ENZYMES FROM CARNIVOROUS PLANTS (NEPENTHES).* ISOLATION OF THE PROTEASE NEPENTHACIN

J. JENTSCH

Max-Planck-Institut für experimentelle Medizin, Abteilung Biochemische Pharmakologie, Göttingen, Hermann-Rein-Str. 3, Germany

Received 9 February 1972

1. Introduction

The investigation of Nepenthes protease occupies an important place among the studies concerning the digestive enzymes of carnivorous plants. In a previous communication [1] we demonstrated that the determination and isolation of proteolytic activity in the viscous secretion of Nepenthes pitchers is more difficult than in animal tissues because of the high concentration of carbohydrates and ultraviolet-absorbing materials that interfere with the direct measurement of ultraviolet absorption, or the gel chromatography of proteins. In our hands the gel filtration experiments described by Nakayama and Amagase [2] did not lead to a useful isolation procedure of the protease.

Another problem arose from the small amount of the protease in the secretion of the pitcher. We have thus developed a new purification method obtained with the secretions of some 20 species of Nepenthes.

De Zeeuw [3] and Steckelberg et al. [4] showed that the proteolytic activity was present in opened pitchers *and* also in the juice of pitchers opened under sterile conditions and could hence not be of bacterial origin. In general agreement with these results, the present paper describes the isolation and purification of the proteolytic enzyme and other unknown protein material obtained with the *sterile* secretion of *closed* pitchers. This alternative method was based on repeated chromatography on highly cross-linked Sephadex and Sephadex ion exchanger.

* Part II, Previous communication see [1].

2. Materials and methods

2.1. Materials

Sephadex G-50 (fine particle size), Sephadex G-75 and DEAE-Sephadex A-50 (capacity 3.5 mequiv/g) were products of Deutsche Pharmacia, Frankfurt, Germany. All other chemicals were of the purest grade available. The anterior parts of Nepenthes leaf blades, which are transformed into pitcher traps, were preferably collected during the summer season. The colorless secretion was taken from the unopened pitcher by a syringe provided with a sterile injection needle. Before injection the pitcher wall was sterilized with ethanol. The secretion was immediately transferred into a round-bottle flask and frozen at -50°.

2.2. Protease assay

Protease was assayed using casein (Hammarsten) as substrate. A 0.3% casein solution was obtained by grinding 150 mg of casein and 50 ml of McIlvaine buffer pH 2.9 [5] with a glass rod. The sediment was shaken before use. The reaction mixture contained 2 ml casein solution pH 2.9 and 0.2 ml enzyme eluate. After 30 min at 40° and cooling in a water bath, 2 ml of 5% trichloroacetic acid was added to the incubation mixture. After 30 min unhydrolyzed casein was removed by centrifugation, and the concentration of hydrolyzed casein in the supernatant was monitored by the absorbancy at 280 nm. The assay was used as the basis for protease purification.

2.3. Carbohydrate assay

Carbohydrate was measured by the method of Yemm and Willis [6].

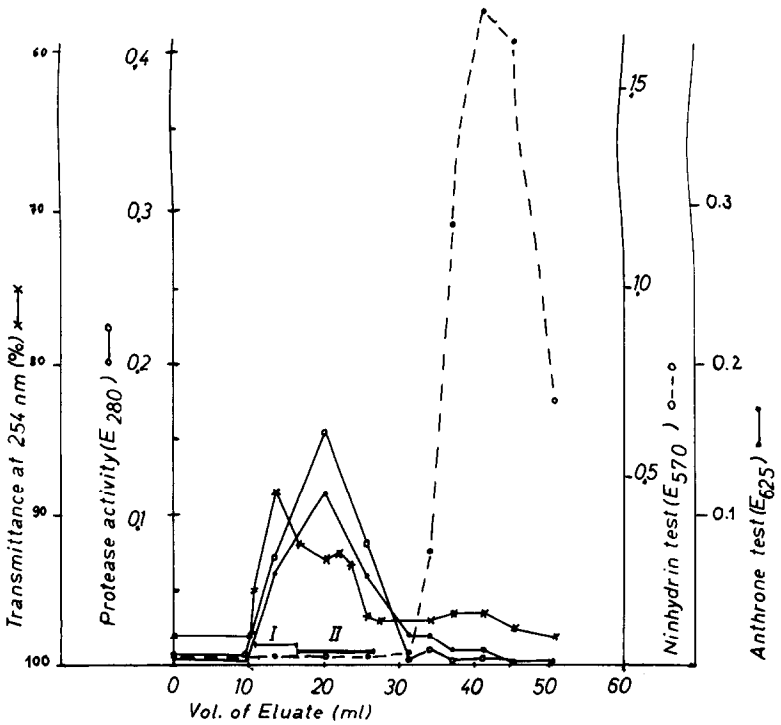

Fig. 1. Gel filtration of the high molecular fraction from the Sephadex G-50 column on Sephadex G-75 in 0.005 M Tris–citrate buffer. (o–o–o): protease activity (extinction at 280 nm) given by 0.2 ml of the effluent; (x–x–x): 254 nm transmittance; (•–•–•): anthrone test (E at 625 nm) 0.2 ml was tested for carbohydrate; (o––o––o): ninhydrin test (E at 570 nm) 0.05 ml was tested for NH_4 formate. Combined fractions II were further purified.

2.4. Chromatography on Sephadex G-50 and G-75

50 ml of Nepenthes secretion were freeze-dried, dissolved in 1 ml water and applied to a G-50 column 0.9 X 95 cm (elution buffer: 0.05 M NH_4 formate pH 4.5). The high molecular fraction [cf. 1] was desalted on a Sephadex G-75 column 1.0 X 50 cm in 0.005 M Tris–citrate pH 7.2 (fig. 1).

2.5. Chromatography on DEAE-Sephadex A-50

Gel filtration on Sephadex G-75 (fig. 1) was carried out separately for 5 portions of the secretion. The combined fractions II (protease and carbohydrate positive) were lyophilized, dissolved in 3 ml water and chromatographed on a DEAE-Sephadex column 0.9 X 11.5 cm (elution buffer: 0.005 M Tris–citrate pH 7.2). Freeze-dried fraction B, which contained the protease activity [1], was dissolved in 30 ml water and applied to the same column of DEAE-Sephadex A-50 equilibrated previously with 0.005 M Tris–citrate pH 7.2 A gradient of 0.005 M Tris–citrate pH 7.2 (in a 50 ml mixing chamber) and 1 M NaCl pH 6.0 (in the reservoir) was applied (fig. 2). The protease was eluted by 0.2 M NaCl. By these purification procedures a large amount of non-proteolytic proteins was removed.

2.6. Chromatography on Sephadex G-75

The eluates from DEAE-Sephadex (fraction B′) were pooled and lyophilized. After dissolving in 1 ml water the carbohydrate-free protease was desalted on a Sephadex G-75 column 1.2 X 38 cm (elution buffer: 0.005 M NH_4 formate pH 4.4). By this way the large amount of ultraviolet-absorbing low molecular material was also removed; it showed no proteolytic activity (fig. 3). Frozen protease solutions are stable for several years.

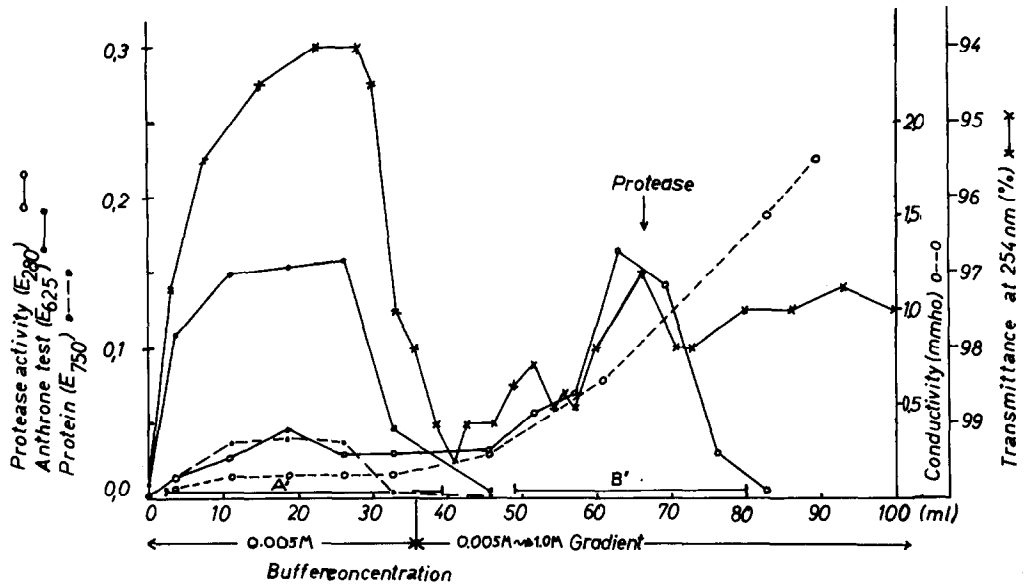

Fig. 2. Fractionation of the acid protease from DEAE-Sephadex A-50 (0.9 × 11.5 cm) [1] on the same column of DEAE-Sephadex A-50. Starting buffer 0.005 M Tris–citrate pH 7.2; gradient to 1 M NaCl pH 6.0. (•—•—•): Anthrone test (E at 625 nm) 0.4 ml was tested; (•– –•– –•): protein in 0.4 ml of the effluent was estimated by Lowry's method (E at 750 nm). Combined fractions B' were further purified (see fig. 3).

3. Results and discussion

A simple method has been developed to purify protease from Nepenthes pitchers in milligram amounts. The purification steps include chromatography on Sephadex G-50 and Sephadex G-75 (fig. 1), a repeated chromatography on DEAE-Sephadex A-50 (fig. 2), a Sephadex G-75 chromatography to remove the salts (fig. 3). The resulting material smoothly splits polypeptides as substrates [7]. See [1] for further details of purification. The acid protease in Nepenthes is named nepenthacin. This name is proposed, designating its origin and acid pH optimum.

Since Nepenthes protease has a number of enzymatic characteristics very similar to those of pepsin (such as the pH optimum), several years ago De Zeeuw [3] and Morrissey [8] postulated analogies between the pitchers of Nepenthes plants and the mammalian stomach. Investigations of Morrissey made it possible to suggest a closer analogy between the animal and vegetable organs. He found that whereas the pH of the secretion in unopened pitchers was 5.5, it was

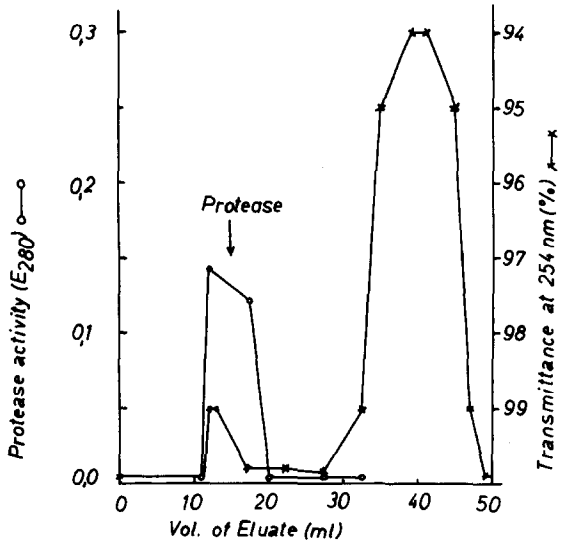

Fig. 3. Chromatography of the acid protease on Sephadex G-75 in 0.005 M NH_4 formate buffer. Symbols as in fig. 1. 0.2 ml was tested for proteolytic activity.

some 2 pH units lower in the secretion of opened pitchers. Moreover, tests with silver nitrate showed Cl^- to be present in the secretions of many pitchers.

Recently, Nakayma and Amagase [2] described the partial purification of the acid protease from opened pitchers and in contrast with the results of earlier authors [3,4] and ours [1], the Japanese workers could not find any proteolytic activity in the secretions of unopened pitchers. Thus, they could not exclude the bacterial origin of their protease. All the experiments were made with this crude enzyme preparation.

Acknowledgement

Nepenthes plants were supplied by the Botanical Gardens of Tübingen and München.

References

[1] J. Jentsch, Botaniker-Tagung, Erlangen, Sept. 1970: Ber. Dtsch. Botan. Ges. 83 (1970) 171.
[2] S. Nakayama and S. Amagase, Proc. Japan Acad. 44 (1968) 358.
[3] J. de Zeeuw, Biochem. Z. 269 (1934) 187.
[4] R. Steckelberg, U. Lüttge and J. Weigl, Planta (Berlin) 76 (1967) 238.
[5] T.C. McIlvaine, J. Biol. Chem. 49 (1921) 183.
[6] E.W. Yemm and A.J. Willis, Biochem. J. 57 (1954) 508.
[7] J. Jentsch, in preparation.
[8] S. Morrissey, Nature 176 (1955) 1220.