

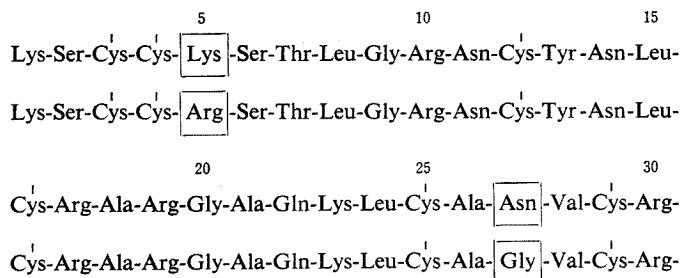
Complete Primary Structures of Two Subunits of Purothionin A, a Lethal Protein for Brewer's Yeast from Wheat Flour

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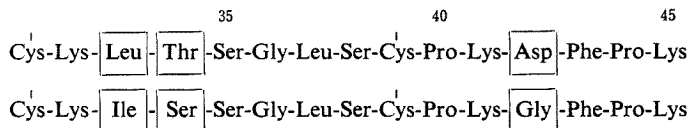
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Received for publication, May 14, 1977

Purothionin (a lethal protein for brewer's yeast) obtained from wheat flour (*Triticum vulgare*, Manitoba No. 3) was separated into two active fractions, A and B, by CM-cellulose column chromatography. The main component, purothionin A, was purified to a homogeneous state as shown by polyacrylamide gel disk electrophoresis and isoelectric focusing. The molecular weight of purothionin A was estimated by the sedimentation equilibrium method to be 11,300 in its native state, 5,500 in 6 M guanidine HCl and after its modification with succinic anhydride. Chemical studies including the determination of N- and C-terminal amino acid residues and amino acid sequence analyses using a combination of automatic Edman degradation and tryptic digestion of purothionin A revealed its dimeric structure composed of nonidentical and closely similar polypeptide chains. Two distinct polypeptides were isolated by DE-52 column chromatography after succinylation. Based on the amino acid composition of each polypeptide, it was possible to align all the tryptic peptides of purothionin A without any ambiguities, establishing the complete primary structures of the purothionin A subunits.



Abbreviations: PA, purothionin A; PB, purothionin B; PAox, performic acid-oxidized purothionin A; PBox, performic acid-oxidized purothionin B; To, tryptic peptide from performic acid-oxidized purothionin A; C, paper chromatography; E, high voltage paper electrophoresis; ND, not determined; PAac, alkylated and citraconylated purothionin A; Cys(CAM), carboxamidomethylated cysteine; PAs, succinylated purothionin A; PAs-I, succinylated purothionin A-I; PAs-II, succinylated purothionin A-II; PA-I, purothionin A-I; PA-II, purothionin A-II.



The two polypeptides in purothionin A have very similar structures, differences occurring at the five positions enclosed by boxes.

The existence of a lethal substance for brewer's yeast in wheat flour was suggested by Jago and Jago (1895) (1). They observed that when brewer's yeast was used for dough fermentation in bread making, production of CO₂ gas was depressed. In 1942, Balls *et al.* (2) extracted the toxic substance with a molecular weight of 12,000 and named it "purothionin." There was, however, some doubt about its homogeneity. Axford *et al.* (1967) (3) showed that purothionin gave a doublet on electrophoresis on a starch gel. Redman and Fisher (1968) (4) obtained two components, purothionin α and β , from the soft flour "Snowdown."

In previous papers, the amounts of the toxic protein for the yeast in various wheats and barleys (5), and the purification and some chemical properties of the toxic protein (6) were reported. Its chemical and biological properties are very similar to "purothionin" and so we call our protein purothionin. Okada *et al.* (1973) (7) described that purothionin bound to the cell membranes of the yeast, causing functional changes in the cytoplasmic membranes and consequent inhibition of sugar incorporation and that leaking of cell constituents was observed. To elucidate the mechanism of the interaction between purothionin and the yeast cell membrane, it is essential to study the molecular structure of purothionin.

This paper deals with the separation of purothionin into two components, A and B, and the determination of the primary structure of one of the components, purothionin A.

MATERIALS AND METHODS

Materials—CM-cellulose, Dowex AG50W-X2 (200–400 mesh) and DE-52 were from Brown Co., BIO RAD Laboratories and Whatman, respectively. Diaflo ultrafilter was from Amicon Far East Ltd. The specially prepared reagents for disk electro-

phoresis and Edman degradation were from Nakarai Chemicals Ltd. and Wako Pure Chemical Industries Ltd., respectively. Carrier ampholytes were from LKB PRODUCTER AB. Carboxypeptidases A [EC 3.4.2.1] and B [EC 3.4.2.2], and trypsin were from SIGMA. Guanidine HCl was recrystallized from hot methanol. Other chemicals were of reagent grade.

Purification of Purothionin—Purothionin was purified according to the method described previously (6) with some modifications. Four kilograms of wheat flour were suspended in 20 liters of 0.05 N H₂SO₄ and kept at 30°C for 3 h. After removal of precipitate by centrifugation (3,000 rpm, 10 min, room temperature), the supernatant was neutralized with 10 N NaOH and stood for 12 h at 5°C. The resulting precipitate was removed by centrifugation. The supernatant was chromatographed on a CM-cellulose column (2.5 × 80 cm) equilibrated with 0.05 M phosphate buffer, pH 7.2 and eluted with a linear gradient of 0.1 to 0.8 M NaCl in the same buffer (700 ml in each reservoir) at room temperature. The flow rate was 55 ml/h. The protein was monitored by the absorbance at 280 nm. The toxic activity of purothionin was measured as described previously (5).

Performic Acid Oxidation—Performic acid-oxidized purothionin was prepared according to the method of Hirs (8) at 0°C.

Alkylation and Citraconylation—Purothionin was alkylated with iodoacetamide after reduction with thioglycol (9) and then subjected to citraconic anhydride treatment (10).

Succinylation—Succinylation of purothionin A was carried out as described by J.H. Freishmeim (11), using 600 mol of succinic anhydride per mole of free amino group. After removal of excess reagents by a Diaflo ultrafilter, UM-2, succinylated purothionin A was lyophilized.

Polyacrylamide Gel Disk Electrophoresis—Disk

electrophoresis was carried out in 7.5% polyacrylamide gel at pH 9.4 (12, 13) and 4.0 (14). The upper electrode was connected to the anode and electrophoresis was carried out at 25 mA/cm² for 50 min at room temperature. The gels were stained with 0.02% coomassie brilliant blue R and destained with 10% trichloroacetic acid (15).

Isoelectric Focusing—Isoelectric focusing was performed with carrier ampholytes from pH 9.0 to 11.0 at 500 volts for 40 h (16).

Analytical Ultracentrifugation—The high speed sedimentation equilibrium method (17) was carried out with a Spinco model E ultracentrifuge, at 17,000 rpm at 26°C in 0.05 M Tris-HCl buffer, pH 7.5 or in the same buffer containing 6 M guanidine HCl. The protein concentration was from 1 to 5 mg per ml. The molecular weight was calculated according to the method of Adams (18) assuming that the partial specific volume was 0.74.

Amino Acid Analysis—Purothionin A and B were hydrolyzed with 6 N HCl in evacuated, sealed tubes at 110°C for 20, 41, and 70 h. The hydrolysates were dried over NaOH pellets in a vacuum desiccator and analyzed with a JEOL 5AH amino acid analyzer, with an accelerated system according to the method of Spackman *et al.* (19). The values of threonine and serine were obtained by extrapolation to zero time of hydrolysis. Other values were the averages of different hydrolysis times. The values of tryptophan were obtained spectrophotometrically (20). For the peptide fragments obtained by tryptic digestion, 20 h hydrolysis was performed and no correction was made for losses of any residues during the hydrolysis.

Determination of N- and C-Terminal Amino Acid Residues—The modified Edman degradation method (21) and the cyanate method (22) were used on performic acid-oxidized purothionin A for quantitative analysis of its N-terminal residue. In the former method, the recovery of phenylthiohydantoin (PTH) amino acid was calculated from the extinction coefficient in the ultraviolet region in absolute ethanol and in the latter, free amino acid regenerated from its hydantoin was analyzed on the amino acid analyzer. Diisopropylfluorophosphate-treated carboxypeptidases A and B were used to analyze the C-terminus of performic acid-oxidized purothionin A (23). The reaction was terminated by transferring the reaction mixture into sodium citrate buffer, pH 2.2. The liberated

amino acids were determined with an amino acid analyzer without removing undigested protein. An aliquot of the reaction mixture was analyzed by the amino acid analyzer after acid hydrolysis to measure the amount of protein used.

Analysis of the N-Terminal Sequence—The N-terminal sequence of performic acid-oxidized purothionin A was determined by the automatic Edman degradation procedure (24) using a JEOL sequence analyzer model JAS-47K. The reagents used for phenylthiocarbamylation were 5% phenylisothiocyanate in heptane and 5% diethylallylamine in 60% pyridine. PTH derivatives of amino acids were identified by thin layer chromatography (25). Thiazolinone derivatives were also hydrolyzed with hydriodic acid to regenerate the original amino acids (26) and they were analyzed with the amino acid analyzer.

Digestion with Trypsin—Tryptic digestions of performic acid-oxidized purothionin A (50 mg) and alkylated and citraconylated purothionin A (25 mg) were carried out in 0.5% NH₄HCO₃ at 30°C for 6.5 h with a weight ratio of trypsin to substrate of approximately 1:50. The reaction was stopped by the addition of glacial acetic acid and then the reaction mixture was lyophilized.

Separation of Peptides—The lyophilized tryptic peptides of performic acid-oxidized purothionin A were chromatographed on a Dowex AG50W-X2 column (1 × 111.5 cm) at 40°C equilibrated with 0.02 M pyridine-acetic acid buffer, pH 3.1. The flow rate was 17 ml/h. The peptides were eluted successively as follows; (a) 300 ml of 0.02 M pyridine-acetic acid buffer, pH 3.1, (b) a linear gradient of 600 ml of 0.02 M pyridine-acetic acid buffer, pH 3.1 to 600 ml of 2 M pyridine-acetic acid buffer, pH 4.1, and (c) 300 ml of 8.5 M pyridine-acetic acid buffer, pH 5.1. Three ml fractions were collected and peptides were monitored at 570 nm for ninhydrin color values after alkaline hydrolysis (27). Further purification of each fraction was carried out with paper chromatography and high voltage paper electrophoresis on Whatman No. 3MM paper. Paper chromatography was performed using *n*-butanol/pyridine/acetic acid/water, 15/10/3/12 as a solvent (28) and run for 20 h or longer at room temperature. High voltage paper electrophoresis was carried out in pyridine-acetic acid buffer, pH 3.6 (29). The peptides were recovered by eluting with 30% acetic acid and in

some cases with 0.05 M ammonia.

The lyophilized tryptic peptides of alkylated and citraconylated puromycin A were chromatographed on a Dowex AG50W-X2 column (1.5×99 cm) essentially the same conditions as described above. Peptides were eluted as follows; (a) 300 ml of the starting buffer (0.02 M pyridine-acetic acid buffer, pH 3.1), (b) a linear gradient of 300 ml of the starting buffer to 300 ml of 2 M pyridine-acetic acid buffer, pH 4.1, (c) a linear gradient of 300 ml of 2 M pyridine-acetic acid buffer, pH 4.1 to 300 ml of 4 M pyridine-acetic acid buffer, pH 5.1, and (d) 300 ml of 8.5 M pyridine-acetic acid buffer, pH 5.1.

Sequence Determination of Tryptic Peptides—

The sequences of the isolated tryptic peptides were analyzed by manual Edman degradation (30). The PTH derivatives of amino acids were identified by thin layer chromatography (23). In some cases, the subtraction method (31) was employed.

Nomenclature—The tryptic peptides from PAox were numbered in the order of their elution from the Dowex column (Fig. 6). The peptides isolated by either paper chromatography or high voltage paper electrophoresis were numbered in the order of their presentation in this paper. The peptide number is followed by the sequence, purification method (*C* refers to paper chromatography and *E* to high voltage paper electrophoresis) and recovery as a percentage of the PAox subjected to the digestion.

Chromatography of Succinylated Puromycin A on DE-52 Column—Succinylated puromycin A (20 mg) dissolved in 1 ml of 0.05 M Tris-HCl buffer, pH 8.0, was applied to a DE-52 column (1.5×36.5 cm) equilibrated with the starting buffer, 0.05 M Tris-HCl, pH 8.0. Chromatography was performed with a linear gradient of 500 ml of the starting buffer to 500 ml of 0.4 M NaCl in the starting buffer at room temperature at a flow rate of 17 ml/h. Peptides in the eluates (2.5 ml each) were monitored for ninhydrin color values after alkaline hydrolysis (27). Fractions collected were lyophilized after desalting with a Diaflo ultrafilter, UM-2.

RESULTS

Isolation of the Two Components of Puromycin

—The toxic activity of puromycin was separated into two fractions by CM-cellulose column chro-

matography as shown in Fig. 1. The major and the minor active fractions designated as puromycin A and puromycin B, respectively, were pooled and condensed by a Diaflo ultrafilter. In some cases, rechromatography of each fraction by a smaller size of column (1.5×75 cm) under the same conditions as those of the first chromatography, was necessary. Figure 2 shows polyacrylamide gel disk electrophoresis patterns of PA and

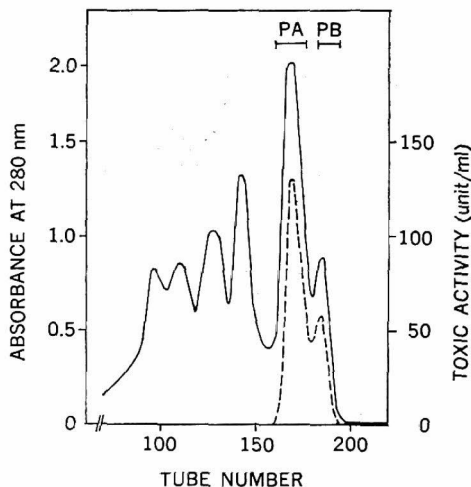


Fig. 1. Chromatography of puromycin on a CM-cellulose column. The experimental conditions are described in the text. —, Absorbance at 280 nm; ---, Toxic activity: Each fraction was 6 ml.

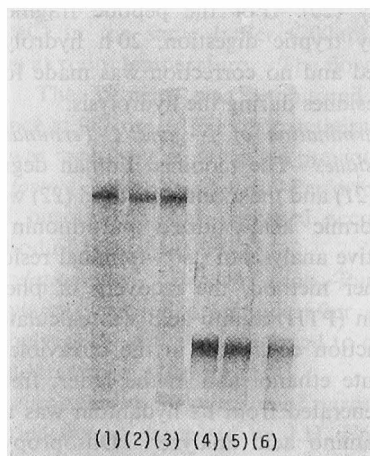


Fig. 2. Polyacrylamide gel disk electrophoresis of PA and PB at pH 9.4 ((1)–(3)) and pH 4.0 ((4)–(6)). Sample (1) and (4), PA (10 μ l); Sample (2) and (5), mixture of PA and PB (10 μ l each); Sample (3) and (6), PB (10 μ l). Conditions are described in the text.

PB at pH 9.4, and 4.0. PA was electrophoretically homogeneous (Fig. 2, (1) and (4)) and this was confirmed by the isoelectric focusing method (pI 10.0). PB was also recovered in a homogeneous state as shown by electrophoresis (Fig. 2, (3) and (6)). PA and PB were indistinguishable on electrophoresis (Fig. 2, (2) and (5)).

Molecular Weight of PA—The estimated molecular weights were plotted against the protein concentration (Fig. 3). Extrapolation showed the molecular weight of PA to be 11,300 in its native state and 5,500 in 6 M guanidine HCl, showing that PA was dissociated into smaller subunits. Dissociation of PA was also achieved by the modification of lysine residues with succinic anhydride (MW 5,500).

Amino Acid Composition—Table I presents the amino acid compositions of performic acid-oxidized

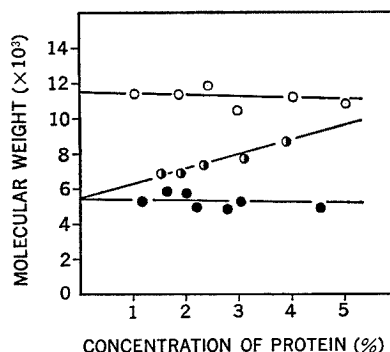


Fig. 3. Variations in the molecular weight of PA as a function of protein concentration. Conditions are described in the text. ○, PA in 0.05 M Tris-HCl buffer, pH 7.5; ●, PA in 6 M guanidine HCl; ◐, PAs in 0.05 M Tris-HCl buffer, pH 7.5.

TABLE I. Amino acid compositions of PAox and PBox. The results are expressed as the number of residues on the basis of the assumption that the native protein of PB has a molecular weight of 11,300.

	PAox					PBox				
	Hydrolysis time (h)			Mean value	Nearest integer	Hydrolysis time (h)			Mean value	Nearest integer
	20	41	70			20	41	70		
Asp	5.82	5.67	5.42	5.63	(6)	4.68	4.74	4.66	4.69	(5)
Thr ^a	2.79	3.30	2.39	3.30	(3)	8.16	7.42	7.58	8.30	(8)
Ser ^a	7.40	5.68	4.13	8.80	(9)	9.64	7.72	7.80	10.00	(10)
Glu	1.98	1.90	1.94	1.94	(2)	1.94	2.36	2.34	2.21	(2)
Pro	3.91	4.14	4.32	4.12	(4)	4.20	4.56	4.58	4.45	(4)
Gly	7.97	7.90	8.14	8.00	(8)	8.66	9.00	8.92	8.86	(9)
Ala	6.20	5.47	6.07	5.91	(6)	2.30	2.52	2.58	2.47	(2)
Cys(O ₃ H)	15.41	15.45	15.79	15.55	(16)	15.96	16.78	16.10	16.28	(16)
Val	2.20	2.02	2.03	2.08	(2)	2.24	2.24	2.12	2.20	(2)
Met	0	0	0	0	(0)	0	0	0	0	(0)
Ile	1.04	0.96	0.91	0.97	(1)	0	0	0	0	(0)
Leu	9.12	8.86	8.74	8.91	(9)	10.50	10.04	10.40	10.31	(10)
Tyr	1.87	1.96	2.05	1.96	(2)	0	0	0	0	(0)
Phe	2.14	2.30	2.25	2.23	(2)	2.16	2.14	2.10	2.13	(2)
Lys	10.60	11.44	11.44	11.16	(11)	10.30	10.80	10.70	10.60	(11)
His	0	0	0	0	(0)	0	0	0	0	(0)
Arg	9.16	8.82	8.67	8.88	(9)	9.28	9.70	10.14	9.71	(10)
Trp ^b	0	0	0	0	(0)	0	0	0	0	(0)

^a The values of threonine and serine were extrapolated to zero hydrolysis time. ^b The values of tryptophan were obtained spectrophotometrically (20).

PA and PB. The amino acid compositions of PBox were calculated on the assumption that the molecular weight of PB was nearly equal to PA (11,300). Characteristic features are an extremely high content of cysteic acid, lysine and arginine, and the lack of methionine, tryptophan and histidine. Marked differences in PAox and PBox are the amounts of threonine and alanine. All cysteic acids detected in PAox must be derived from intramolecular disulfide bonds, because free sulfhydryl groups were not titrated by Ellman's reagent (32) either in the native state or in 6 M guanidine HCl.

Terminal Amino Acids—Using the modified Edman degradation method and the cyanate method, the N-terminal amino acid of PAox was determined to be solely lysine with the recovery of 1.31 mol (Edman degradation) and 1.37 mol (cyanate method) per mole of the protein (molecular weight, 11,300) without any correction of the yields during the procedures. Figure 4 shows the results of carboxypeptidase B digestion of PAox, showing that the C-terminal amino acid was lysine, and the amount recovered was 1.83 mol per mole of the protein (molecular weight, 11,300). No significant amount of amino acids was released by carboxypeptidase A digestion. These results suggested that the protein is composed of two polypeptide chains having lysine residues on both termini.

Sequence Analysis of PAox—PAox (7.5 mg, 0.75 μ mol) was subjected to 28 cycles of automatic Edman degradation (Fig. 5). Two kinds of amino acids were obtained in nearly equal quantities at

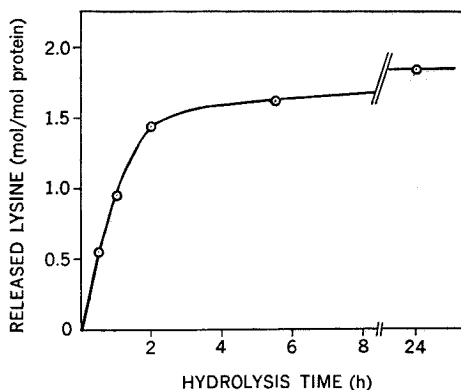


Fig. 4. Rate of release of C-terminal lysine from performic acid-oxidized purothionin A by incubation with carboxypeptidase B. For conditions, see the text.

the 5th (lysine and arginine) and the 21th (aspartic acid or asparagine and glycine) positions, suggesting the nonidentical subunit structure of PA.

Isolation and Sequence Determination of Tryptic Peptides—The tryptic digests of PAox were fractionated by chromatography on a Dowex AG50W-X2 column (Fig. 6). Peaks 3, 4, 5, 6, 8, 9, and 11 were shown to be homogeneous by paper chromatography and high voltage paper electrophoresis. These peaks were used for sequence determination without further purification. Peaks 1, 2, 7, and 10 containing at least two ninhydrin positive substances were purified by paper chromatography, high voltage paper electrophoresis or a combination of both.

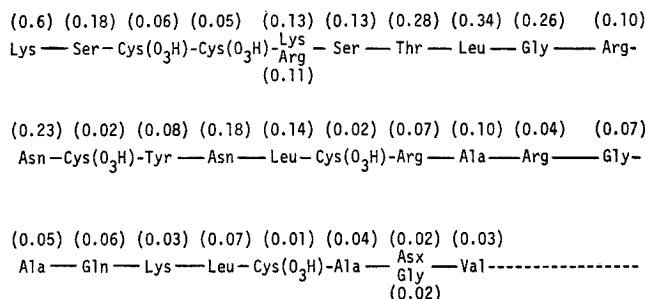


Fig. 5. The results of sequencer analysis of PAox. The estimated molar recoveries at each step are shown above or below the residues in parentheses. The values were obtained from amino acid analysis after HI hydrolysis. No corrections were made for losses of any residues during the extraction step of thiazolinone derivatives and the hydrolysis procedure.

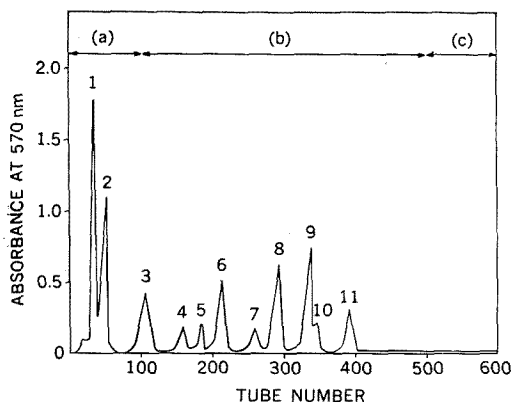


Fig. 6. Chromatography of tryptic hydrolysates of PAox on a Dowex AG50W-X2 column under the conditions described in the text (each fraction was 3 ml).

To-1-1 (Leu-Cys(O₃H)-Ala-Asn-Val-Cys(O₃H)-Arg), *E, C*, 3.5%: Six steps of Edman degradation established the sequence as shown in Table II.

To-1-2 (Leu-Cys(O₃H)-Ala-Gly-Val-Cys(O₃H)-Arg), *E, C*, 4.3%: The results of six steps of Edman degradation are shown in Table III.

To-1-3 (Ser-Cys(O₃H)-Cys(O₃H)-Lys), *E, C*, 1.1%: The amino acid composition of this peptide is Ser 1.00 (1), Cys(O₃H) 2.18 (2), and Lys 0.82 (1). After one step of Edman degradation, Ser 0.10, Cys(O₃H) 2.18 (2), and Lys 0.62 (1) were obtained. Lysine was placed at the C-terminus from the specificity of trypsin and thus the sequence was concluded to be as shown above.

To-1-4 (Ser-Cys(O₃H)-Cys(O₃H)-Arg), *E, C*, 3.8%: The composition is Ser 0.95 (1), Cys(O₃H) 2.05 (2), and Arg 1.01 (1). Three steps of Edman degradation were performed; [step 1] Ser 0.08, Cys(O₃H) 2.09 (2), Arg 0.99 (1) and after step 3, free arginine was found with amino acid analysis without acid hydrolysis of the residue.

To-2 (Asn-Cys(O₃H)-Tyr-Asn-Leu-Cys(O₃H)-Arg), *C, E*, 4.4%: Paper chromatography and high voltage paper electrophoresis were carried

TABLE II. Edman degradation of To-1-1. The amino acid composition of peptides in this and succeeding tables is expressed in terms of the molar ratios per amino acid residues.

	Leu	Cys(O ₃ H)	Ala	Asx	Val	Arg	PTH
Composition	0.98 (1)	1.97 (2)	0.98 (1)	1.02 (1)	1.04 (1)	1.02 (1)	
Edman degradation							
step 1	0.10	2.00 (2)	1.01 (1)	0.87 (1)	1.08 (1)	1.08 (1)	Leu
step 2	0.11	1.25 (1)	1.00 (1)	0.90 (1)	1.05 (1)	1.09 (1)	ND
step 3	0.22	1.24 (1)	0.28	0.84 (1)	1.10 (1)	1.12 (1)	Ala
step 4	0.11	1.26 (1)	0.27	0.26	1.09 (1)	1.11 (1)	Asn
step 5	0.11	1.28 (1)	0.25	0.28	0.33	0.99 (1)	Val
step 6	0	0.10	0	0	0	0.99 (1)	ND

TABLE III. Edman degradation of To-1-2.

	Leu	Cys(O ₃ H)	Ala	Gly	Val	Arg	PTH
Composition	0.99 (1)	1.88 (2)	1.00 (1)	1.02 (1)	1.02 (1)	1.08 (1)	
Edman degradation							
step 1	0.17	1.99 (2)	1.06 (1)	0.99 (1)	1.02 (1)	1.05 (1)	Leu
step 2	0.17	1.02 (1)	0.91 (1)	1.02 (1)	1.00 (1)	1.07 (1)	ND
step 3	0.15	0.95 (1)	0.31	0.95 (1)	1.07 (1)	1.06 (1)	Ala
step 4	0.12	0.88 (1)	0.27	0.38	1.00 (1)	1.12 (1)	Gly
step 5	0.17	0.92 (1)	0.46	0.55	0.43	1.06 (1)	Val
step 6	0	0.21	0	0	0	1.06	ND

TABLE IV. Edman degradation of To-3

	Ile	Leu	Ser	Thr	Gly	Cys(O ₃ H)	Pro	Lys	PTH
Composition	1.06 (1)	3.06 (3)	4.68 (5)	0.90 (1)	2.06 (2)	2.00 (2)	2.18 (2)	2.00 (2)	
Edman degradation									
step 1	0.05	1.82 (2)	4.83 (5)	0.92 (1)	2.14 (2)	2.04 (2)	1.93 (2)	2.00 (2)	Ile & Leu
step 2	0.04	1.82 (2)	4.04 (4)	0.31	2.18 (2)	2.05 (2)	2.02 (2)	1.86 (2)	Ser & Thr
step 3	0	1.85 (2)	2.42 (2)	0	2.16 (2)	2.03 (2)	1.48 (2)	1.89 (2)	Ser
step 4	0	1.74 (2)	2.36 (2)	0	0.48	2.02 (2)	2.29 (2)	1.89 (2)	Gly
step 5	0	0.44	2.26 (2)	0	0.35	1.93 (2)	2.35 (2)	1.74 (2)	Leu
step 6	0	0.33	0.76	0	0.31	1.79 (2)	1.66 (2)	1.80 (2)	Ser
step 7	0	0.37	0.84	0	0.40	0.33	2.25 (2)	1.77 (2)	ND
step 8	ND	ND	ND	ND	ND	ND	ND	ND	Pro

out to remove minor contamination. The composition of this peptide is Asx 2.07 (2), Cys(O₃H) 2.02 (2), Tyr 0.77 (1), Leu 1.06 (1), and Arg 1.08 (1). Six steps of Edman degradation were performed: [step 1] Asx 1.31 (1) (PTH-Asn), Cys(O₃H) 2.12 (2), Tyr 1.05 (1), Leu 0.93 (1), Arg 1.16 (1), [step 2] Asx 1.31 (1), Cys(O₃H) 1.59 (1), Tyr 1.14 (1), Leu 0.97 (1), Arg 1.05 (1), [step 3] (PTH-Tyr), [step 4] (PTH-Asn), and [step 5] (PTH-Leu). After step 6, free arginine was detected without acid hydrolysis of the residue. Thus, the sequence of this peptide was determined to be as described above.

To-3 (Leu/Ile - Thr/Ser - Ser - Gly - Leu - Ser-Cys(O₃H)-Pro-Lys), 32.2%: The amino acid composition and the results of Edman degradation are presented in Table IV. Two kinds of amino acids were obtained at the first two steps of Edman degradation (Leu and Ile at step 1 and Thr and Ser at step 2), showing that this fraction is probably a mixture of two peptides which were unseparable by any of the purification methods used. The subtraction procedures revealed that the amount of each peptide was nearly equal. The succeeding six steps of Edman degradation provided the remaining sequence, Ser-Gly-Leu-Ser-Cys(O₃H)-Pro-Lys in both of the peptides. By an additional experiment (tryptic digestion of alkylated and citraconylated PA (PAac)) as described later, it was established that To-3 is composed of two peptides and the definite sequences are (a) Leu-Thr-Ser-Gly-Leu-Ser-Cys(O₃H)-Pro-Lys and (b) Ile-Ser-Ser-Gly-Leu-

TABLE V. Results of Edman degradation of To-5.

	Lys	Ser	Cys(O ₃ H)	PTH
Composition	2.00 (2)	0.90 (1)	2.00 (2)	
Edman degradation				
step 1	0.72 (1)	1.00 (1)	2.01 (2)	Lys
step 2	0.80 (1)	0.08	1.78 (2)	Ser
step 3	0.80 (1)	0	0.86 (1)	ND

Ser-Cys(O₃H)-Pro-Lys.

To-4 (Cys(O₃H)-Lys), 48.1%: This peptide is composed of two amino acids, Cys(O₃H) 0.95 (1) and Lys 1.04 (1). Based on the specificity of trypsin, the sequence must be Cys(O₃H)-Lys.

To-5 (Lys - Ser - Cys(O₃H) - Cys(O₃H) - Lys), 31.9%: The amino acid composition and the results of three steps of Edman degradation are shown in Table V. By placing lysine at the C-terminus from the specificity of trypsin, the sequence of this peptide was established to be as shown above. This peptide must be from the N-terminal position of PAox because the corresponding sequence has been found by analysis of PAox using the sequencer (Fig. 5).

To-6 (Lys - Ser - Cys(O₃H) - Cys(O₃H) - Arg), 30.5%: The sequence of this peptide was established by four steps of Edman degradation (Table VI). This peptide also correspondings to the N-terminal sequence of PAox (Fig. 5).

TABLE VI. Results of Edman degradation of To-6.

	Lys	Ser	Cys(O ₃ H)	Arg	PTH
Composition	1.01 (1)	0.95 (1)	1.98 (2)	1.05 (1)	
Edman degradation					
step 1	0.04	0.96 (1)	1.99 (2)	1.04 (1)	Lys
step 2	0.03	0.08	1.91 (2)	1.08 (1)	Ser
step 3	0	0.16	1.14 (1)	1.08 (1)	ND
step 4	Arginine was found on amino acid analysis without acid hydrolysis of the residue.				

To-7-1 (Asp-Phe-Pro-Lys), C, 25.2%: The composition of this peptide is Asx 0.93 (1), Phe 1.21 (1), Pro 0.97 (1), and Lys 0.91 (1). Three steps of Edman degradation were carried out; [step 1] (PTH-Asp), [step 2] (PTH-Phe), and [step 3] (PTH-Pro).

To-7-2 (Lys), C, 12%: Lysine was the only amino acid detected in this fraction.

To-8 (Gly-Ala-Gln-Lys), 63.5%: The amino acid composition of this peptide is Gly 0.99 (1), Ala 1.01 (1), Glx 1.00 (1), and Lys 1.01 (1). Three steps of Edman degradation were carried out; [step 1] (PTH-Gly), [step 2] (PTH-Ala), and [step 3] (PTH-Gln).

To-9 (Ser-Thr-Leu-Gly-Arg), 63.0%: The composition of this peptide is Ser 0.90 (1), Thr 0.96 (1), Leu 1.02 (1), Gly 1.05 (1), and Arg 1.06 (1). Four steps of Edman degradation were carried out; [step 1] (PTH-Ser), [step 2] (PTH-Thr), [step 3] (PTH-Leu), and [step 4] (PTH-Gly).

To-10 (Gly-Phe-Pro-Lys), 23.7%: The composition of this peptide is Gly 1.01 (1), Phe 0.88 (1), Pro 1.04 (1), and Lys 1.07 (1). Three steps of Edman degradation were performed. [step 1] (PTH-Gly), [step 2] (PTH-Phe), and [step 3] (PTH-Pro).

To-11 (Ala-Arg), 54.7%: The composition of this peptide is Ala 0.98 (1) and Arg 1.02 (1). One step of Edman degradation yielded PTH-Ala and free arginine.

These results are summarized in Fig. 7.

Tryptic Digests of PAac—Tryptic digestion of PAac was performed in the same way as described above. After digestion, the reaction mixture was brought to pH 3.0 by adding glacial acetic acid and

- To-1-1 : Leu-Cys(O₃H)-Ala-Asn-Val-Cys(O₃H)-Arg
 To-1-2 : Leu-Cys(O₃H)-Ala-Gly-Val-Cys(O₃H)-Arg
 To-1-3 : Ser-Cys(O₃H)-Cys(O₃H)-Lys
 To-1-4 : Ser-Cys(O₃H)-Cys(O₃H)-Arg
 To-2 : Asn-Cys(O₃H)-Tyr-Asn-Leu-Cys(O₃H)-Arg
 To-3 : Leu/Ile-Thr/Ser-Ser-Gly-Leu-Ser-Cys(O₃H)-Pro-Lys
 To-4 : Cys(O₃H)-Lys
 To-5 : Lys-Ser-Cys(O₃H)-Cys(O₃H)-Lys
 To-6 : Lys-Ser-Cys(O₃H)-Cys(O₃H)-Arg
 To-7-1 : Asp-Phe-Pro-Lys
 To-1-2 : Lys
 To-8 : Gly-Ala-Gln-Lys
 To-9 : Ser-Thr-Leu-Gly-Arg
 To-10 : Gly-Phe-Pro-Lys
 To-11 : Ala-Arg

Fig. 7. Amino acid sequences of tryptic peptides of PAox.

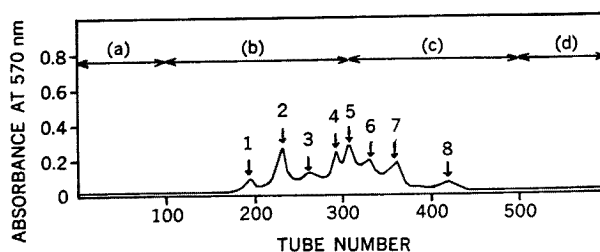


Fig. 8. Chromatography of tryptic fragments of PAac on a Dowex AG50W-X2 column according to the method described in the text (each fraction was 3 ml).

stood overnight at room temperature to remove the citraconyl groups. Isolation of the tryptic peptides of PAac (Tac) was performed by chromatography on a Dowex AG50W-X2 column (Fig. 8) and then by paper chromatography and/or high voltage paper electrophoresis. Seven peptides were isolated in a homogeneous state and their amino acid compositions are shown in Table VII. The amino acid compositions of Tac-2-1, Tac-4-1, Tac-7-1, Tac-8-1, and Tac-6-1 corresponded to those of To-9, To-11, To-2, To-6, and the sum of those of To-8 and To-1-2, respectively, and no further

sequence studies were performed. Tac-3-1 and Tac-5-1 must be C-terminal peptides of PA because of the absence of arginine residues in them and their structures were determined as follows.

Tac-3-1 (Cys(CAM)-Lys-Leu-Thr-Ser-Gly-Leu-Ser-Cys(CAM)-Pro-Lys-Asp-Phe-Pro-Lys): Four steps of Edman degradation yielded the N-terminal sequence, X-Lys-Leu-Thr. Three useful fragments were isolated from the tryptic digests of this peptide by high voltage electrophoresis (T(Tac-3-1)-1~3). Their amino acid compositions are shown in Table VIII. The sum of the compositions

TABLE VII. Amino acid compositions of tryptic peptides obtained from PAac.

	Tac-2-1	Tac-3-1	Tac-4-1	Tac-5-1	Tac-6-1	Tac-7-1	Tac-8-1
Asx		1.07 (1)				2.22 (2)	
Thr	0.99 (1)	0.99 (1)					
Ser	0.84 (1)	1.66 (2)		2.33 (3) ^b			0.93 (1)
Glx					0.92 (1)		
Pro		2.04 (2)		1.99 (2)			
Gly	1.07 (1)	0.96 (1)		2.02 (2)	1.92 (2)		
Ala			1.00 (1)		1.88 (2)		
Cys(CAM) ^a		1.52 (2)		1.69 (2)	1.60 (2)	1.78 (2)	2.23 (2)
Val					1.17 (1)		
Ile				0.91 (1)			
Leu	1.01 (1)	2.00 (2)		1.12 (1)	1.03 (1)	1.13 (1)	
Tyr						0.90 (1)	
Phe		0.99 (1)		1.00 (1)			
Lys		2.89 (3)		2.85 (3)	1.10 (1)		1.15 (1)
Arg	1.09 (1)		1.00 (1)		0.99 (1)	0.97 (1)	0.91 (1)

^a "Cys(CAM)" means "carboxamidomethylated cysteine." ^b The value was obtained from the results of amino acid sequencing.

TABLE VIII. Amino acid compositions of tryptic peptides obtained from Tac-3-1.

	Tac-3-1	T(Tac-3-1)-1	T(Tac-3-1)-2	T(Tac-3-1)-3
Asx	1.07 (1)			1.05 (1)
Thr	0.99 (1)		0.87 (1)	
Ser	1.66 (2)		1.98 (2)	
Pro	2.04 (2)		1.37 (1)	1.22 (1)
Gly	0.96 (1)		1.31 (1)	
Cys(CAM)	1.52 (2)	0.91 (1)	0.95 (1)	
Leu	2.00 (2)		1.74 (2)	
Phe	0.99 (1)			0.81 (1)
Lys	2.89 (3)	1.10 (1)	1.25 (1)	0.94 (1)

TABLE IX. Amino acid compositions of tryptic peptides obtained from Tac-5-1.

	Tac-5-1	T(Tac-5-1)-1	T(Tac-5-1)-2	T(Tac-5-1)-3
Ser	2.33 (3)		2.21 (3)	
Pro	1.99 (2)		1.01 (1)	0.98 (1)
Gly	2.02 (2)		0.99 (1)	1.05 (1)
Cys(CAM)	1.69 (2)	1.15 (1)	1.15 (1)	
Ile	0.91 (1)		0.70 (1)	
Leu	1.12 (1)		1.02 (1)	
Phe	1.00 (1)			0.98 (1)
Lys	2.85 (3)	0.94 (1)	1.13 (1)	1.09 (1)

of these three fragments corresponds to that of Tac-3-1. From its amino acid composition, T(Tac-3-1)-1 must be the N-terminal fragment, corresponding to the sequence to To-4. T(Tac-3-1)-3, corresponds in composition to To-7-1, must be placed at the C-terminal position of this peptide because of the lack of leucine and threonine residues. Thus, T(Tac-3-1)-2 could be placed at the middle and its N-terminal sequence must be Leu-Thr. The composition of the remaining residues corresponding to the C-terminal seven residues of To-3, establish the structure of To-3-(a) to be Leu-Thr-Ser-Gly-Leu-Ser-Cys-Pro-Lys. Therefore the sequence of Tac-3-1 is Cys(CAM)-Lys-Leu-Thr-Ser-Gly-Leu-Ser-Cys(CAM)-Pro-Lys-Asp-Phe-Pro-Lys.

Tac-5-1 (Cys(CAM)-Lys-Ile-Ser-Ser-Gly-Leu-Ser-Cys(CAM)-Pro-Lys-Gly-Phe-Pro-Lys): Edman degradation established the sequence of the first four residues, X-Lys-Ile-Ser. Tryptic digestion gave three fragments and their amino acid compositions are presented in Table IX. T(Tac-5-1)-1 and T(Tac-5-1)-3 were placed at the N- and C-terminal regions of this peptide for the same reason as described above for the determination of the sequence of Tac-3-1. The amino acid composition of T(Tac-5-1)-2 and the 3rd and the 4th residues of the original peptide confirmed the structure of To-3-(b) to be Ile-Ser-Ser-Gly-Leu-Ser-Cys(O₃H)-Pro-Lys. Therefore, the sequence was determined to be Cys(CAM)-Lys-Ile-Ser-Ser-Gly-Leu-Ser-Cys(CAM)-Pro-Lys-Gly-Phe-Pro-Lys.

Figure 9 summarizes the sequence studies. Based on the results of the sequencer analysis, eleven of the tryptic peptides derived from the

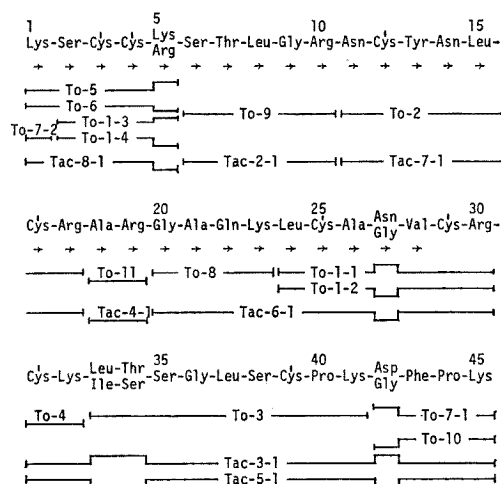


Fig. 9. Summary of the sequence studies of PA. Arrows (→) just below the sequence show sequencer analysis. To and Tac represent the peptides derived by tryptic digestions of PAox and PAac, respectively.

performic acid-oxidized purothionin A were put in order as shown in the figure, determining the sequence of the N-terminal thirty residues. Tryptic digestion of alkylated, citraconylated purothionin A established the primary structure of the remaining C-terminal position.

Isolation of the Succinylated Subunits—PA and succinylated PA showed clearly different migrations and PAs yielded two distinct bands (Fig. 10), suggesting that the protein consists of nonidentical subunits (PAs-I and PAs-II). Citraconylation of PA was also carried out to facilitate dissociation,

however, analytical electrophoresis yielded a single broad band with increasing anodic mobility. PA was chromatographed on a DE-52 column (Fig. 11). Two fragments were collected and lyophilized after desalting with a Diaflo ultrafilter. Extraction of both fractions by analytical polyacrylamide gel disk electrophoresis revealed that fraction I contained PAs-I and fraction II contained PAs-II (Fig. 10). Recoveries of PAs-I and PAs-II calculated from the amino acid composition (Table X) were 28% and 29.5%, respectively.

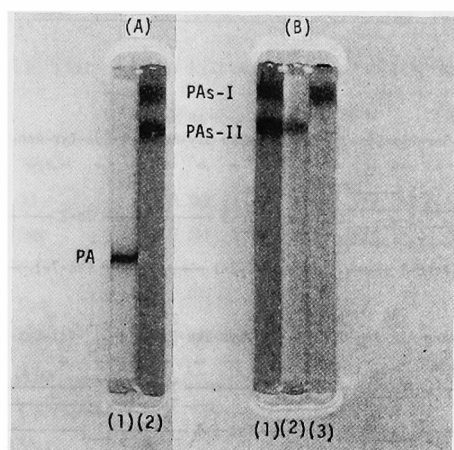


Fig. 10. Polyacrylamide gel disk electrophoretic patterns of PA and PAs. Conditions are described in the text. (A): Sample (1) and (2) were from PA and PAs, respectively. (B): Sample (1), (2), and (3) were from PAs before separation by DE-52 column, fraction I and fraction II, respectively.

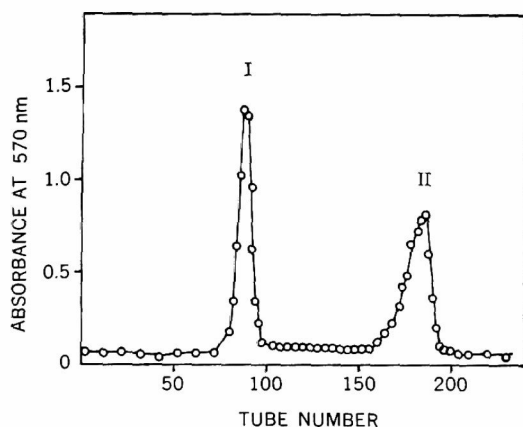


Fig. 11. Chromatography of PAs on a DE-52 column. Experimental conditions are described in the text (each fraction was 2.5 ml). ○, Absorbance at 570 nm.

Amino Acid Compositions and Sequences of the Isolated Subunits—The amino acid compositions of PAs-I and PAs-II are compared in Table I, showing distinct differences in the numbers of Asx, Thr, Ser, Gly, Ile, Leu, Lys, and Arg. In the proposed structure shown in Fig. 9, only five of the forty-five positions are different between the two polypeptides; position 5 (Lys, Arg), 27 (Asn, Gly), 33 (Leu, Ile), 34 (Thr, Ser), and 42 (Asp, Gly). Lack of Ile in PA-I suggests that its C-terminal peptides is Tac-3-1 and that of PA-II is Tac-5-1. Presence of more Asx and less Gly in the composition of PA-I indicates that To-1-1 is in PA-I and To-1-2 in PA-II. Presence of more Lys in PA-I shows that To-5 is the N-terminal peptide of PA-I. The complete

TABLE X. Amino acid compositions of PA, PA-I, and PA-II. The results are expressed as the number of residues on the basis of the assumption that the native protein has a molecular weight of 11,300 and the two subunits 5,500.

	PA	PA-I	PA-II
Asx	5.63 (6)	4.02 (4)	2.16 (2)
Thr	3.30 (3)	1.94 (2)	1.00 (1)
Ser	8.80 (9)	4.00 (4)	4.92 (5)
Glx	1.94 (2)	1.01 (1)	1.04 (1)
Pro	4.12 (4)	2.11 (2)	2.13 (2)
Gly	8.00 (8)	3.11 (3)	4.88 (5)
Ala	5.91 (6)	3.12 (3)	3.05 (3)
1/2Cys	15.55 (16) ^b	ND (8) ^a	ND (8) ^a
Val	2.08 (2)	0.88 (1)	0.84 (1)
Met	0 (0)	0 (0)	0 (0)
Ile	0.97 (1)	0 (0)	0.95 (1)
Leu	8.91 (9)	4.86 (5)	3.89 (4)
Tyr	1.96 (2)	ND (1) ^a	ND (1) ^a
Phe	2.23 (2)	1.01 (1)	1.00 (1)
Lys	11.16 (11)	6.08 (6)	4.81 (5)
His	0 (0)	0 (0)	0 (0)
Arg	8.88 (9)	4.11 (4)	4.95 (5)
Trp ^c	0 (0)	0 (0)	0 (0)

^a The values were obtained from the results of amino acid sequencing. ^b The value was determined as cysteic acid. ^c The value was obtained spectrophotometrically (20). () represents the nearest integer.

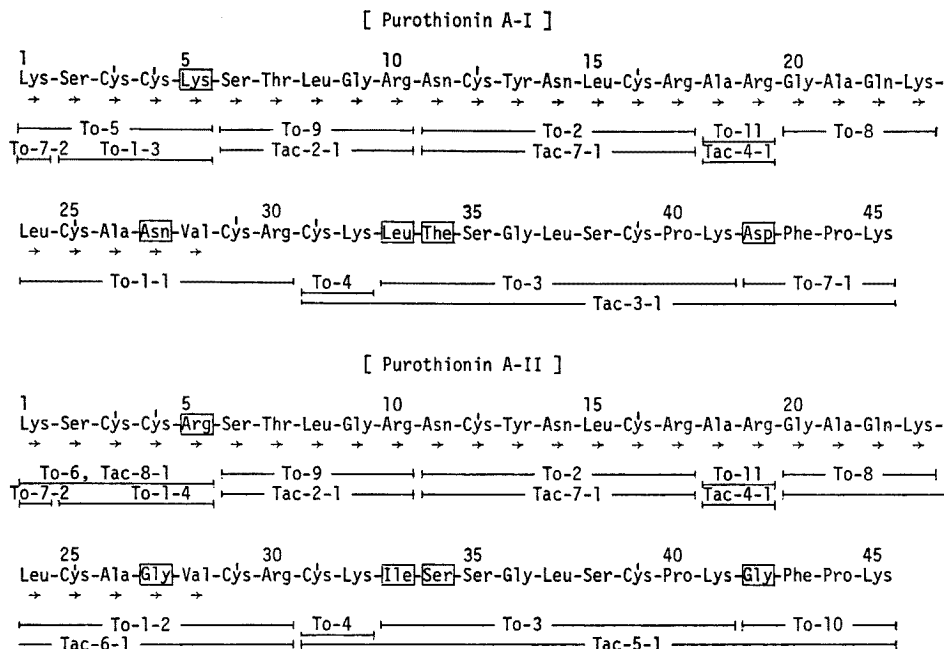


Fig. 12. Complete amino acid sequences of PA-I and PA-II. Arrows (→) just below the sequence represent sequencer analysis. Different residues are boxed (). For other symbols, see the text.

amino acid sequences of PA-I and PA-II are outlined in Fig. 12.

DISCUSSION

The modified, simpler purification method permitted higher recovery of the two forms of purothionin at the final step, purothionin A and B, in a 4 : 1 ratio, respectively. Although they showed similar specific activities and similar rates of migration on acrylamide gel disk electrophoresis, some differences were found in their amino acid compositions. Chemical and physical analysis of purothionin B will be reported in the near future. Purothionin (from soft flour "Snowdown") has also been separated into α and β by Redman and Fisher (4), but whether they are identical to our purothionin A and B is doubtful, because their amino acid compositions are different from ours.

Purothionin A (MW 11,300) was dissociated to monomers (MW 5,500) in 6 M guanidine HCl. Lysine was the only residue found at the N- and C-termini with recoveries of over 100% without

any correction during the procedures. These results gave the impression that purothionin A is a dimer composed of identical polypeptide chains. However, studies on the primary structure created the possibility of a nonidentical subunit structure. Twenty-eight cycles of automatic Edman degradation liberated single amino acid residues at each step except for the 5th and the 27th steps. Pairs of amino acids were obtained at these two steps with nearly equal recoveries (Lys and Arg at the 5th, and Asn and Gly at the 27th step). Alignment of all the tryptic peptides of PAox was made as shown in Fig. 9. The nearly equal recoveries of paired peptides; To-1-1 and To-1-2, To-1-3 and To-1-4, To-5 and To-6, and the presence of two C-terminal peptides, Tac-3-1 and Tac-5-1, support the proposed nonidentical dimeric structure.

Two subunits were isolated from the succinylated purothionin A chromatographically and electrophoretically, and their amino acid compositions were analyzed. Investigation of the differences in the amino acid compositions of these subunits, as shown in Table X, revealed that PAS-

I contains more Asx, Thr, Leu, and Lys whereas it contains less Ser, Gly, Arg and no Ile. These facts made deduction of the primary structures of PA-I and PA-II possible.

Strongly succinylated purothionin A was biologically inactive. Although the recoveries of PA-I and PA-II were nearly equal, it is uncertain whether the dimer is composed of PA-I and PA-II alone (homodimer), or the combined form of PA-I and PA-II (heterodimer). During the preparation of this paper, and after publishing a preliminary report of the primary structures of PA-I and PA-II (33), the amino acid sequence of wheat β -purothionin was elucidated by Mak and Jones (34), revealing its similarity to purothionin A-I and confirming our sequence.

Viscotoxins (35, 36), a group of pharmacologically active proteins for heart muscle, extracted from European mistletoe, have closely similar amino acid sequences to purothionins. It is of great interest that similar proteins have been extracted from two plants (wheat and European mistletoe) that are quite different from a taxonomical point of view. No free SH groups were found in purothionin A as well as in viscotoxins, and determination of the positions of intrapeptide disulfide bonds is now in progress.

The authors are grateful to Suntory Ltd., for approval to publish this paper and Dr. S. Senoh, Director of the Institute, for his encouragement. We wish to thank Prof. H. Matsubara, Faculty of Science, Osaka University, for his advice throughout this work. We also thank Prof. H. Wada, School of Medicine, Osaka University, for his advice and his permission to use the protein sequencer. Thanks are due to Dr. K. Kakiuchi, the Institute for Protein Research of Osaka university, for measurement of molecular weights.

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