Bradykinin-Degrading Glycoprotein in *Aloe arborescens* var. *natalensis*

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Abstract: A homogeneous glycoprotein (aloe glycoprotein; mol. mass 40,000) containing 50.7 % of protein was isolated from an extract of *A. arborescens* var. *natalensis* by precipitation with 60 % ammonium sulfate. Aloe glycoprotein had bradykinin-degrading activity on an isolated guinea pig ileum *in vitro*. Peptide analyses using a reversed-phase, high performance liquid chromatography coupled with amino acid analysis showed that aloe glycoprotein cleaves the Pro^7-Phe^8 and Phe^5-Arg^9 bonds of the bradykinin molecule. The proteolytic action suggests that aloe glycoprotein has carboxypeptidase *N*- and *P*-like activity.

Introduction

The anti-inflammatory action of *Aloe* extracts has been reported but without sufficient pharmaceutical evidence (1). The bradykinin-degrading or anti-bradykinin activity of *Aloe arborescens* Mill. var. *natalensis* Berger was examined to validate this anti-inflammatory action. In this report, data on the isolation of a glycoprotein (aloe glycoprotein) with bradykinin-degrading activity on an isolated guinea pig ileum *in vitro* and a proteolytic activity against bradykinin are presented.

Material and Methods

Plant material

Fresh *Aloe* leaves, five to six years old, were harvested at the green house of the herbal garden of Kyushu University in June, 1983.

Methods of analysis

The protein and carbohydrate content of samples were determined by the Lowry (2) and phenol-sulfuric acid (3) methods. Elution of DEAE-cellulofine (Seikagaku Kogyo, Co. Ltd.) and Sepharose 6B (Pharmacia Fine Chemicals) columns was monitored by measuring the absorbance at 260 nm, and at 490 nm by the phenol-sulfuric acid method. Following column chromatography, the active glycoprotein was dialyzed overnight at 4° C against 20 mM phosphate buffer, pH 7.4. For amino acid analyses, samples were hydrolyzed in 6N HCl at 110° C for 20 h in evacuated sealed tubes. The hydrolysates were evaporated to dryness, dissolved in 0.02N HCl, and analyzed on an amino acid analyzer (Hitachi 835 amino acid analyzer). The molecular mass was determined from gel filtration on a Sepharose 6B column (1.5 × 51.5 cm) eluted with 0.3N NaCl at a flow rate of 26 ml/h. A series of dextrans, 10, 20, 40, 70, and T-500 (Pharmacia Fine Chemicals) was used as standards and the molecular mass was estimated from the linear correlation between the logarithm of the molecular masses of the standards and ratios of their elution volumes to the void volume of the column (4). Ultracentrifugation was performed on an analytical ultracentrifuge at 2 × 10^5 g with an optical system (Schlieren Spinco Model E, Beckman Instrument Inc.) in a single sector at 20° C. Analytical SDS-polyacrylamide gel (PAGE) was performed on 10 % acrylamide slab gels (10 × 13.7 × 0.2 cm) by the Laemmli method (5). Samples were suspended in a solution containing 1 % SDS, 25 mM Tris-HCl, pH 8.3, 2.4 M urea, and 1 % dithiothreitol, and the suspension was heated in boiling water for 5 min. After electrophresis the gels were stained with Coomassie brilliant blue and periodate-Schiff's (6) reagent. Aloe glycoprotein (200 µg) was hydrolyzed in 2N H_2SO_4 at 100° C for 3 h in a sealed tube. Excess sulfuric acid was removed by precipitation as barium sulfate. The filtrate was passed through a column of Dowex 50 W (H^+-form), and neutral and amino sugars were obtained. Gas chromatography of the trimethylsilylated sugars from the hydrolysate was performed on a column of 2 % silicone SE 52 (3 mm × 1 m) on Diasolid LP (60–80 mesh) at 160° C with a helium flow rate of 45 ml/min. The hexoses and hexosamines had the following retention times (min): mannose 5.30, 7.80; galactose 7.18; glucose 8.00, 12.6; in the hydrolysate, 5.30, 7.10, 7.30, 12.6; galactosamine 6.68; glucosamine 7.85; *N*-acetylglucosamine 14.0; in the hydrolysate 6.70, 7.90, 14.0. Acid hydrolysis of aloe glycoprotein (400 µg) was carried out in 6N HCl at 100° C for 6 h, and quantitative analysis of the hexosamine was done by the Elson-Morgan method (7).

High-performance liquid chromatography

Peptides were separated by a reversed-phase (TSK gel ODS-120A, Toyo Soda), high-performance liquid chromatography (HPLC) (Toyo Soda, HPLC-830A). The assay conditions were as previously described (8). Ten µg of bradykinin (1 mg/ml in H_2O) was incubated with 1 mg of aloe glycoprotein dissolved in 0.2 ml of 10 mM phosphate buffer, pH 7.4, at 30° C for 10 min. The reaction was stopped by heating at 100° C for 10 min and the mixture was then lyophilized. The dried sample was dissolved in 0.5 ml of H_2O and applied to the Sep-pak C_{18}-cartridge (Waters Associates Inc.). The sample adosorbed on the Sep-pak C_{18}-cartridge was washed with 2 ml of H_2O and then eluted with 80 % acetonitrile (2 ml) containing 0.1 % HCl. The solution was concentrated at 30° C by flash evaporation and lyophilized. The residue, dissolved in an appropriate volume of H_2O, was filtered through a millipore filter (Millipore Corp.) and applied to a reversed-phase column (300 × 4 mm) for HPLC. The column was eluted with a linear gradient (40 ml) of acetonitrile from 1 % to 40 %, containing 0.1 % HCl, at a flow rate of 1 ml/min. The peptide elution was monitored by measuring the absorbance of the effluents at 210 nm (Toyo Soda UV-8 Model 2). The elution volumes for des-Phe^8-Arg^9-bradykinin, bradykinin, and des-Arg^9-bradykinin were 23, 27, 31 ml, respectively.

Assay method

Bradykinin-degrading or anti-bradykinin activity was estimated from the biological assay on an isolated guinea pig ileum as previously reported (8). Briefly, a 2 to 3 cm strip of guinea pig ileum was suspended in 10 ml of magnesium ion-free Tyrode solution which was oxygenated with oxygen/carbon dioxide (95 : 5) in an organ bath at 30° C. The contractile responses of various concentrations of bradykinin were measured with a mechanoelectric transducer equipped with a recorder for 45 sec after injection. At the beginning of the assay, responses to a series of four doses of bradykinin (usually 25, 50, 75, and 100 ng dissolved in 1 ml of 10 mM phosphate buffer at pH 7.4) were checked.

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and each of them was repeated two or three times to obtain a standard dose-response curve. For measurement of bradykinin-degrading or anti-bradykinin activity, 0.2 ml of bradykinin solution (1 μg/ml) was incubated with 0.2 ml of a sample for 5 min at 30° C, and the contractile response by 0.2 ml of this mixture (100 ng bradykinin equivalent) was measured. When the activity of a sample was so potent that more than 70 % of bradykinin was inactivated, the sample was diluted with the same buffer to give at least 30 ng of bradykinin remaining after 5 min of incubation.

Extraction and purification of the bradykinin-degrading glycoprotein

The fresh leaves (7 kg) were homogenized and centrifuged at 9×10^3 g for 30 min. The supernatant was precipitated with 20 % and 60 % ammonium sulfate, and each precipitate was dialyzed (Visking tube, Union Carbide Co.) against distilled water for 48 h. Each nondialyzable fraction was lyophilized to a colorless material. The crude material from the 20 % ammonium sulfate precipitation was dissolved in 0.02M NH_4HCO_3, pH 7.8, and applied to a column of DEAE-cellulofine (500 ml, 4.5 × 28 cm). The column was eluted with the same solution at a flow rate of 36 ml/h. The pooled eluate was dialyzed and lyophilized to a colorless polysaccharide fraction (217 mg). The column was further eluted with 0.3M NaCl in 0.02M NH_4HCO_3, and the pooled eluate was dialyzed and lyophilized to a pale brown glycoprotein fraction (47 mg). The crude material precipitated at 60 % ammonium sulfate saturation was treated by the same procedure and yielded a colorless polysaccharide fraction (1.3 g) and a pale brown glycoprotein fraction (1.7 g). The glycoprotein fraction obtained from the 20 to 60 % ammonium sulfate precipitation was dissolved in 0.02 M NH_4HCO_3 containing 0.3M NaCl and applied to a column of Sepharose 6B (4.5 × 27.5 cm). The column was eluted with the same solution. The effluents were fractionated into two portions. Each was dialyzed and lyophilized to obtain glycoprotein fractions 1 (44 mg) and 2 (212 mg).

Chemicals

Bradykinin and des-Arg^9-bradykinin were obtained from the Protein Research Foundation, Osaka and bromelain was purchased from Nakarai Chemical Co. Ltd., Kyoto, Japan.

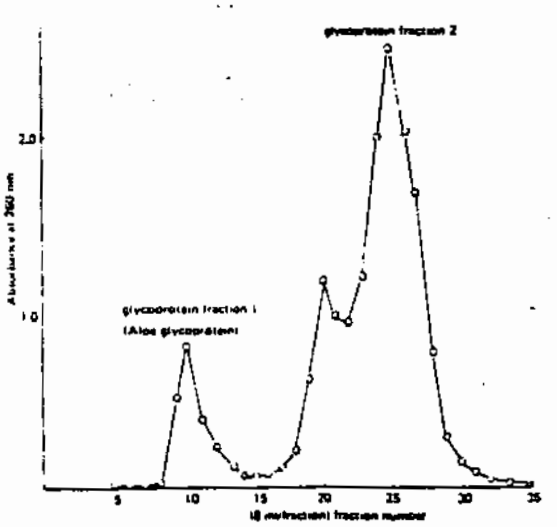

Fig. 1. Chromatography of 20–60 % ammonium sulfate precipitate

Results and Discussion

Extraction and concentration of the bradykinin-degrading aloe glycoprotein

The nondialyzable fraction from the supernatant inhibited the contractile response to bradykinin by an isolated guinea pig ileum *in vitro*, but the dialyzable fraction did not show any bradykinin-degrading or anti-bradykinin activity. Since it had been previously reported that the crude extract contained an anti-tumor aloemannan with a molecular mass greater than 10,000, the neutral polysaccharides were separated (9). After removal of the neutral polysaccharide fraction from the 20 to 60 % ammonium sulfate precipitation by gel filtration on DEAE-cellulofine, the glycoprotein fraction was isolated. As shown in Table I, bradykinin-degrading activity appeared in the crude glycoprotein fraction, which was composed of carbohydrate (34 %) and protein (47.4 %). Further purification of this crude glycoprotein fraction was accomplished by gel-filtration on Sepharose 6B to obtain glycoprotein fractions 1 and 2. Glycoprotein fraction 1 (carbohydrate 28.5 % and protein 50.7 %, aloe glycoprotein) had a specific activity 10-times higher than that of the crude nondialyzable extract. Glycoprotein fraction 2 was inactive. Bromelain (10) was used as a positive control and showed activity similar to aloe glycoprotein.

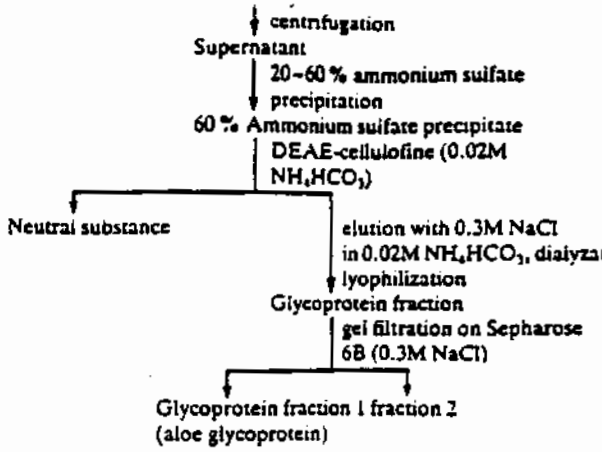

Chart 1. Fractionation scheme of aloe glycoprotein

Characterization of the bradykinin-degrading glycoprotein (aloe glycoprotein)

The molecular mass was estimated to be 40,000 by gel-filtration on Sepharose 6B with a series of dextrans as standards. The bradykinin-degrading glycoprotein (24 mg in 0.02 M NH_4HCO_3 (2 ml) showed a single symmetrical peak with $S_{20,w} = 3.34$ S on ultracentrifugation, and thus appeared to be homogeneous. On vertical slab electrophoresis with 10 % SDS-PAGE at pH 8.8, aloe glycoprotein showed a single band when stained with Coomassie brilliant blue or periodate-Schiff's reagent. Gas chromatographic analysis of the hydrolysate of aloe glycoprotein showed the presence of mannose, galactose, glucose, glucosamine, galactosamine, and *N*-acetylglucosamine in a ratio of 2:2:1:1:4:1. The hexosamine content was 5 %. Amino acid analysis of aloe glycoprotein revealed the following amino acids (%): Asp (6), Thr (4), Ser (12), Glu (8), Gly (15), Ala (9), Val (8), Ile (5), Leu (8), Phe (4), Lys (10), Arg (4), and Pro (7). The chemical and physical properties of aloe glycoprotein were very similar to aloelectin (11).

Activity of the bradykinin-degrading aloe glycoprotein

In a preliminary experiment the degradation of bradykinin with time was determined (5, 10, 15, 20, 30, and 60 min). The half

Table I. Bradykinin-degrading activity of fractions on an isolated guinea pig ileum

Sample	Carbohydrate (%)	Protein	Unit/g[a]	Ratio	Yield (%)
Crude Extract (Nondialyzable material)	50.0[b]	23.6	130.4	1.00	10^{-1}
20 % Ammonium sulfate precipitate					
0.02 M NH_4HCO_3 eluate	78.0	0.2	16.0	0.12	3×10^{-3}
0.3 M NaCl eluate	34.7	40.8	1148.0	8.8	6×10^{-4}
20 to 60 % Ammonium sulfate precipitate					
0.02 M NH_4HCO_3 eluate	94.7	3.0	131.0	1.01	10^{-2}
0.3 M NaCl eluate	34.0	47.4	1020.0	7.85	2×10^{-2}
Glycoprotein fraction 1 (aloe glycoprotein)	28.5	50.7	1400.0	10.7	4×10^{-4}
fraction 2	20.3	51.8	87.0	0.66	6×10^{-4}
Bromelain[c]			1180.0	9.05	

[a] One kininase unit is defined as the amount of enzyme necessary to degrade 1 μg of bradykinin in 1 min at 30° C at pH 7.4. For statistical purposes, 4–5 guinea pigs were used for each assay.

[b] The content of carbohydrate and protein does not account for 100 % of the material. This may be due to water, since this polymer is extremely hygroscopic.

[c] See Ref. (10).

time for substrate degradation was about 7 min. Bradykinin dissolved in 10 mM phosphate buffer, pH 7.4, was incubated with aloe glycoprotein at 30° C for 10 min. After the Sep-pak C_{18}-cartridge and millipore treatments, the lyophilized metabolite was subjected to HPLC on a reversed-phase column. Three major peaks were detected at elution volumes of 23,27, and 31 ml. The peaks were collected and amino acid analysis showed the following compositions: Ser, Gly, Phe, Arg, Pro, (1:1:1:1:3); Ser, Gly, Phe, Arg, Pro (1:1:2:2:3); and Ser, Gly, Phe, Arg, Pro (1:1:2:1:3); respectively. These compositions are consistent with the structures: Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7; Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9 (bradykinin); and des-Arg (Arg^1 or Arg^9)-bradykinin, respectively. The peak at an elution volume 31 ml was identified as des-Arg^9-bradykinin by cochromatography with a des-Arg^9-bradykinin standard on HPLC. When a sample of des-Arg^9-bradykinin was incubated with aloe glycoprotein, a peak of des-Phe^8-Arg^9-bradykinin was detected on HPLC. These results indicated that aloe glycoprotein cleaves the Phe^8-Arg^9 and Pro^7-Phe^8 bonds of the bradykinin molecule. No peaks due to the two major metabolites were detected on HPLC in the incubation mixture of bradykinin with glycoprotein fraction 2. Proteases of plant origin, bromelain, papain, and ficin, cleave the Gly^4-Phe^5 and Phe^5-Ser^6 bonds of the bradykinin molecule, whereas both shimeji kininase (12) from mushrooms and kinonase AI (13) from microbes cleave the Gly^4-Phe^5 and Pro^7-Phe^8 bonds. These experiments demonstrated the proteolytic activity of aloe glycoprotein, which contains protein, carbohydrate, and hexosamine (50.7 %, 28.5 % and 5 %, respectively), against bradykinin to produce des-Phe^8-Arg^9- and des-Arg^9-bradykinin, suggesting the presence of carboxypeptidase *N*- and *P*-like enzymes (14). These results may provide a pharmaceutical basis for the anti-inflammatory action of *Aloe arborescens* var. *natalensis*.

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