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Modification of Phytolacain with TPCK

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Abstract

A radioactive tosyl-L-phenylalanine chloromethyl ketone (^{3}H-TPCK) was synthesized. When phytolacain was incubated with 25-fold molar excess of ^{3}H-TPCK for 60 min, the phytolacain activity was completely inhibited and about one mol of ^{3}H-TPCK was incorporated in one mol of phytolacain. The ^{3}H-TPCK modified phytolacain was then reduced, pyridylethylated and digested by lysyl endopeptidase. The radioactive peptide fragment was isolated and determined its amino acid sequence. The radioactive fragment contained 38 amino acid residues. The N-terminal amino acid sequence of the radioactive fragment was found to be Asn-Gln-Gly-Glu-PECys-Gly-Ser-X-Trp-Ala-(X did not detect as a PTH-amino acid). It was concluded that X was active cysteine residue and TPCK reacted with this residue.

Key words: TPCK, phytolacain, cysteine protease,

Phytolacain is a cysteine protease isolated from fruits of Pokeweed (*Phytolacca americana L.*) (*1*). It is well known that some cysteine proteases are inhibited by tosyl-L-phenylalanine chloromethyl ketone (TPCK) and tosyl-L-lysine chloromethyl ketone (TLCK) (*2*). In the case of serine proteases, TPCK and TLCK react with the active site histidine residues (*3, 4*). But in the case of cysteine proteases, TPCK dose not react with the active site histidine residue. When papain, which is a typical cysteine protease, was incubated with TPCK, one mol of sulfhydryl group disappeared and it was estimated that TPCK reacted with active cysteine residue (*2*). However, the fragment which contained TPCK modified active cysteine residue had not been isolated and it was not established by chemical method that TPCK reacted with the active cysteine residues of cysteine proteases. Then we synthesized radioactive TPCK and determined the modification site of phytolacain by TPCK.

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Results and Discussion

^{3}H-TPCK was prepared from ^{3}H-L-phenylalanine (3 mmol, 500 μCi) by the method of Schoellmann (*3*). The specific radioactivity of the obtained ^{3}H-TPCK was 2,000 dpm/10 μg. As shown in Fig. 1, phytolacain was completely inhibited with 20-fold molar excess of TPCK at a reaction time of 60 min. Then phytolacain (986 nmol) was modified with 25-fold molar excess of ^{3}H-TPCK for 60 min. The modified phytolacain was isolated by gel filtration and determined the amount of protein and radioactivity. The amount of protein obtained was 22.3 mg (892 nmol) and the radioactivity of 0.21 mg of modified phytolacain (8.4 nmol) was 600 dpm. The specific activity of ^{3}H-TPCK was 70 dpm/nmol, then the protein contained 8.6 nmol of ^{3}H-TPCK. Consequently, 1.02 mol of ^{3}H-TPCK was incorporated in one mol of phytolacain. This result indicated that complete inactivation was achieved upon the introduction of one molecule of TPCK per molecule of phytolacain.

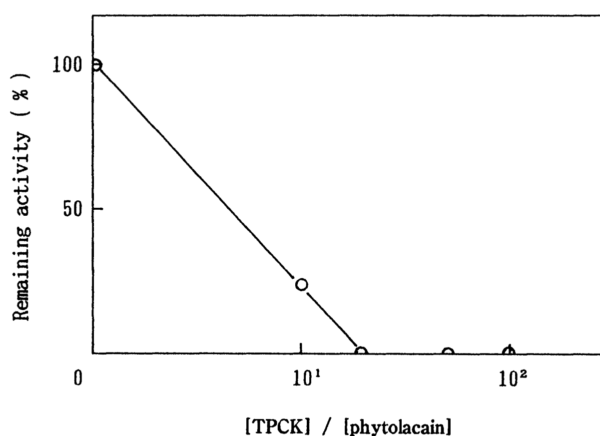

Fig. 1. Inhibition of phytolacain with TPCK. Phytolacain solution was incubated with various concentrations of TPCK solution for 60 min and remaining activity was measured.

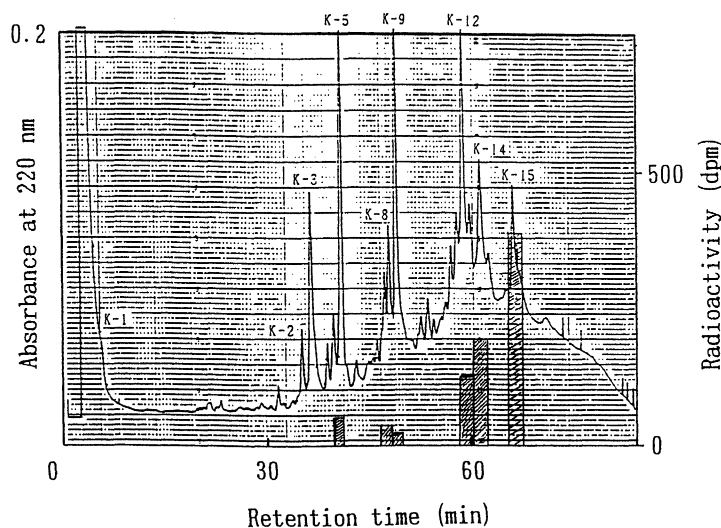

Fig. 2. The elution profile of lysyl endopeptidase digest of S-pyridylethylated ^{3}H-TPCK-phytolacain.

In order to determine the TPCK binding site, 21 mg (840 nmol) of modified phytolacain was reduced and pyridylethylated. And then, modified protein was digested by lysyl endopeptidase and resulting peptide fragments were separated with RP-HPLC using a C_8 column (Fig. 2). The radioactive fraction (k-14) was collected and rechromatogrphed. As shown in Table I, k-14 was made up of 38 amino acid residues. Then, the radioactive peptide was subjected to automated Edman degradation (Fig. 3). No amino acid PTH derivative was found at 8th cycle, this showed that the amino acid residue presented in position 8 of the radioactive fragment was modified by TPCK. The amino acid sequence around position 8 was analogous to that of active cysteine residues of other cysteine proteases (Fig. 4) (5-8). Then it was concluded that the amino acid residue at position 8 of the radioactive fragment was active cysteine residue of phytolacain and TPCK reacted with this residue.

Table I. Amino acid composition of radioactive fragment

Asx	4.4	Val	4.0
Thr	2.7	Met	0.9
Ser	2.9	Ile	2.2
Glx	4.5	Leu	3.0
Gly	5.2	Phe	1.0
Ala	3.4	Lys	1.0
1/2-Cys	2.5	Arg	2.5

Cycle	1	2	3	4	5	6	7	8	9	10
Detected Amino Acid	Asn-	Gln-	Gly-	Glu-	PECys-	Gly-	Ser-	X	-Trp-	Ala

Fig. 3. Amino acid sequence of N-terminal portion of radioactive fragment.

papain	: -Asn-Gln-Gly -Ser-Cys-Gly-Ser-Cys-Trp-Ala-
actinidin	: -Ser -Gln-Gly -Glu-Cys-Gly-Gly-Cys-Trp-Ala-
S. bromelain	: -Asn-Gln-Asn-Pro-Cys-Gly-Ala-Cys-Trp-Ala-
catepsin B	: -Asp-Gln-Gly -Ser-Cys-Gly-Ser-Cys-Trp-Ala-
catepsin H	: -Asn-Gln-Gly -Ala-Cys-Gly-Ser-Cys-Trp-Thr-
phytolacain	: -Asn-Gln-Gly -Glu-Cys-Gly-Ser- X -Trp-Ala-

Fig. 4. Amino acid sequence of active cysteine residue region of cysteine proteases.

Experimental

Synthesis of ^{3}H-TPCK

^{3}H-Tos-Phe (1) To a solution of ^{3}H-L-phenylalanine (495 mg, 3 mmol, 500 μCi) in 1 M NaOH (6.6 ml), was added tosyl chloride (690 mg, 3.6 mmol) in dioxane (5 ml) and the mixture was stirred for 4 h at room temperature, and dioxane was removed by evaporation. The solution was washed with ether and acidified by 6 M HCl. The resulting precipitate was collected by filtration and washed with cold water. The product was recrystallized from ethanol-water; yield, 798 mg (83%).

^{3}H-Tos-Phe-Cl (2) To the chilled suspension of compound 1 (798 mg, 2.5 mmol) in 16 ml of ether, was added PCl_5 (582 mg, 2.8 mmol) and stirred for 10 min at 0℃ and for 10 min at room temperature and for 1 h at 0℃. The resulting precipitate was collected by filtration, washed with ether and cold water; yield, 637 mg (72%).

^{3}H-Tos-Phe-CHN_2 (3) To the chilled suspension of compound 2 (636 mg, 1.8 mmol) in 18 ml of dry ether, was added CH_2N_2 (3.6 mmol) in cold dry ether (10 ml) and stirred for 1 night at 0℃. The solution was refluxed for 15 min and evaporated to dryness; yield, 620 mg (100%).

^{3}H-TPCK (4) The suspension of compound 3 (620 mg, 1.8 mmol) in dry ether (10 ml) was treated with hydrogen chloride for 2 h. The solution was evaporated and the resulting crystals were collected by filtration with the aid of petroleum ether. The product was recrystallized from 95% ethanol; yield 170 mg (27%).

Enzymic studies

Phytolacain was prepared in our laboratory. Radioactivity was measured in 2 ml scintillation solution (Scintisol EX-H, Dojin Chemical Co.) using a liquid scintillation counter Beckman LS-3150T.

Measurement of Enzyme Activity Three ml of Bz-Tyr-pNA solution (1mM, 0.05 M Tris-HCl buffer pH 7.2) was placed in a spectrophotometric cuvette and 0.1 ml of enzyme solution was added and the absorbance at 410 nm was measured continuously.

Inhibition of Phytolacain by TPCK The 0.5 ml of phytolacain solutions (20 μM, 0.05 M Tris-HCl buffer, pH 7.2) were incubated with 0.5 ml of TPCK solutions (methanol) of various concentrations for 60 min at 37℃ and the remaining activities were measured as described above.

Modification of Phytolacain with ^{3}H-TPCK To 36 ml of the phytolacain solution (27.4 μM, 0.05 M phosphate buffer, pH 7.2), was added 36 ml of ^{3}H-TPCK solution (685 μM, 0.05 M phosphate buffer, pH 7.2 containing 10% of methanol) and then incubated for 60 min at 37℃. The reaction mixture was lyophilized and the protein was separated by gel filtration on Sephadex G-25 column (1.8 × 86 cm) equilibrated with 1 M AcOH. The fractions containing protein were collected, lyophilized and the amounts of protein and radioactivity were measured.

Preparation of S-Pyridylethylated Modified Phytolacain The obtained ^{3}H-TPCK-phytolacain (21 mg, 0.82 μmol) was dissolved in 2.3 ml of 0.5 M Tris-HCl buffer, pH 8.5 containing 6 M

guanidine·HCl and 10 mM EDTA, and the solution was kept under N_2 gas for 30 min at 40℃. Then, dithiothreitol (46 mg, 300 μmol) was added and kept under N_2 gas for 3 h at 40℃. Then, 4-vinyl pyridine (94 μl, 900 μmol) was added and kept under N_2 gas for 2 h at 40℃. The mixture was dialyzed against 0.1 M acetic acid for 5 days. The solution was lyophilized; yield, 16 mg.

Enzymic Digestion The obtained PE-^{3}H-TPCK-phytolacain (7 mg) was dissolved in 0.7 ml of 50 mM Tris-HCl buffer (pH 9.0) containing 6 M urea. Digestion was initiated by addition of 0.7 ml of lysyl endopeptidase solution (0.04% by w/w, 50 mM Tris-HCl buffer, pH 9.0). The reaction mixture was incubated for 24 h at 37℃. The reaction was stopped by boiling for 1 min.

Separation of Radioactive Fragment The lysyl endopeptidase digests were chromatographed on a RP-HPLC C_8 column (Aquapore RP-300, 4.6 × 100 mm, Applied Biosystems) equilibrated in solvent A (0.1 % trifluoroacetic acid). The column was eluted for 5 min with solvent A and then with a linear gradient of from 0% solvent B [CH_3CN/2-propanol (3/7. v/v) containing 0.07% trifluoroacetic acid] to 40% solvent B for 40 min and to 60% solvent B for 20 min at a flow rate of 0.5 ml/min. The elute was monitored at 220 nm. Each peak was collected manually, and the peptides were recovered by lyophilization. Each radioactive component was further purified on the same conditions.

Amino Acid Composition and Amino Acid Sequencing Amino acid analyses were carried out on a HITACHI L-8500 analyzer. The peptide (approximately 3 nmol) was hydrolyzed in 6 M HCl containing 1% phenol for 24 h at 110℃. Automated Edman degradation was performed with an Applied Biosystems 470 A protein sequencer.

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