Journal of Chromatography, 489 (1989) 384–389
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4644

Note

Purification of the decomposing enzyme from *Nepenthes alata* against glycophorin B of human red blood cells by high-performance liquid chromatography

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(First received August 16th, 1988; revised manuscript received December 7th, 1988)

Nepenthes alata is a tropical insectivorous plant with pitchers in which insects are caught and digested. The secretion from these pitchers has been shown to contain some properties of proteolytic enzymes [1,2]. We have already reported the alteration of blood group antigens on human red blood cells (RBCs) treated by the *Nepenthes* extract [3]. These changes suggested that several enzyme activities were included in the extract. However, few studies were made on the secretion and extract from *Nepenthes* in relation to RBCs. Treatments of RBCs with various enzymes have been recognized to be useful in structural studies on the antigens of RBC membranes [4–7]. In this study we report the purification, by means of high-performance liquid chromatography (HPLC), of the decomposing enzyme against glycophorin B antigen from the *Nepenthes* extract.

EXPERIMENTAL

Nepenthes extraction

Leaves (160 g) of *N. alata* were extracted with 480 ml of distilled water with a blender for 10 min. After centrifugation at 1500 *g* for 15 min, powdered ammonium sulphate was added to the supernatant with stirring until 100% saturation. The supernatant was centrifuged, and the precipitate was diluted in 20 m*M* phosphate buffer (pH 7.5) and dialysed against the same buffer.

Size-exclusion high-performance liquid chromatography

HPLC analyses were performed with a Model 342 gradient liquid chromatographic system (Beckman, Berkeley, CA, U.S.A.). The column was a TSK-GEL

0378-4347/89/$03.50

3000SW (600 mm × 7.5 mm I.D., Toyo Soda, Tokyo, Japan). The system included Model 114M pumps, a loop-type 210A injector and a Model 160 UV variable-wavelength detetor operated at 280 nm (all from Beckman). The eluent was a phosphate buffer (20 m*M*, pH 6 or 100 m*M*, pH 7), and the injection volume was 100 μl. The molecular mass marker was obtained from Bio-Rad (Richmond, CA, U.S.A.).

Isoelectrophoretic analysis

All electrophoresis material was purchased from Pharmacia (Uppsala, Sweden). Gels, 0.5 mm thick and containing 7.3% acrylamide and 7.6% ampholine (pH 3.5–9.5), were prepared, and 15-μl samples were applied and run for 1.5 h at a constant current of 11 mA. The system was operated at a constant voltage of 1300 V for 3 h according to Righetti [8], and the gel was stained with a silver-staining kit (Wako, Tokyo, Japan).

Treatment of red blood cells by the Nepenthes extract

One volume of packed RBCs was washed, then incubated with ten volumes of *Nepenthes* extract for 2 h at 37°C. The RBCs were washed four times with saline and used for further experiments as *Nepenthes*-treated RBCs in 3% suspension.

Agglutination test

Monoclonal anti-glycophorin B antibody produced in our laboratory was used [9]. One drop of 3% RBC suspension was mixed with one drop of anti-glycophorin B antibody in a test-tube, and incubated according to the method of the American Association of Blood Banks [10]. The mixture was maintained at 37°C for 30 min, then centrifuged at 100 *g* for 1 min before the agglutination was tested. The activity of the enzyme was expressed as the decrease of the titre, the reciprocal of the greatest dilution of the extract at which the agglutination with monoclonal anti-glycophorin B antibody could be detected.

RESULTS AND DISCUSSION

The enzymes from *Nepenthes* leaves were extracted with distilled water or phosphate buffer. This is the method usually used to extract the enzyme. After this step, water or phosphate buffer was used as the mobile phase in the size-exclusion HPLC purifications. When distilled water was used, the resolution was very poor (Fig. 1A). In contrast, phosphate buffer with a molarity of 20 or 100 m*M* and a pH of 6 or 7, respectively, gave good resolution of the components of the crude extract (Fig. 1B and C).

Low flow-rates generally give better resolution. Two flow-rates, 0.5 and 0.1 ml/min, were tested; the latter produced a broad peak whereas the former gave rapid resolution, and so was adopted for further experiments.

Final purification schedule

Two purification cycles on the TSK-GEL 3000SW column were effective in obtaining the main enzyme from *Nepenthes* (Fig. 2). The position of the enzyme

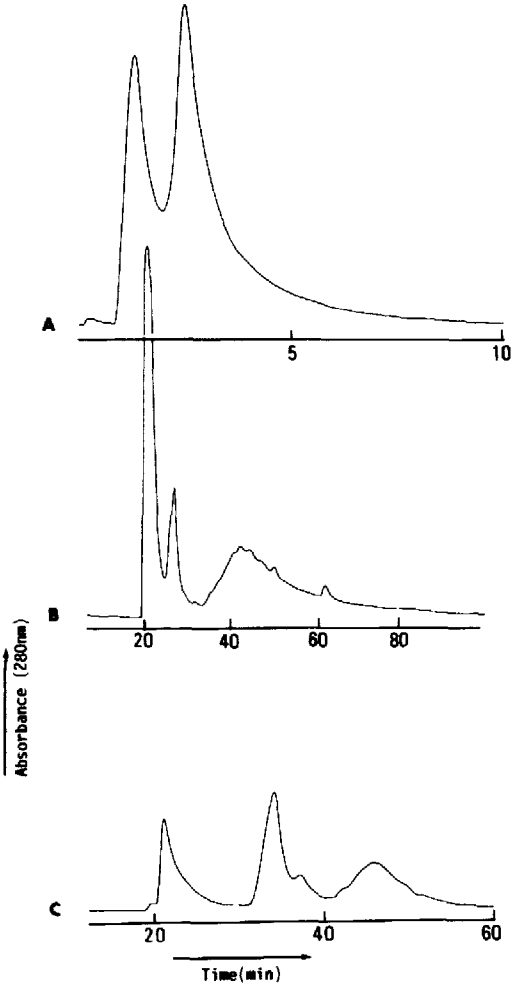

Fig. 1. HPLC of the *Nepenthes* extract fractionated by ammonium sulphate on a TSK-GEL 3000SW column. Eluent: (A) distilled water; (B) 20 m*M* phosphate buffer (pH 6); (C) 100 m*M* phosphate buffer (pH 7).

was determined by performing agglutination tests of the monoclonal anti-glycophorin B antibody against RBCs treated with each peak (Table I). The enzyme from the shaded peak in Fig. 2B was concentrated ca. 50-fold with Minicon (Amicon, Danvers, MA, U.S.A.), and run again on the same column. The molecular mass of this peak, as determined by comparison with molecular mass standards (proteins), was ca. 784 000 (Fig. 3).

Isoelectric focusing

In order to characterize further the purified enzyme from a biochemical point of view, we analysed it by polyacrylamide gel isoelectric focusing using silver staining for detection (Fig. 4). The isoelectric point of this enzyme was estimated to be a. 5.2.

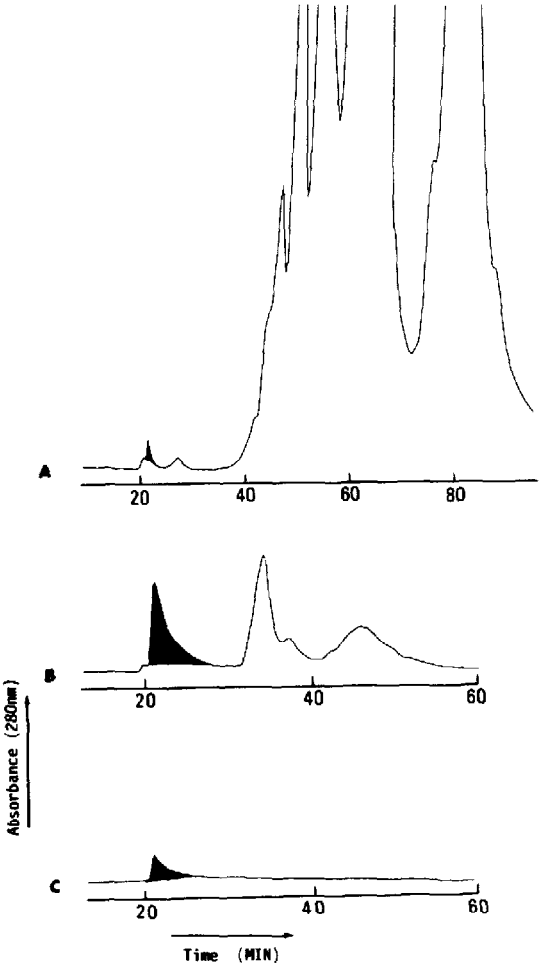

Fig. 2. Final enzyme purification scheme; the shaded area in A was precipitated by ammonium sulphate and recycled in the run shown in B. The shaded area in B contains the enzyme, the purity of which was checked in C.

TABLE I

AGGLUTINATION TEST OF THE TREATED RBCs WITH MONOCLONAL ANTI-GLYCOPHORIN B ANTIBODY

Peak number in HPLC of the extract of *Nepenthes*	Hemagglutination titre		
	×2	×4	×8
1	−	−	−
2	+	+	−
3	+	+	−
4	+	+	−

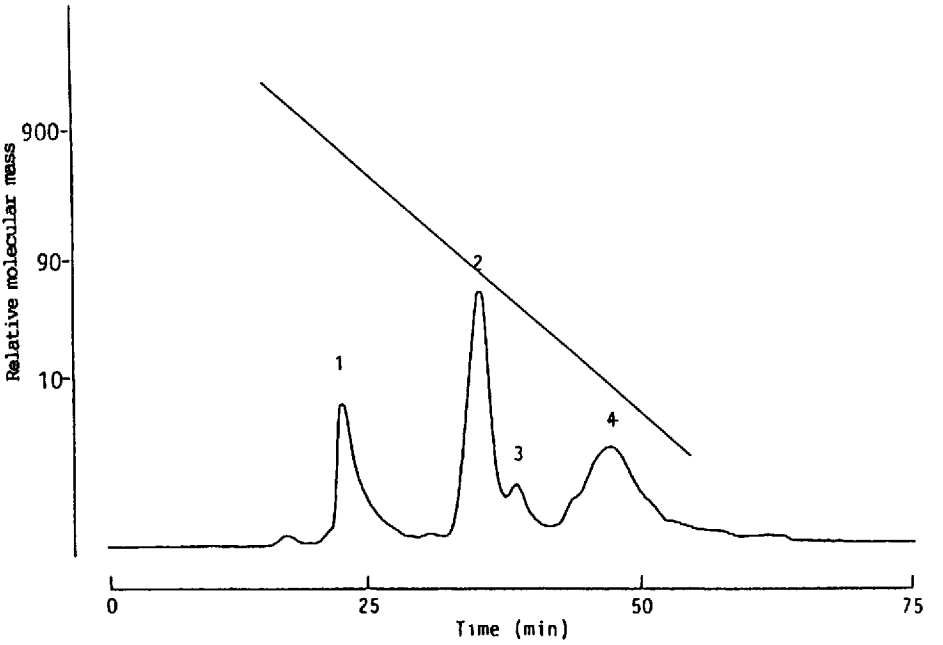

Fig. 3. Molecular mass estimations of the *Nepenthes alata* enzyme. See text for details of the chromatographic separation. The column was calibrated with (A) immunoglobulin M (M_r 900 000), (B) thyroglobulin (M_r 670 000), (C) immunoglobulin G (M_r 158 000), (D) ovalbumin (M_r 44 000), (E) myoglobulin (M_r 17 000) and (F) vitamin B_{12} (M_r 1350). The molecular masses of individual peaks were estimated to be ca. (1) 784 000, (2) 83 600, (3) 47 300 and (4) 10 600.

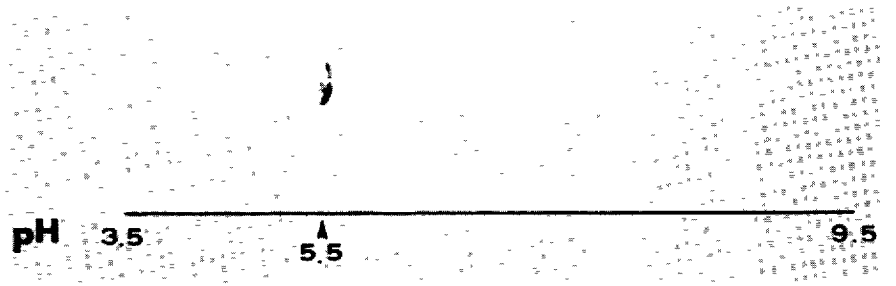

Fig. 4. Isoelectric focusing of the enzyme purified by HPLC; detection by silver staining. The isoelectric point is indicated by an arrow.

In this study we separated the enzyme decomposing glycophorin B and determined its molecular mass and isoelectric point. It appears that the *Nepenthes* extract has other enzyme activities as well because of the losses and decreases of several blood group antigens that belong to glycolipid and glycophorin A-like blood group systems [3].

Further experiments are now in progress to separate other enzymes from *N. alata* using this HPLC technique.

ACKNOWLEDGEMENT

Part of this research was supported by scientific research funds (01 570 295 and 62 304 039) from the Ministry of Education, Science and Culture, Japan.

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