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Identification of Essential Amino Acid Residues of an α-Amylase Inhibitor from *Phaseolus vulgaris* White Kidney Beans[1]

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Kidney bean (*Phaseolus vulgaris*) α-amylase inhibitors, which are bivalent inhibitors with the subunit stoichiometry of $(\alpha\beta)_2$ complex, have been inferred to contain unique arginine, tryptophan, and tyrosine residues essential for the inhibitory activity. To test the validity of this inference, an attempt was made to identify the essential amino acid residues of a white kidney bean (*P. vulgaris*) α-amylase inhibitor (PHA-I) by using the chemical modification technique combined with amino acid sequencing and mass spectrometry. Exhaustive modification of the arginine residues by phenylglyoxal did not lead to a marked loss of activity, suggesting that no arginine residue is directly associated with the inhibitory activity. *N*-Bromosuccinimide treatment of PHA-I in the presence or absence of a substrate α-amylase revealed the involvement of two tryptophan residues in α-amylase inhibition, and they were identified as Trp188 of the β-subunit by amino acid sequencing and mass spectrometry of lysylendopeptidase peptides. Further, two tyrosine residues were preferentially modified either by *N*-acetylimidazole or by tetranitromethane, resulting in a concomitant loss of most of the PHA-I activity. Amino acid sequencing of the lysylendopeptidase peptides from a tetranitromethane-modified PHA-I identified Tyr186 of the β-subunit as an essential residue.

Key words: α-amylase inhibitor, chemical modification, essential amino acid residues, kidney bean, *Phaseolus vulgaris*.

α-Amylase inhibitors from kidney beans (*Phaseolus vulgaris*) have been shown to bind and inhibit two α-amylase molecules on the basis of their tetrameric structure, $(\alpha\beta)_2$ (*1, 2*), which is formed by the post-translational processing of the precursors (*3*). Only a little, however, is known about their active site structure and, consequently, their inhibition mechanism. Mirkov *et al.* suggested a triad structure composed of Arg74, Trp188, and Tyr190 (in the sequence of the precursor protein) constituting the active site, by site-directed mutagenesis based on knowledge about the three-dimensional model of legume lectins, which have high homology to *P. vulgaris* α-amylase inhibitors (*4*). Considerable doubt remains, however, as to whether this triad structure is valid. From the X-ray crystal structure of a complex of an α-amylase inhibitor with porcine pancreatic α-amylase (PPA), Bompard-Gilles *et al.* doubted whether Arg74 plays a direct role in PPA inhibition (*5*). Figure 1, prepared according to the data of Bompard-Gilles *et al.* (*5*), shows the amino acid residues involved in the interactions with PPA. Nakaguchi *et al.* also implied that Arg74 is not involved in the direct interaction with PPA but essential for the post-translational processing of the precursors to form an active tetramer (*2*). It is interesting, therefore, to see whether the amino acid residues so far suggested to be essential can be confirmed to be so. Here, an attempt was made to identify the amino acid residues essential for the inhibitory activity of a white kidney bean (*P. vulgaris*) α-amylase inhibitor (PHA-I). Chemical modification experiments with amino acid-specific reagents, combined with amino acid sequencing and mass spectrometry, identified Trp188 and Tyr186 of the β-subunit as the amino acid residues directly involved in PPA inhibition.

MATERIALS AND METHODS

Materials—PHA-I was purified from kidney beans (*P. vulgaris*) (*6*), and its concentration was calculated using $A_{280}[1\%/\text{cm}] = 11.8$ (*1*). The α- and β-subunits of PHA-I were separated from each other by the method described in a previous paper (*7*). The preparative procedure of a complex of PHA-I with two PPA molecules was also previously described (*1*). PPA (Type I-A) was purchased from Sigma and its concentration was obtained using $A_{280}[1\%/\text{cm}] = 24.0$ and $M_r = 52{,}000$ (*8*). Phenylglyoxal (PGO) and

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Abbreviations: NAI, *N*-acetylimidazole; NBS, *N*-bromosuccinimide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PGO, phenylglyoxal; PHA-I, α-amylase inhibitor of *Phaseolus vulgaris* white kidney bean; PPA, porcine pancreatic α-amylase; TNM, tetranitromethane.

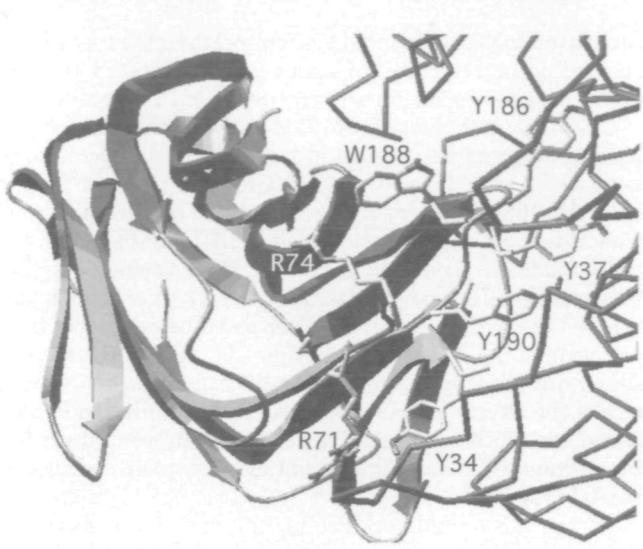

Fig. 1. **Schematic representation of the amino acid residues involved in the interactions with PPA.** PHA-I is depicted with a ribbon diagram and PPA with a wireframe model. The figure was drawn with Swiss-Model (*19*).

N-bromosuccinimide (NBS) were obtained from Aldrich, and NBS was recrystallized from water. *N*-Acetylimidazole (NAI) and tetranitromethane (TNM) were from Nacalai Tesque, and lysylendopeptidase from *Achromobacter lyticus* M497-1 was a product of Wako Pure Chem. Aqueous urea was always treated with ion-exchange resin AG501-X8D (Bio-Rad) just before its use. Other chemicals used here were described in a previous paper (*1*).

Chemical Modification of Arginine Residues—The arginine residues of PHA-I were modified with PGO essentially as described previously (*9*). A PHA-I solution (2 mg/0.9 ml, 40 μM) in 50 mM sodium phosphate buffer (pH 7.9) was mixed with 100 mM PGO (0.1 ml) in the same buffer and incubated at 37°C for 20 to 120 min. The modified protein was passed through a Sephadex G-25 (fine) column (1.2×30 cm) equilibrated and eluted with 40 mM glycerophosphate buffer (pH 6.9), then assayed for residual activity. Part of the modified protein solution was dialyzed against water and assayed for amino acid composition.

Chemical Modification of Tryptophan Residues—Oxidation of tryptophan residues with NBS was performed by a modification of the method previously described (*10*). A PHA-I solution (0.5 mg/0.9 ml, 10 μM) in 0.1 M sodium acetate buffer (pH 5.0) containing 50 mM NaCl and 1 mM $CaCl_2$ was mixed with 5-μl portions of 2 mM NBS in the same buffer at 1-min intervals. After cessation of the decrease in the absorbance at 280 nm, the reaction solution was mixed with tryptophan (10 μmol), then passed through a Sephadex G-25 (fine) column (1.2×30 cm) equilibrated and eluted with 40 mM glycerophosphate buffer (pH 6.9). The product thus purified was assayed for residual activity. Part of the product was exhaustively dialyzed against water with the intention of assaying amino acid composition.

Chemical Modification of Tyrosine Residues—Acetylation of tyrosine residues was performed essentially according to the method of Simpson and Vallee (*11*). A PHA-I solution (1 mg/0.9 ml, 20 μM) in 40 mM sodium phosphate buffer (pH 6.9) containing 25 mM NaCl was brought to 0.5 to 5 mM NAI by adding an NAI solution in the same buffer (0.2 ml), and incubated at 37°C for 1 h. The modified protein was purified by passing through a Sephadex G-25 (fine) column (1.2×30 cm) equilibrated and eluted with 40 mM glycerophosphate buffer (pH 6.9), and assayed for residual activity. The amount of tyrosyl residues modified was determined by the method of Balls and Wood (*12*) using 1 M hydroxylamine hydrochloride at pH 7.5.

Nitration of tyrosine residues was performed using TNM by a modification of the method of Riordan and Vallee (*13*). A PHA-I solution (2.8 mg/0.5 ml, 0.1 mM) in 0.1 M Tris-HCl buffer (pH 8.0) was mixed with 10 to 300 mM TNM (25 μl) in 95% ethanol, allowed to stand at 25°C for 1 h with stirring, then dialyzed against water. After being lyophilized, the products were dissolved in a small amount of 20 mM ammonium acetate buffer (pH 6.8) and developed on a Sephadex G-75 (superfine) column (1.2×95 cm) equilibrated and eluted with the same buffer to remove the polymerized products (*14*). The purified protein was dialyzed as described above with the intention of assaying of residual activity and amino acid composition.

Chemical Modifications of Amino Acid Residues of PHA-I Complexed with PPA—Amino acid chemical modifications of a complex of PHA-I with two PPA molecules were carried out under high ionic strength conditions which stabilize the oligomeric structure of the complex and have no unfavorable effects on the chemical modifications of tryptophan and tyrosine residues. A PPA-PHA-I complex solution was dialyzed against 0.1 mM sodium acetate buffer (pH 5.0; for NBS modification) or against 0.1 M Tris-HCl buffer (pH 8.0; for TNM modification), both containing 1 mM $CaCl_2$, 1 M NaCl, and 0.5 M ammonium sulfate, and then subjected to the chemical modifications as described above. After dialysis against 0.1 M Tris-HCl buffer (pH 8.5), the solution was mixed with an equal volume of 0.1 M Tris-HCl (pH 8.5) containing 1 M NaCl and 5 M guanidine hydrochloride and allowed to stand at 37°C for 3 h. PHA-I released from the complex was separated by gel-filtration HPLC on a TSK-GEL G-3000SW (0.75×30 cm) (Tosoh) operated with 40 mM glycerophosphate buffer (pH 6.9) containing 2 M urea, then dialyzed as described above for the purpose of assaying residual activity and amino acid composition.

PPA-PHA-I Complex Formation—Binding of PHA-I to PPA was estimated essentially by the procedures of Kasahara *et al.* (*1*) but with some modifications as follows. A mixture of 72 μM PPA and 32 μM PHA-I in 40 mM glycerophosphate buffer (pH 6.9) containing 10 mM calcium acetate was incubated at 37°C for 2 h, then the reaction solution was developed on a HW-55F (Tosoh) column (1.2×95 cm) equilibrated and eluted with the same buffer. The elution of peptides was monitored by measuring the absorbance at 280 nm.

Lysylendopeptidase Digestion and Peptide Separation—Lysylendopeptidase digestion of the α- and β-subunits was carried out as performed previously, *i.e.*, at 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 peptides obtained were separated by HPLC on a reversed-phase column (Cosmosil $5C_{18}$-AR, 4.6×150 mm) developed with a linear acetonitrile gradient.

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 with a Shimadzu PSQ-1 gas-phase protein sequencer. The carboxyl-terminal amino acid residues were determined by hydrazinolysis according to the method of Fraenkel-Conrat and Tsung (*15*).

Mass Spectrometry of Peptides—Lysylendopeptidase peptides were analyzed using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Finnigan MAT VISION 2000) operated in the positive-ion reflectron mode. A peptide sample (1 pmol or so) was mixed with a matrix material (2,5-dihydroxybenzoic acid, 10 mg/ml) in 30% acetonitrile/70% water/0.1% trifluoroacetic acid.

RESULTS

Chemical Modification of Arginine Residues—Incubation of PHA-I with PGO, an arginine-specific reagent, ultimately modified 7 of the 16 arginine residues. The inhibitor, however, still retained over half its original activity (Fig. 2A) and displayed a high level of PPA-binding ability (Fig. 2B). This finding, together with the fact that the inhibitor submitted to the same modification treatment without PGO showed an activity loss of about 25%, suggested that no arginine residues are directly involved in PPA inhibition.

Chemical Modification of Tryptophan Residues—NBS, although convenient for use in tryptophan modification, is known to oxidize tyrosine residues under certain conditions (*16*). We first examined, therefore, the conditions under which the tryptophan residues of PHA-I can be preferentially modified. As shown in Fig. 3A, the tyrosine oxidation was greatly affected by a change in NBS concentration and seemed to be limited at a NBS concentration of lower than approximately 75 μM. In addition, the pH dependence of the tryptophan and tyrosine modifications, which should reflect the effects of pH on the structural stability of PHA-I as well as on the modification reactions, presented pH 5.0 for a preferential modification of the tryptophan residues of PHA-I (Fig. 3B).

On the basis of these results, the correlation between number of NBS-modified tryptophan residues and activity loss of PHA-I was examined in further detail. Figure 4 shows that 2 of the 8 tryptophan residues were modified with a concomitant disappearance of the ability of PHA-I to complex with PPA. On the other hand, PHA-I complexed with two PPA molecules was barely susceptible to the

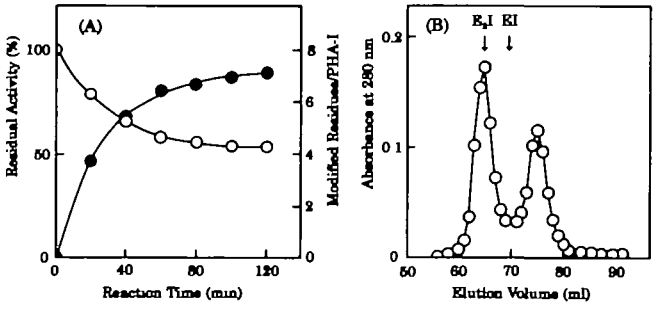

Fig. 2. **Correlation between number of PGO-modified arginine residues and activity loss.** (A) At the indicated times aliquots were withdrawn and assayed for residual activity (open circles) and number of modified arginine residues (closed circles). (B) PHA-I treated with PGO for 120 min was assayed for PPA-binding activity as described under "MATERIALS AND METHODS." The arrows EI and E_2I indicate the elution positions of complexes of PHA-I with one and two PPA molecules, respectively.

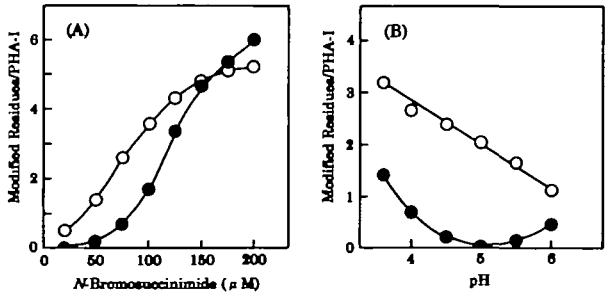

Fig. 3. **The dependence of NBS modification of PHA-I on the NBS concentration and pH.** (A) PHA-I was modified with various concentrations of NBS in 0.1 mM sodium acetate buffer at pH 4.0. (B) PHA-I was modified with 75 μM NBS in 0.1 mM sodium acetate buffer at various pH values. The modified proteins obtained in (A) and (B) were subjected to further treatments as described under "MATERIALS AND METHODS," then assayed for tryptophan (open circles) and tyrosine (closed circles) contents by amino acid analysis.

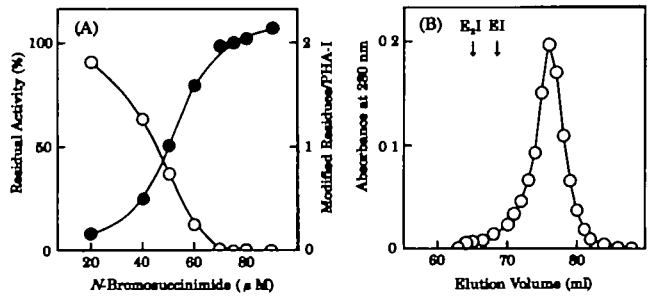

Fig. 4. **Correlation between number of NBS-modified tryptophan residues and activity loss.** (A) The proteins modified with various concentrations of NBS in 0.1 mM sodium acetate buffer at pH 5.0 were assayed for residual activity (open circles) and number of modified tyrosine residues (closed circles). (B) The protein modified with 75 μM NBS was assayed for PPA-binding activity. The arrows EI and E_2I indicate the elution positions of complexes of PHA-I with one and two PPA molecules, respectively.

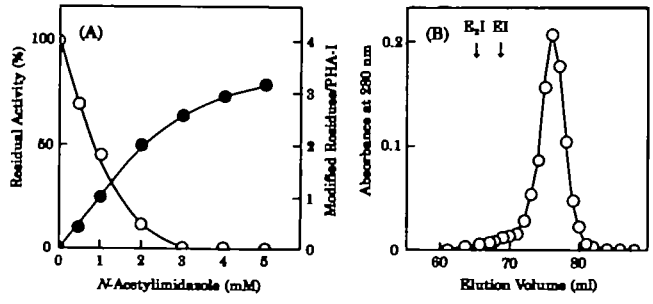

Fig. 5. **Correlation between number of NAI-modified tyrosine residues and activity loss.** (A) The proteins modified with various concentrations of NAI were assayed for residual activity (open circles) and number of modified tyrosine residues (closed circles). (B) The protein modified with 2 mM NAI was assayed for PPA-binding activity. The arrows EI and E_2I indicate the elution positions of complexes of PHA-I with one and two PPA molecules, respectively.

tryptophan modification, and PHA-I separated from the NBS-treated complex retained about 70% of the original activity (data not shown). It is probable, therefore, that one tryptophan residue essential for the inhibitory activity is situated in each of the two active sites of PHA-I.

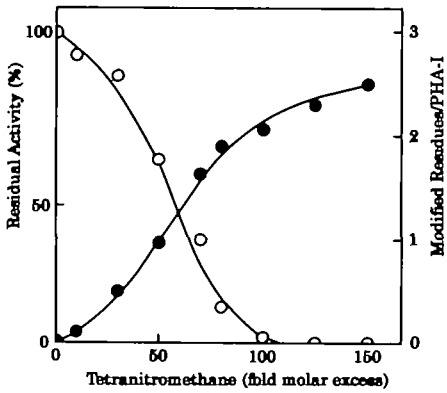

Fig. 6. **Correlation between number of TNM-modified tyrosine residues and activity loss.** PHA-I was modified with TNM at concentrations up to 150-fold molar excess over the protein, then assayed for residual activity (open circles) and number of modified tyrosine residues (closed circles).

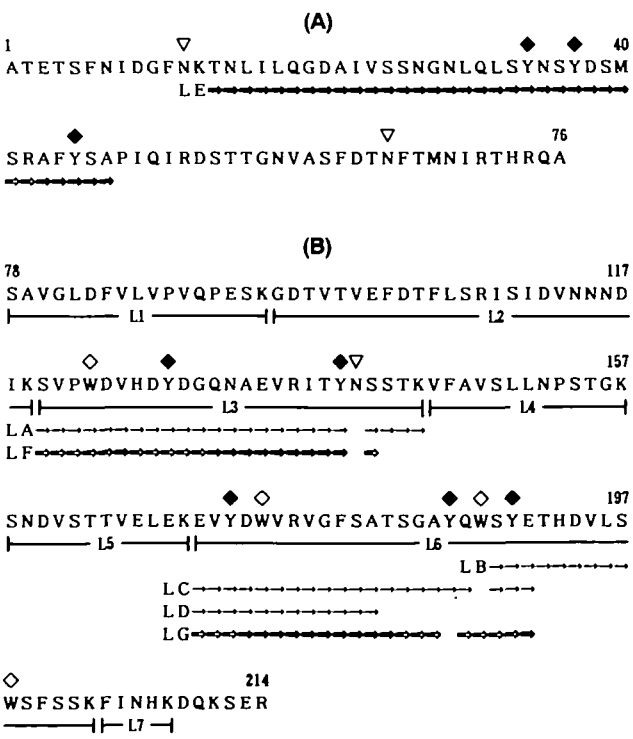

Fig. 7. **Alignment of known amino acid sequences of the α- (A) and β-subunits (B) of PHA-I and identifying strategies of modified tryptophan and tyrosine residues.** L1-L7, lysylendopeptidase peptides previously analyzed for amino acid sequences (*1*); ◇, tryptophan residues; ◆, tyrosine residues; ▽, *N*-glycan attachment sites; →, automated Edman degradation of the peptides from the NBS-modified PHA-I; ⇒, automated Edman degradation of the peptides from the TNM-modified PHA-I. Residues are numbered according to the sequence of the precursor protein, starting at the N-terminus of the α-subunit.

Chemical Modification of Tyrosine Residues—To determine the number of tyrosine residues directly involved in PHA-I function, chemical modification was performed with a tyrosine-specific reagent, NAI. This labile reagent modified the tyrosine residues primarily depending upon its concentration, and a 120-fold excess of reagent modified 2 tyrosine residues with a concomitant activity loss of approximately 90% (Fig. 5), suggesting that the active site of PHA-I contains one functional tyrosine residue. Although NAI has been favorably used for the quantification of solvent-exposed tyrosine residues of proteins, the acetyl group of the resulting *O*-acetyltyrosine is readily hydrolyzed under the conditions, for example, of amino acid sequencing and MALDI-TOF mass spectrometry. With a view to locating the functional tyrosine residues and confirming their number, TNM modification, which forms a relatively stable tyrosine derivative, was applied to PHA-I. Like NAI acetylation, the nitration of the tyrosine residues of PHA-I was dependent on the TNM concentration. With a 100-fold molar excess of TNM, 2.1 residues of 3-nitrotyrosine were produced with a concomitant inactivation of PHA-I, similarly to the results from the NAI modification

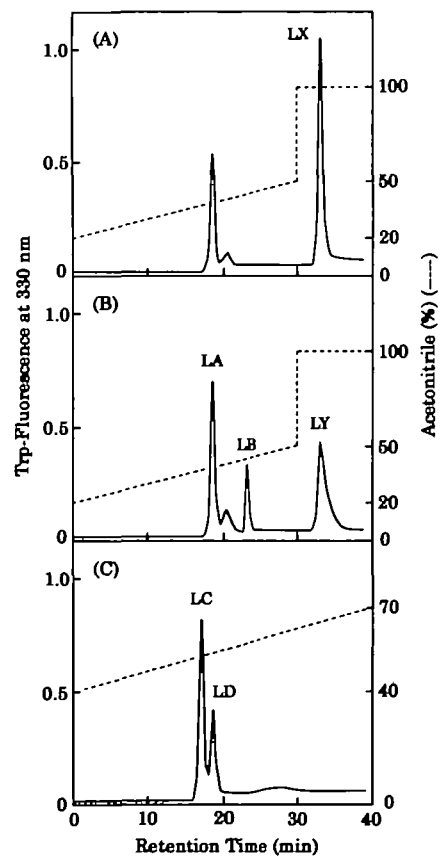

Fig. 8. **Reversed-phase HPLC of the lysylendopeptidase peptides of the β-subunit separated from the NBS-modified PHA-I.** The peptides obtained before (A) and after (B) NBS-modification of PHA-I were separated on a reversed-phase column eluted with a linear gradient of 20-50% acetonitrile in 0.1% trifluoroacetic acid, then with 100% acetonitrile in 0.1% trifluoroacetic acid. The peak fraction LY was subjected to further separation on the same column eluted with a linear gradient of 40-70% acetonitrile in 0.1% trifluoroacetic acid (C). The elution of peptides was monitored by tryptophan fluorescence at 330 nm (excitation at 280 nm).

(Fig. 6). Under these conditions, close to 60% of PHA-I was converted to polymerized products by intermolecular cross-linking. Under the same conditions, PHA-I complexed with two PPA molecules was not modified by TNM, and PHA-I separated from the TNM-treated complex retained over 80% of the original activity (data not shown).

Identification of the Essential Tryptophan and Tyrosine Residues—The complete amino acid sequences of the α- and β-subunits of PHA-I have previously been determined (*1*). As shown in Fig. 7, all of the tryptophan residues of PHA-I are contained in the β-subunit. To locate the NBS-modified tryptophan residues, the β-subunit was separated from the NBS-modified PHA-I, digested with lysylendopeptidase, and fractionated by reversed-phase HPLC. Figure 8, A and B, shows the elution profiles of the peptides obtained before and after NBS modification of PHA-I, respectively. The NBS treatment toward tryptophan modification led to the production of a new peak LB and a deformed peak LY. First, automated N-terminal sequencing identified peak LA as L3, Ser120-Lys144 (Fig. 7). In this sequencing, phenylthiohydantoin-tryptophan was detected at the 4th cycle in a 39% yield. The minor peptide eluted immediately after LA was found to have the same amino acid sequence as LA, suggesting that it derived from the microheterogeneity of the *N*-glycan attached to Asn140 (*1, 18*). Next, peptide LB, obtained in a 37% yield, was revealed to have the complete sequence of Ser189-Lys203 including Trp198 unmodified (Fig. 7). In agreement with this a pseudomolecular ion of m/z 1,774.3 was detected, deviating from the expected molecular mass by 0.024% (Fig. 9A). Mass spectrometry further showed that LY is a mixture of two peptides (Fig. 9B), which were separated into peaks LC and LD (34 and 25% yields, respectively) by rechromatography (Fig. 8C). An N-terminal sequence of Glu170-Glu191 was obtained for peptide LC, and a tryptophan residue was detected at the 5th cycle in a 41% yield, but no phenylthiohydantoin derivative at the 19th cycle. These findings, together with the mass signal at m/z 3,995.7 (Fig. 9B), deviating from the expected molecular mass for NBS-modified peptide L6 by 0.087%, indicated that LC corresponds to peptide L6, Glu170-Lys203, including NBS-modified Trp188 (Fig. 7). On the other hand, the N-terminal sequence of LD was determined up to the 12th residue, alanine, showing the same amino acid sequence as found for LC. The mass signal m/z 2,255.7 found for LD, together with its N-terminal sequence, suggested that the NBS modification of Trp188 led to the production of LD and LB. The mass signal expected for Trp188-modified peptide, Glu170-hydroxytryptophan188, is 2,238.4, and the considerable difference between these values may be due mainly to a lack of the knowledge of the exact molecular structure of the oxidatively cleaved tryptophan residue at the C-terminus of peptide LD. In fact, C-terminal analysis of LD by hydrazinolysis failed to detect hydroxytryptophan. NBS modification has a tendency to cleave the peptide bonds including the carboxyl groups of tryptophan residues (*17*). The production of LD and LB, therefore, is not unexpected, but rather helpful in indicating the site of the NBS-modified tryptophan residue. Taken together, there seems no doubt that Trp188 is accessible to the NBS modification and directly involved in PPA inhibition.

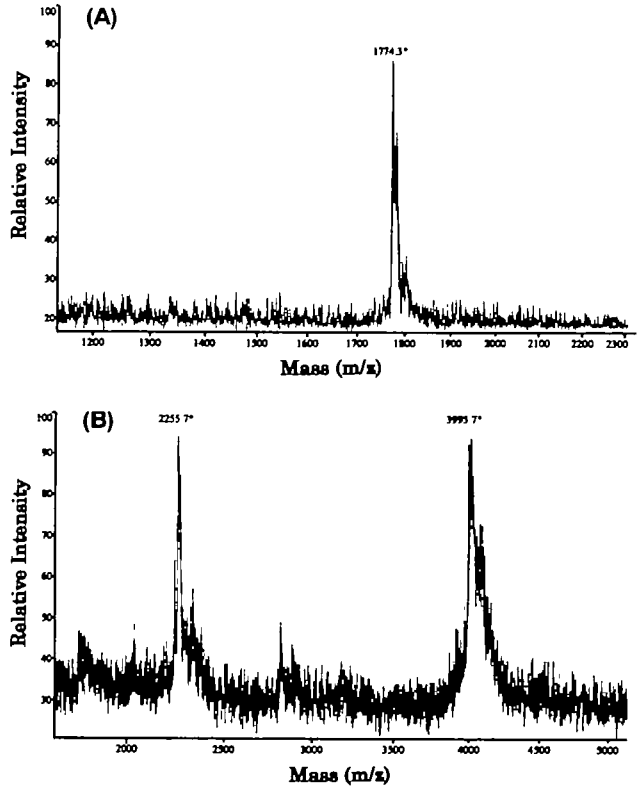

Fig. 9. **MALDI-TOF mass spectrometry of the lysylendopeptidase peptides of the NBS-modified β-subunit.** Peptides LB (A) and LY (B) in Fig. 8B were analyzed by MALDI-TOF mass spectrometry. Accelerating voltage, 20,000; grid voltage, 93.5% of the accelerating voltage; guide wire voltage, 0.050% of the accelerating voltage; delay, 50 ns; laser step, 1,930; scan average, 108. See the text for more details.

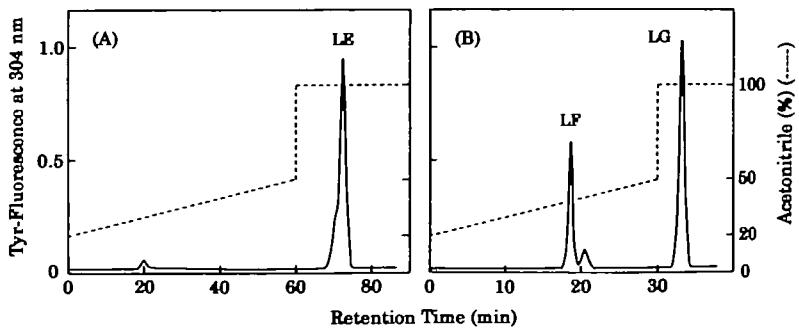

Fig. 10. **Reversed-phase HPLC of lysylendopeptidase peptides of the subunits separated from the TNM-modified PHA-I.** The peptides of the α- (A) and β-subunits (B) were separated on a reversed-phase column eluted with a linear gradient of 20-50% acetonitrile in 0.1% trifluoro acetic acid, then with 100% acetonitrile in 0.1% trifluoroacetic acid. The elution of peptides was monitored by tyrosyl fluorescence at 304 nm (excitation at 275 nm).

As described above, one tyrosine residue essential for the inhibitory activity is situated in the active site of PHA-I. To identify this unique tyrosine residue, the α- and β-subunits separated from TNM-modified PHA-I were extensively digested with lysylendopeptidase and fractionated by reversed-phase HPLC. α-Subunit gave one fluorescent peak LE (Fig. 10A), and its N-terminal amino acid sequence Thr14–Ala47 was revealed by automated sequencing, as would be expected from the known amino acid sequence of this subunit (Fig. 7). In this sequencing, tyrosine residues were detected at the 21st, 24th, and 32nd cycles in yields of 14, 11, and 7%, respectively, showing that none of the tyrosine residues (Tyr34, Tyr37, and Tyr45) in the α-subunit are TNM-modified. The shoulder of LE peak seemed likely to derive from the microheterogeneity of the *N*-glycan attached to Asn65 (*1, 18*). The β-subunit gave two fluorescent peaks LF and LG (Fig. 10B), in accord with expectations based on the previous results from the complete amino acid sequencing of the β-subunit (*1*) (Fig. 7). The small peak following LF, corresponding to the minor peak following LA (Fig. 8B), seemed to derive from the microheterogeneity of the *N*-glycan attached to Asn140 (*1, 18*). To locate the TNM-modified tyrosine residue, the N-terminal sequences of LF and LG were determined with the sequencer up to the 22nd residue each (Fig. 7). Sequencing of peptide LF distinctly revealed tyrosine residues at the 9th and 20th cycles in yields of 29 and 12%, respectively. In the sequencing of LG (71% yield), however, only a trace of phenylthiohydantoin derivative was detected at the 17th cycle, though a tyrosine residue was identified in an 8% yield at the 21st cycle. It follows from these results that TNM preferentially modified Tyr186, which is essential for PPA inhibition.

DISCUSSION

Site-directed mutagenesis has been widely used to identify the amino acid residues directly involved in protein functions. Substitution of a whole amino acid residue, however, would unexpectedly change protein stability and even conformation. In addition, substitution of amino acid residues of precursor proteins would abolish their post-translational processing into active oligomers, presenting a puzzling situation. Chemical modification experiments in which only the side groups of amino acid residues of matured proteins are modified would eliminate these difficulties in site-directed mutagenesis. Such seems to be the case with the Arg modification of PHA-I in this study. Although mutation of Arg74 abolished the expression of an active inhibitor (*4*), the exhaustive PGO modification of the Arg residues of matured PHA-I did not lead to a marked decrease in the inhibitory activity of PHA-I. It seems most likely, therefore, that the processing at Asn77 (*3*) needs the Arg74 residue and, consequently, the disappearance of Arg74 precluded the processing into an active tetramer, supporting the previous suggestions (*2, 5*).

The combined use of amino acid sequencing and mass spectrometry revealed that the PHA-I inactivation by NBS is due to the selective modification of Trp188, consistent with the information presented by Mirkov *et al.* (*4*) and Bompard-Gilles *et al.* (*5*). From the X-ray crystal structure of an inhibitor-PPA complex, Bompard-Gilles *et al.* have identified Trp188 as the only tryptophan residue directly interacting with the amino acid residues of the PPA active site (*5*). There seems no doubt, therefore, that Trp188 is one of the amino acid residues essential for the inhibitory activity of PHA-I.

The results obtained by NAI and TNM modifications indicated that Tyr186 is directly involved in PPA inhibition. It seems likely, however, that PHA-I inactivation by the preferential modification of Tyr186 does not necessarily allow us to view Tyr186 as the only tyrosine residue essential for the PHA-I function, for the following reasons. (i) Treatment of PHA-I with a relatively high concentration of NAI resulted in modification of over three tyrosine residues (Fig. 5A). (ii) PHA-I complexed with two PPA molecules was not modified by TNM, and its activity was not impaired. (iii) PHA-I with two tyrosine residues modified still contained a small amount of unmodified Tyr186 and exhibited a slight inhibitory activity. These results, together with the finding that the yield of the phenylthiohydantoin derivative at the 21st cycle of peptide LG was somewhat lower than expected, suggest that Tyr190 may be another tyrosine residue involved in PHA-I function. Recently Bompard-Gilles *et al.* have also suggested the importance of Tyr190 as well as Tyr186 in the active site (*5*), in agreement with the results previously obtained by site-directed mutagenesis of Tyr190 (*4*). Thus, Tyr190 might also play an essential role in PPA inhibition, even though it is not very accessible to chemical modification.

In conclusion, these findings indicate a structurally essential, nonfunctional role of Arg74, and direct participation of Trp188 and Tyr186 in PPA inhibition. The information obtained here are expected to facilitate further studies toward a full understanding of the inhibition mechanism.

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