsin digestion of α- and β-subunits was performed similarly, but at an enzyme/substrate molar ratio of 1 : 200. Lysyl endopeptidase digestion of the β-subunit was carried out 37°C for 4 h at an enzyme/substrate molar ratio of 1 : 800 in 50 mM Tris-HCl buffer (pH 9.1) containing 2 M urea. The β-subunit was also digested with V8 protease at 30°C for 4 h at an enzyme/substrate molar ratio of 1 : 100 in 50 mM ammonium bicarbonate containing 2 M urea.

Separation of Peptides—The peptides obtained were purified by HPLC (Irica 852) on a C_{18} reversed-phase column (Develosil ODS-5, 4.6×150 mm; Nomura Chemical) developed with a linear gradient of acetonitrile (1%/min) containing 0.1% trifluoroacetic acid or 10 mM ammonium acetate (pH 5.6) at a flow rate of 1 ml/min. The elution of peptides was monitored at 220 nm and the peptides obtained were numbered in the order of their alignment from the amino terminus according to the established sequence.

SEC/Light-Scattering—SEC/light-scattering experiments were carried out according to the procedures described previously (*12-14*). The SEC columns (1×30 cm), Superdex-75 or Superdex-200, were run with Dulbecco's phosphate-buffered saline (pH 7.1) at a flow rate of 0.5 ml/min. Stock solutions of PPA (2.99 mg/ml) and PHA-I (2.99 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 coefficient 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 (*15*). Extinction coefficients, ε, of complexes, AmBn, were calculated using

$$\varepsilon = \frac{m\varepsilon_A M_A + n\varepsilon_B M_B}{mM_A + nM_B}$$

from the molecular weight M and the extinction coefficient ε of each protein, A (*e.g.*, PPA) and B (*e.g.*, PHA-I), in the complex.

PHA-I and PPA Assays—The PHA-I inhibitory activity was assayed exactly as described previously (*6*). The activity of PPA (65 ng) was determined using soluble starch (2.0 mg in 0.5 ml of 40 mM sodium phosphate buffer (pH 6.9) containing 25 mM NaCl and 10 mM $CaCl_2$). The reaction was allowed to proceed at 37°C and stopped by adding 1 M acetic acid (0.5 ml) after 10 min. After mixing with the iodine reagent (*16*) (0.5 ml), the reaction solution was allowed to stand at room temperature for 25 min and its absorbance at 680 nm was measured.

Isolation of PPA-PHA-I Complexes—To prepare a 1 : 1 complex of PPA and PHA-I, 30 μM PPA was incubated with 100 μM PHA-I in 40 mM sodium phosphate buffer (pH 6.9) (0.3 ml) containing 25 mM NaCl and 10 mM $CaCl_2$ at 37°C for 1.5 h. A 2 : 1 complex was prepared by incubating a mixture of 132 μM PPA and 32 μM PHA-I under the same conditions. Preparation of these complexes was also attempted at protein concentrations of 1/20 those in 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.

Polyacrylamide Gel Electrophoresis—Polyacrylamide disc gel electrophoresis under native conditions was performed using 7% polyacrylamide gels (pH 4.3) in the cold as described by Reisfeld *et al.* (*17*). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli (*18*) 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.

RESULTS

Amino Acid Sequences of α- and β-Subunits—First, the N-terminal sequence of the α-subunit was directly determined with the automated sequencer up to the 42nd residue as shown in Fig. 1A. Subsequently, two C-terminal residues were identified as arginine and alanine by hydrazinolysis, indicating heterogeneity in the C-terminal region. Trypsin digestion of α-subunit yielded five major peptides, T1-T5, which were subjected to amino acid and N-terminal sequence analyses (Fig. 1A) (HPLC and amino acid analysis data not shown). On the Edman degradation of T1 and T4, no phenylthiohydantoin amino acids were detected in the 12th and 13th cycles, respectively. The sequences around the 12th residue of T1 and 13th residue of T4, XXX-Lys-Thr and XXX-Phe-Thr, suggested that these sequences correspond to the consensus sequence Asn-X-Ser/Thr (where X is any amino acid except proline and perhaps aspartic acid) and that both amino acid residues represented by XXX are asparagine with a carbohydrate chain. Since α-subunit is known to have two Asn-linked sugar chains on its polypeptide (*6, 7*), there is little doubt that these sequences represent the *N*-glycosylation sites of α-subunit. The alignment of the tryptic peptides was established by overlapping the amino acid sequences of chymotryptic peptides. Four chymotryptic peptides, C1-C4, isolated by HPLC were analyzed for amino acid composition and sequenced. On the basis of these sequences, the order of the tryptic peptides was determined as shown in Fig. 1A. In addition, the sequences of peptides C3 and C4 clearly explained the heterogeneity at the C-terminal region of the α-subunit. Thus, the complete amino acid sequence of the α-subunit was determined as shown in Fig. 1A, and two *N*-glycosylation sites were found in the sequencing process.

Next, the N-terminal amino acid sequence of the β-sub-

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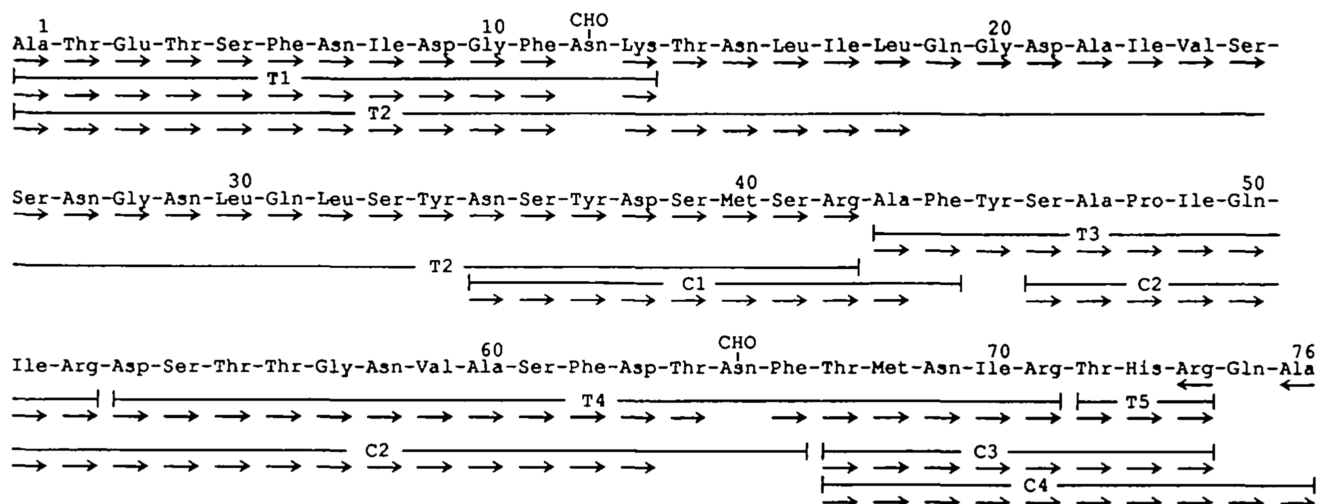
(A)



(B)

1 10 20
Ser-Ala-Val-Gly-Leu-Asp-Phe-Val-Leu-Val-Pro-Val-Gln-Pro-Glu-Ser-Lys-Gly-Asp-Thr-Val-Thr-Val-Glu-Phe-
L1 L2
V1 V2

30 40 50
Asp-Thr-Phe-Leu-Ser-Arg-Ile-Ser-Ile-Asp-Val-Asn-Asn-Asn-Asp-Ile-Lys-Ser-Val-Pro-Trp-Asp-Val-His-Asp-
L2 L3
V3

60 CHO 70
Tyr-Asp-Gly-Gln-Asn-Ala-Glu-Val-Arg-Ile-Thr-Tyr-Asn-Ser-Ser-Thr-Lys-Val-Phe-Ala-Val-Ser-Leu-Leu-Asn-
L3 L4
V4
β-C1

80 90 100
Pro-Ser-Thr-Gly-Lys-Ser-Asn-Asp-Val-Ser-Thr-Thr-Val-Glu-Leu-Glu-Lys-Glu-Val-Tyr-Asp-Trp-Val-Arg-Val-
L5 L6
V5
β-C1

110 120
Gly-Phe-Ser-Ala-Thr-Ser-Gly-Ala-Tyr-Gln-Trp-Ser-Tyr-Glu-Thr-His-Asp-Val-Leu-Ser-Trp-Ser-Phe-Ser-Ser-
L6
V6

130 137
Lys-Phe-Ile-Asn-His-Lys-Asp-Gln-Lys-Ser-Glu-Arg
L7
V6

Fig. 1. **Complete amino acid sequences and sequencing strategies of PHA-I α- (A) and β-subunits (B).** "CHO" denotes *N*-glycosylation site. →, automated Edman degradation; ←, hydrazinolysis.

unit was directly determined with the sequencer up to the 25th residue, phenylalanine (Fig. 1B). The C-terminal amino acid residue of the β-subunit was identified as arginine by hydrazinolysis. When digested with lysyl endopeptidase, the β-subunit gave seven peptides, L1-L7, and they were analyzed for composition and sequenced

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(Fig. 1B). To align the lysyl endopeptidase-digested peptides, the β-subunit was digested with V8 protease and the resulting peptides were separated by reversed-phase HPLC. Six peptides, V1-V6, thus obtained were analyzed for composition and sequenced (Fig. 1B). In sequencing of peptide V4, no phenylthiohydantoin derivative was detected at the 6th cycle and the sequence around the 6th residue of V4, XXX-Ser-Ser, proved to be a candidate for the consensus sequence Asn-X-Ser/Thr. This finding, together with the fact that the β-subunit has an Asn-linked sugar chain (*6, 7*), indicates that the 6th residue of V4 is probably the *N*- glycosylation site. To complete the sequencing of β-subunit, chymotrypsin digestion of the β-subunit was performed and the sequence of peptide β-C1 was found to overlap peptides L5 and L6 (Fig. 1B). The determined complete amino acid sequence of β-subunit is shown in Fig. 1B along with the sequencing strategy.

Molecular Weight and Subunit Structure of PHA-I—Figure 2 shows the SEC elution patterns on Superdex-75 of PHA-I, *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 PHA-I 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 PHA-I (Table I). This value is exactly twice the sequence molecular weight of the heterodimer $\alpha\beta$, *i.e.*, 23,705 (corrected for

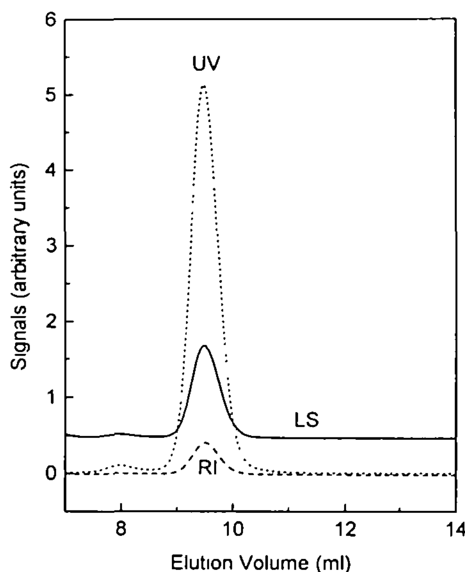

Fig. 2. **SEC elution profile of PHA-I.** PHA-I (0.1 ml of 2.99 mg/ml) was injected into a Superdex-75 column and the effluent was monitored by absorbance at 280 nm (UV, dotted line), light-scattering (LS, solid line), and refractive index (RI, dashed line).

TABLE I. **Molecular weights of PHA-I and PPA measured by light-scattering.**[a] Molecular weights of PHA-I and PPA determined using the light-scattering data were compared with their sequence molecular weights.

Protein	ε^b (ml/mg·cm at 280 nm)	Theoretical molecular weight from amino acid sequence	Experimental molecular weight from light-scattering
α-Subunit	0.47	8,301	
β-Subunit	1.92	15,404	
α-Subunit+β-Subunit	1.41	23,705	
PHA-I			48,600[c] 47,000[d]
PPA	2.52	51,300	50,000[d]

[a]All molecular weights are given without carbohydrate contributions. [b]Calculated from amino acid compositions as described under "MATERIALS AND METHODS." [c]Performed using a Superdex-75 column. [d]Performed using a Superdex-200 column.

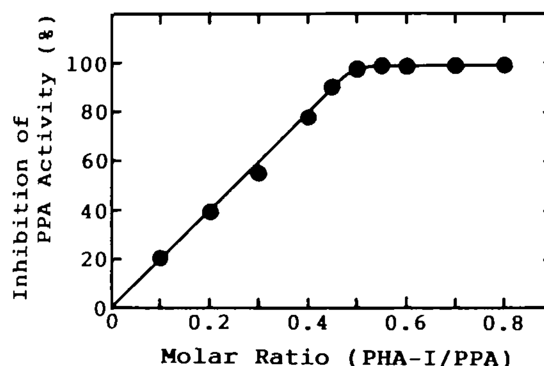

Fig. 3. **Inhibition of PPA by PHA-I.** PPA (5 μM) was incubated with increasing amounts of PHA-I in 40 mM sodium phosphate buffer (pH 6.9) (0.1 ml) containing 25 mM NaCl and 10 mM $CaCl_2$ at 37°C for 30 min. After diluting 200-fold with the same buffer, the remaining activity was determined as described under "MATERIALS AND METHODS." The protein concentrations were calculated using the adsorptivities and molecular weights of PPA and PHA-I. The PHA-I molecular weight 56,400 used here was obtained by correcting the polypeptide molecular weight 47,400 for its carbohydrate content (*6, 7*).

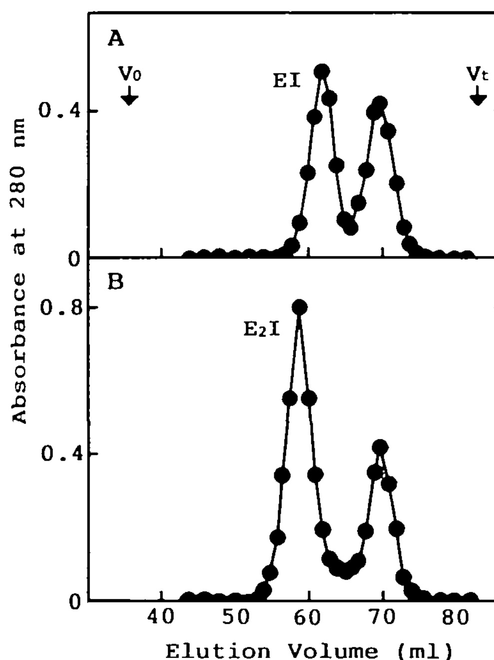

Fig. 4. **Separation of PPA-PHA-I complexes by gel filtration.** Reaction mixtures (0.3 ml) containing 30 μM PPA and 100 μM PHA-I (A), and 132 μM PPA and 32 μM PHA-I (B), were developed on a TOYO PEARL HW55F column.

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the heterogeneity of the α-subunit and for isotopic composition), demonstrating that PHA-I is a tetrameric complex, $(\alpha\beta)_2$, in solution.

Stoichiometry of PPA Inhibition by PHA-I—The inhibitory equivalent of PHA-I to PPA was estimated by measurement of the inhibition extent, using the PPA molecular weight 52,000 (*10*) and the PHA-I molecular weight of 56,400 obtained by correcting the polypeptide molecular weight of 47,400 for its carbohydrate content (*6, 7*). Figure 3 indicates that an inactive 2 : 1 complex could be formed between PPA and PHA-I, suggesting that PHA-I is a bivalent inhibitor.

Isolation and Characterization of PPA-PHA-I Complexes—Attempts to isolate PPA-PHA-I complexes by gel filtration were made in order to get more insight into the interaction between PPA and PHA-I. Reaction mixtures involving a large excess of PHA-I (Fig. 4A) or PPA (Fig. 4B), were submitted to gel filtration after incubation. The second peaks in Fig. 4, A and B, were found to correspond to free PHA-I and PPA, respectively, by SDS-PAGE (data not shown). The fractions of the first peaks (designated EI and E_2I as shown in Fig. 4, A and B, respectively) were combined separately. These complexes, EI and E_2I, appeared to be homogeneous on native polyacrylamide gel disc electrophoresis at an acidic pH (data not shown); but the native gel electrophoresis at a higher pH was unsuccessful, owing to the unavoidable dissociation of the complexes into their components under alkaline conditions. Lowering the PPA and PHA-I concentrations of the reaction mixtures to a 1/20 level produced no detectable variations in the gel filtration patterns obtained above, showing that PHA-I binds PPA without any change of its oligomeric structure even in such a dilute solution.

Complex E_2I exhibited no detectable inhibitory activity, whereas complex EI still retained about 50% of the activity of the native PHA-I on a molar basis. To compare the compositions of E_2I and EI, they were submitted to SDS-PAGE and the band intensities of PHA-I and PPA were estimated by densitometry (Fig. 5). PHA-I was quantitated by summing the intensities of the two bands, which had been separated from each other owing mainly to the heterogeneity of the *N*-glycan chains (*6*). The relative band intensity PPA/PHA-I from E_2I was exactly twice that from EI, in agreement with the result from the inhibition test of PHA-I with PPA (Fig. 3).

Molecular Weights of PPA-PHA-I Complexes—To obtain corroborating evidence for the bivalent inhibition by PHA-I, an attempt was made to determine the molecular weights of the PPA-PHA-I complexes. We first looked at the molecular weights of PHA-I and PPA by the same technique as described above, but with Superdex-200. Figure 6, panels A and B, show the elution profiles of PHA-I and PPA, respectively. PHA-I exhibited a molecular weight closely similar to that obtained with Superdex-75, and the molecular weight obtained for PPA was essentially identical to the theoretical one, indicating that PPA is a monomer (Table I).

Next, PPA and PHA-I were mixed in a molar ratio of 2 : 1 and allowed to complex with each other. The elution profile of this mixture, shown in Fig. 6C, demonstrated a single symmetrical peak eluting earlier than either component alone, indicating formation of a complex. The absence of peaks corresponding to each individual protein suggests that both proteins were eluted as a 2 : 1 complex. To calculate the molecular weight of this complex from the three signals, we need to calculate the protein concentration from the absorbance trace and hence we also need the extinction coefficient of this complex. The extinction coefficient, however, depends on the stoichiometry of the complex, which we are now determining. This dilemma was solved by assuming the stoichiometry, *i.e.*, the extinction coefficient of the complex. Based on the assumed stoi-

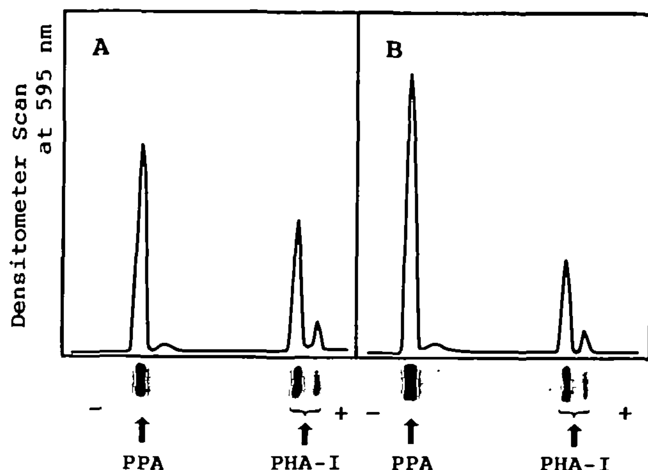

Fig. 5. **SDS-PAGE of PPA-PHA-I complexes, EI (A) and E_2I (B).** Each complex (9 μg) purified by gel filtration on HW55F was analyzed as described in the text and the band intensity was quantitated with a densitometer.

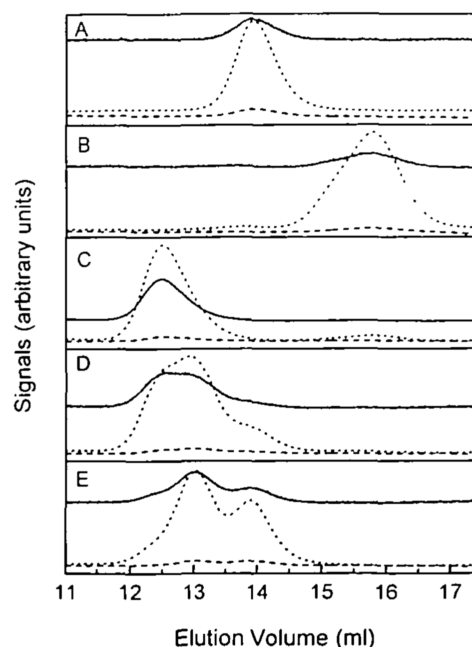

Fig. 6. **SEC elution profiles of PHA-I (A), PPA (B), and mixtures of PPA and PHA-I (C, D, and E).** The stock solutions of PPA and PHA-I were used to prepare individual protein samples and the mixtures. PPA and PHA-I was mixed at a molar ratio of 2 : 1 (C), 1 : 1 (D), or 1 : 2 (E) and allowed to complex each other at 37°C for 1.5 h. These samples were injected into a Superdex-200 column. Lines are as defined in Fig. 4.

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TABLE II. **Molecular weights of PPA-PHA-I complexes measured by light-scattering.** Experimental molecular weights of the complexes were obtained from light-scattering signals, using the extinction coefficients calculated on the basis of assumed stoichiometries of the complexes. In order to find a valid assumption, theoretical molecular weights were also calculated based on the assumed stoichiometries.

Assumed stoichiometry	ε[a] (ml/mg·cm at 280 nm)	Theoretical molecular weight from amino acid sequence	Experimental molecular weight from light-scattering	Valid assumption?
Complex from a mixture of PPA and PHA-I at a 2 : 1 molar ratio				
1 PPA : 1 PHA-I (dimer)	1.99	98,700	167,000	No
2 PPA : 1 PHA-I (trimer)	2.17	150,000	154,000	Yes
3 PPA : 1 PHA-I (tetramer)	2.26	201,300	148,000	No
Complex from a mixture of PPA and PHA-I at a 1 : 2 molar ratio				
1 PPA : 1 PHA-I (dimer)	1.99	98,700	99,300	Yes
2 PPA : 1 PHA-I (trimer)	2.17	150,000	91,100	No
3 PPA : 1 PHA-I (tetramer)	2.26	201,300	88,500	No

[a]Calculated as described under "MATERIALS AND METHODS."

chiometry, we calculated the theoretical molecular weight, and based on the assumed extinction coefficient, we calculated the experimental molecular weight. It is evident from Table II that only the complex composed of two PPA molecules and one PHA-I molecule gives a consistent result between theoretical and experimental molecular weights.

When a mixture of equimolecular amounts of PPA and PHA-I was analyzed, light-scattering data gave an elution profile comprised of three peaks (Fig. 6D). The last peak is eluted at the position of free PHA-I, and the first peak is similar in elution position and molecular weight from light-scattering analysis to the peak shown in panel C; *i.e.*, it is a 2 : 1 complex. Because of the formation of this complex from a 1 : 1 mixture, the excess PHA-I seemed to elute as a free form. The second peak, which has higher absorbance (dotted line) but lower light-scattering (solid line) than the first peak, seems to correspond to a complex comprising one molecule each of PPA and PHA-I.

Further, when PPA was mixed with PHA-I in a molar ratio of 1 : 2, a marked decrease in the 2 : 1 complex accompanying a significant increase in the free PHA-I was observed, as was expected (Fig. 6E). The major peak was eluted at the position of the second peak in Fig. 6D, suggesting a predominant formation of a complex comprising one molecule each of PPA and PHA-I. As in the case of the 2 : 1 mixture of PPA and PHA-I, the theoretical and experimental molecular weights of this complex were calculated based on the assumed stoichiometry. As summarized in Table II, only the stoichiometry of one molecule each of PPA and PHA-I clearly offered a consistent result between theoretical and experimental molecular weights, confirming the composition suggested for the second peak in Fig. 6D.

DISCUSSION

The establishment of the primary structures of these subunits provided a basis essential for developing subsequent studies: the precise compositions and sequence molecular weights of both of the subunits are indispensable for elucidation of the subunit structure of and of the stoichiometry of PPA inhibition by PHA-I.

One of the authors (H.Y.) previously suggested a heterodimeric $\alpha\beta$ structure for PHA-I using a chemical cross-linking technique (*19*). However, in studying the complexing of PHA-I and PPA, we obtained data which appeared to be inconsistent with the dimeric structure of PHA-I, leading us to reinvestigate the oligomeric structure of PHA-I. Since PHA-I is known to be composed of α- and β-subunits in a molar ratio of 1 : 1 (*6, 9*), and the sequence molecular weights of these subunits are also now known, estimation of the polypeptide molecular weight of PHA-I must reveal its oligomeric structure. Light-scattering photometry, which is applicable to estimation of the polypeptide molecular weight of glycoproteins (*20*), clearly presented PHA-I with an integral multiple of the sequence molecular weight of a heterodimer $\alpha\beta$, as might have been expected (Table I). To our knowledge, this is the first demonstration of the tetrameric structure of an α-amylase inhibitor. The dimeric structure $\alpha\beta$ previously suggested for PHA-I was based on the observation that only one cross-linked species was produced and accumulated as the cross-linking reaction proceeded (*19*). Since the cross-linking reaction, as compared with the light-scattering analysis, was carried out at a significantly lower concentration of PHA-I, the possibility exists that PHA-I might dissociate into dimers during the reaction. This possibility, however, was ruled out by the gel filtration patterns from PPA-PHA-I complexing at such a low PHA-I concentration. It appears, therefore, that the chemical cross-linking between α- and β-subunits to form a heterodimer $\alpha\beta$ proceeded relatively easily, whereas the cross-linking between these dimers to form a tetramer was hardly possible, owing to the selective cross-linking between particular subunits by the cross-linker. The close similarity of amino acid composition, N-terminal sequence, SDS-PAGE pattern, *etc.* to those of PHA-I suggests that the α-amylase inhibitors from other varieties of *P. vulgaris* kidney beans (*2, 21-25*) also have a tetrameric structure similar to PHA-I.

The finding that PHA-I has a tetrameric structure aroused our interest in the stoichiometry of PPA inhibition by PHA-I. The result from the inhibition test of PHA-I with increasing amounts of PHA-I is consistent with the suggestion previously offered on the basis of the difference spectra observed for the binding of PPA and PHA-I.[2] Other workers, however, have reported an equimolecular inhibition of PPA by α-amylase inhibitors from several kinds of *P. vulgaris* kidney beans (*1, 21, 26-29*). The higher concentrations of PPA and PHA-I used in this study, as well as the addition of Ca^{2+}, may explain the more efficient

[2] Murosaki, S. (1982) Thermodynamic properties of the interaction between porcine pancreatic α-amylase and white kidney bean (*Phaseolus vulgaris*) α-amylase inhibitor. Master of Agriculture thesis. Osaka Prefecture University, Japan.

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complex formation.

Strong support for the above suggestion that one molecule of PHA-I binds and inhibits two molecules of PPA has been furnished by characterization of PPA-PHA-I complexes separated by gel filtration. Further, it is noteworthy that a significant amount of a 2 : 1 complex, along with a 1 : 1 complex, between PPA and PHA-I was produced from a mixture of equimolecular amounts of PPA and PHA-I (Fig. 6D). Although the absorbance difference between the 2 : 1 and 1 : 1 complex peaks is slight, the molar concentration of the 1 : 1 complex is greater than that of the 2 : 1 complex, since the 1 : 1 complex has one less PPA which has a higher extinction coefficient than PHA-I. The binding of PPA to PHA-I, therefore, may follow simple binomial statistics, *i.e.*, an expected ratio 1 : 2 : 1 of (2 : 1 complex) : (1 : 1 complex) : free PHA-I when a mixture of equimolecular amounts of PPA and PHA-I was incubated. This means that two binding sites on PHA-I are independent and of high affinity. This suggestion is in fair agreement with the above observation that a 1 : 1 complex between PPA and PHA-I still retains about 50% of the inhibition activity of the native PHA-I on a molar basis.

The information obtained here, including the procedures for preparative separation of the PPA-PHA-I complexes, can be expected to facilitate succeeding studies on the intersubunit relationship and inhibition mechanism of PHA-I.

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