ISOLATION OF AN ACTIVE GLUCOSIDE IN BRACKEN FERN, *PTERIDIUM AQUILINUM*

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Abstract...An active glucoside that enhanced release of histamine from rat peritoneal mast cells was isolated from a toxic fraction of bracken fern, *Pteridium aquilinum*, by partition column chromatography on Sephadex G-25. It was a yellowish amorphous powder on freeze-drying, readily soluble in water or ethanol, and insoluble in ether. It was hydrolysed with β-glucosidase to release D-glucose. It was assumed to be a kind of β-glucopyranoside with a carboxyclic structure in the aglycone moiety.

Key words: bracken fern, *Pteridium aquilinum*, glucoside, histamine release, mast cell.

INTRODUCTION

Bracken fern (*Pteridium aquilinum*), abbreviated BF, is known to induce serious poisoning in domestic animals. Cattle bracken poisoning, of which the characteristic findings are edema and hemorrhage with a blood coagulation disorder, has been extensively investigated (Heath and Wood, 1958; Umeda et al., 1963; Evans, 1964; Kitahara et al., 1968; Yamane et al., 1975). However, no active substance has yet been isolated. Among experimental animals, the guinea pig was reported to be most susceptible to bracken poisoning, with edema and hemorrhage in the urinary bladder as in cattle (Maeda, 1975; Morita et al., 1976, 1977; Ushijima et al., 1983). The clinical symptoms resemble an early stage of inflammation, with histamine release from mast cells. In previous investigations, we found that acetone-dried powder and water and ethanol extracts from BF caused the release of histamine from rat peritoneal mast cells *in vitro*, and we suggested that the active principles were glucosides (Ishii et al., 1974, 1979; Saito et al., 1979). Furthermore, several glucosides were separated from water and ethanol extracts by column gel chromatography on Sephadex G-10, but no histamine-releasing activity was detected, probably because the glucosides were denatured during the purification procedures (Saito et al., 1979). Recently we obtained a toxic glucoside...
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fraction from the ethanol extract by n-butanol-water partition column chromatography on Sephadex G-25, which produced symptoms in guinea pigs similar to those in cattle with bracken poisoning (Saito et al., 1982).

We did this experiment to get more direct evidence of histamine release caused with glucoside from BF.

MATERIALS AND METHODS

Crude extract of bracken fern: The stems and young folding fronds of fresh BF were collected from the Mitani farm in a rural area of Tottori City in Japan, where bracken poisoning in cattle has occurred. The plants were stored in a freezer at −20°C. The stored BF was freeze-dried and milled to a fine powder before use. About 990 g of the powder was obtained from 8.3 kg of raw material. The procedures of extraction and fractionation are shown in Fig. 1. One hundred grams of the dried BF material was extracted twice, each with 1 liter of ice-cold ethanol, with mixing for 20 min, and the mixture filtered with suction. The pooled filtrate was vacuum dried below 0°C and then dissolved in 100 ml of ice-cold distilled water. Insoluble materials were removed by filtration. The soluble fraction was then freeze-dried, and this material was called the crude extract (BE). About 3 g of the BE was obtained from 100 g of powdered BF material.

Fig. 1. Flow diagram illustrating preparation of bracken fern fractions and glucoside.
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Partition chromatography: Sephadex G-25 (superfine, Pharmacia Fine Chemicals, Sweden) was used as the bed material. The chromatographic gel bed (2.5 × 30 cm) was equilibrated with the water phase of a mixture of n-butanol–water (1:1). Some or all of the BE or its fraction was dissolved in 2 ml of the organic phase of the same mixture, applied to the column, and eluted with the organic phase at a flow rate of 13 ml/hr. Fractions (2.5 ml) were collected using a fraction collector (SF-160K, Toyo-Kagaku, Japan), and vacuum dried. An aliquot of each fraction was used for the determination of ultraviolet absorbance at 260 nm and sugar content. The determination of sugar content was performed according to a colorimetric method (Dubois et al., 1956).

Ascending paper chromatography: This was done to identify the sugar in the glucosides isolated from BF. The isolated glucosides were treated with an enzyme, β-glucosidase (sweet almond, P–L Biochemical, USA), before paper chromatography. It was developed with a system of n-butanol–acetic acid–water (4:1:5) and sugar spots were revealed with AgNO₃ reagent.

Determination of histamine release: Male Wistar albino rats weighing 300-400 g were used. The animals were anesthetized with ether and bled by cutting the carotid arteries. Then 20 ml of a 9.25% sucrose solution containing 40 μM EDTA were injected into the peritoneal cavity and the abdomen was gently massaged for 2 min. The peritoneal cavity was opened by cutting the abdominal wall, and then the peritoneal washings were collected and filtered with a metallic filter (25 μm pore size). The peritoneal washings from two animals were pooled and centrifuged at 1600 rev/min (470 × g) for 5 min. The resultant pellet was resuspended in 2 ml of ice-cold 0.9% NaCl solution containing 2.15 mM phosphate buffer (pH 6.8). This cell suspension was divided into 10 samples (0.2 ml). Each sample contained approximately 1.8 × 10⁷ mast cells. The extracts or isolated glucosides were dissolved in an ice-cold saline solution containing phosphate buffer, 0.2 ml of the test solutions was added and the mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 3.6 ml of ice-cold 0.9% saline solution, and each sample was centrifuged at 3000 rev/min (1660 × g) for 5 min. The remaining histamine in the pellet was extracted with 1 ml of 0.4 NHClO, for 3 min, and each suspension had 3 ml more of distilled water added before being centrifuged at 3000 rev/min (1660 × g) for 10 min. Each supernatant was used in determining histamine content.

Histamine was purified using an anion exchange resin (Dowex 1×8, Dow Chemical, USA) according to the method described by Ando et al. (1973) with minor modifications. The glass column (0.8 × 25 cm) was packed with 6 ml of anion exchange resin. The sample was applied on the column and eluted at a flow rate of 2.5 ml/min using a micro-tube pump (MP-1001 Tokyo-Rika, Japan). Histamine in the eluate was determined fluorometrically according to the method described by Shore et al. (1959). The amount of histamine released during incubation was calculated from the reduction in the initial histamine content in sample, and expressed as a percentage.
RESULTS

Separation of glucosides: The crude extract (BE) from bracken fern was dissolved in 2 ml of the n-butanol phase of a n-butanol-water (1:1) system, applied on a Sephadex G-25 gel column that had been equilibrated with the water phase of that system, and eluted with an n-butanol phase followed by a water phase. Figure 2 shows the separation of the glucosides from a glucide mixture. The elution pattern of glucide with the n-butanol phase was approximately parallel with that of UV absorbance at 260nm, whereas with water phase it was not. The pooled eluate with the n-butanol phase (20 - 125 ml in elution volume) was referred to as BE-I and that with the water phase as BE-II.

Fig. 2. Elution patterns of UV absorbance and glucide of the crude extract (BE) by partition column chromatography on a Sephadex G-25. Sample: 72 mg of the BE. Column: 2.5 × 30 cm. Solvent: n-butanol-water (1:1). Flow rate: 13 ml/hr. Fraction volume: 2.5 ml. UV absorbance (260 nm) was measured with effluent which was diluted 12.5 times with water.

Histamine-releasing activity of the crude extract (BE) and of the fractions (BE-I, BE-II) obtained by partition column chromatography. BE-I represents the fraction eluted with butanol phase. BE-II represents the fraction eluted with the water phase. The histamine release was calculated as a percentage of the total histamine content of each cell sample. Each value represents the mean ± S.E. of 8 observations.
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BE-II (180-260 ml in elution volume). The BE-I fraction was found to have a potent histamine-releasing activity on rat peritoneal mast cells at a concentration of $10^{-2}$ g/ml, whereas the BE-II had little up to $4 \times 10^{-2}$ g/ml (Fig. 3); thus, the activity in BE-I was concentrated some 8 times. The yield of BE-I was approximately 7% in dry weight of the BE.

Isolation of active glucoside: In five separate experiments the BE-I fractions, obtained by the same kind of partition column chromatography of BE, were divided successively into 8 subfractions (A-H), and the histamine-releasing activity of each subfraction was assayed. Figure 4 shows the distribution of histamine-releasing activity in the BE-I fractions. Most of the activity was recovered in subfraction D and E. These active subfractions were further reapplied on the same kind of partition column chromatography, and two glucoside fractions (D-I and D-II) were obtained from

Fig. 4. Distribution of the histamine-releasing activity and elution patterns of UV absorbance and dry weight of BE-I fractionated by partition column chromatography. The chromatographic separation was carried out as that shown in Fig. 1. Each value represents the mean ± S.E. of 5 observations.

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Fig. 5. Histamine-releasing activity and elution pattern of UV absorbance of subfractions D and E by partition column chromatography on Sephadex G-25. The chromatographic procedure and the measurement of UV absorbance were carried out as that shown in Fig. 2. Sample: subfraction D, 11 mg; subfraction E, 13 mg. In histamine-releasing activity each value represents the mean ± S.E. of measurement from four experiments made on different preparations. Dose: $1.5 \times 10^{-2} \text{g/mL}$. **: statistically significant at $p<0.01$ as compared with saline response.

Fig. 6. Paper chromatogram of the active glucoside obtained by repeated chromatography of subfraction E. Chromatogram developed with n-butanol-acetic acid-water (4:1:5, upper layer). Glucide detected with silver nitrate acetone. A: glucose. B: the active glucoside. C: the active glucoside hydrolysed by $\beta$-glucosidase.
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subfraction D and a single glucoside fraction (E-III) from subfraction E. These glucoside fractions were collected only at the sides of the peak of UV absorbance (shaded area in Fig. 5) to have less contamination. The histamine-releasing activity was recovered mostly in the E-III fraction of glucosides, whereas the D-I and D-II had slight or no activity (Fig. 5).

Some properties of the active glucoside: The active glucoside was purified by repeating the partition column chromatography of the E-III fraction several times. When freeze-dried, it was a slightly yellowish powder, was readily soluble in water, methanol, ethanol, and n-butanol, soluble in acetone and ethyl acetate, and insoluble in ethyl ether, chloroform, and benzene.

To confirm that the active substance is a glucoside, the isolated active glucoside and its hydrolysed sample were subjected to paper chromatography with a solvent system of n-butanol-acetic acid-water (4 : 1 : 5). As shown in Fig. 6, the active glucoside gave only one spot at Rf about 0.8 and the hydrolysed glucoside gave a spot corresponding to that of D-glucose. The UV spectra of the isolated active glucoside in water or ethanol showed an absorption maximum at 315 nm. The active glucoside was relatively stable in acidic, but not alkaline solution.

DISCUSSION

We demonstrated that water or ethanol extracts of BF had released histamine from rat peritoneal mast cells in vitro, and that several glucosides were separated by gel filtration on a Sephadex G-10 using water or phosphate buffer (pH 6.5) as the eluent. However, we failed to detect histamine release with these glucosides (Ishii et al., 1979; Saito et al., 1979). We later found that the glucosides in BF were conveniently and effectively separated by n-butanol-water partition column chromatography on Sephadex G-25, and demonstrated that the glucoside fraction (BE-I) had acute toxicity in guinea pigs, producing the characteristic symptoms (Saito et al., 1982).

In these experiments, the BE-I fraction appeared to be composed of about 5 kinds of glucosides with similar partition coefficients (Fig. 2). The activity in the BE-I fraction was recovered in subfractions D and E. The active glucoside was purified from subfraction E by repeating the partition column chromatography, whereas the activity of the subfraction D appears to be due to contaminating active glucoside. Paper chromatography showed that the active glucoside had D-glucose as a sugar component (Fig. 6). The UV spectrum of the active glucoside had an absorption maximum at 315 nm. These results suggest that the active glucoside is a β-glucopyranoside with a carbocyclic structure in the aglucone moiety and is structurally somewhat different from active principle in rhizomes of BF.

It is known that the toxicity of BF for cattle and guinea pigs is unstable and decreases on drying in air or outdoors. In our previous work (Saito et al., 1982), the activity of the purified glucoside was completely lost during gel filtration. In these experiments, the preparation of the crude extract and the purification of the active
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glucoside were carried out at as low temperature as possible. However, considerable loss in the activity was observed during purification, particularly in the process of repeated partition column chromatography on Sephadex G-25. These facts suggest the active principle in BF is very unstable even in mild conditions, though the cause of loss in activity is not known. Also, the active glucoside may not be the only toxic component, although it is an active principle in BF.

It has been reported that the characteristic findings of bracken poisoning in cattle are edema and hemorrhage of the mucosa or submucosa with a blood coagulation disorder (Heath and Wood, 1958: Umeda et al., 1963: Evans, 1964: Kitahara et al., 1968). Similar characteristic symptoms were observed in guinea pigs fed bracken (Maeda, 1975: Morita et al., 1976, 1977: Ushijima et al., 1983), and by single oral administrations of the BE-I fraction (Saito et al., 1982). Thus the active glucoside could be responsible for the acute toxicity in guinea pigs and probably in cattle, and these clinical symptoms are probably mediated by the release of histamine, heparin, and other granule-associated mediators from the mast cells.

BF is also known to be carcinogenic in various animals (Evans and Mason, 1965: Widdop, 1967: Pamukcu et al., 1967: Pamukcu and Price, 1969: Hirono et al., 1970: Pamukcu et al., 1972: Yoshikawa et al., 1981). However, the nature of the carcinogenic principle in BF has not been appreciably elucidated, although shikimic acid (Evans and Osman, 1974) or tannin (Wang et al., 1976) have been isolated from BF as carcinogens. Recently, as a new carcinogenic glucoside, ptaquiloside (Niwa et al., 1983a,b; Hirono et al., 1984) and as a new mutagenic glucoside, aquilide A (Van der Hoeven et al., 1983) have been isolated from BF, respectively. Almost the same chemical structure of both the glucosides has been described independently. Ptaquiloside was isolated by different method from ours: it has been extracted with boiling water, but our active glucoside was extracted with ice-cold ethanol because of the appreciable inactivation during extraction with hot water. There also found some difference in stability under various pH medium: it was reported to be unstable under both acidic and basic conditions, but our active glucoside is relatively stable under acidic, but not under basic conditions. On the other hand, aquilide A was reported to be extracted with light petroleum and methanol and to be activated by alkaline treatment for mutagenicity. Further analysis of our active glucoside was difficult because of its very instability, but we succeeded in isolation and identification of active principleples in the rhizomes of BF, which were found to be structurally somewhat different from the those in the fronds of BF (unpublished data).

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REFERENCES


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