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A NON-DISCRIMINATING PROTEINASE OF *PENICILLIUM NOTATUM*. PARTIAL PURIFICATION AND SPECIFICITY

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SUMMARY

1. A new extracellular proteolytic enzyme has been isolated from the culture medium of *Penicillium notatum*. The fractionation procedure makes use of anion-exchange chromatography and column electrophoresis.

2. The rate and extent of digestion of the proteinase was tested against albumin, α_1-acid glycoprotein, bacitracin, casein, ceruloplasmin, collagen, γ-globulin, a serum macroglobulin, bovine ribonuclease, transferrin, total serum proteins and a soybean protein. In every instance, except casein, the proteinase showed a greater rate and extent of digestion than did Pronase using both enzymes at their pH optima. In no case was free sialic acid liberated or the appearance of reducing sugars observed.

INTRODUCTION

A non-specific proteinase capable of hydrolyzing peptide bonds in complex proteins was needed to initiate a study of glycoprotein structure. Proteins containing large amounts of oligosaccharides which are covalently bound to the peptide chain are known to be quite resistant to proteolysis. The α_1-acid glycoprotein (orosomucoid) of human serum which contains 44% carbohydrate has been said to be quite resistant to bromelin, chymotrypsin, papain, pepsin, Pronase, subtilisin, and trypsin[1-5]. Furthermore, Pronase has been shown to be active against the carbohydrate-amino acid bond as well as able to liberate free sialic acid from glycoproteins. We therefore considered that a proteinase system capable of extensively hydrolyzing the α_1-acid glycoprotein and free of carbohydrase activity would be of general value in studies concerned with the number, nature and distribution of the oligosaccharide side chains of glycoproteins. Such a proteinase was found in the dried culture medium of *Penicillium notatum* kindly supplied by Astra AB, Sodertalje, Sweden.

This report presents data on the production of the proteinase from various strains of *P. notatum* under different conditions of growth as well as partial purification, specificity and use of the enzyme in hydrolyzing most native and denatured proteins to the

peptide or amino acid level in a short period of time. Two reports on the use of this proteinase in glycoprotein structural analyses have been reported[6,7].

MATERIALS AND METHODS

Growth conditions

Spore suspensions of six strains of *P. notatum* (five were obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, one was a wild strain) were prepared by first inoculating the surface of Sabouraud maltose agar (Difco) with a stock spore suspension of *P. notatum* and incubating for 7 days at 23°; and then harvesting the spores by adding 100 ml of an aqueous solution of 1.0% Tween 80 and 0.85% NaCl.

Surface cultures were grown in Roux bottles containing 175 ml of broth at 20–23°. The inoculum for each bottle was 5.0 ml of the spore suspension. A bacto-peptone–yeast extract, Czapek–Dox, reconstituted powdered milk, tryptone–yeast extract, and a wort broth were evaluated as surface culture media at pH values from 4 to 8.

Submerged cultures were made in 1-l or 10-l flasks (Microferm Apparatus, New Brunswick, N.J.) with agitation of 125 rev./min and sterile air of 2 cubic feet per min at 25°. The inoculum was 10 ml of the standard spore suspension per l of media. In addition to the above mentioned media, soy flour and corn steep liquor were evaluated as culture media at pH values from 4 to 8.

Detection of proteolytic activity

An assay based on the reaction of primary amines with ninhydrin was employed[8]. An aliquot of the culture was filtered first through a gauze pad and secondly through filter paper. A 0.2-ml sample of the filtered culture was mixed with 0.8 ml of a solution of 1% albumin (0.1 M acetate, pH 4.0) and allowed to stand at 37°. At zero time, 0.1 ml was withdrawn and frozen; after 4 h, a second 0.1-ml sample was withdrawn. To each tube in duplicate was added 1 ml of ninhydrin reagent. The tubes were heated for 20 min in a boiling-water bath, cooled and 5 ml of diluent (ethanol–water, 1:1, v/v) was added. After standing at room temperature for 20 min, $A_{570\ m\mu}$ was read in a cell of 1 cm in a spectrophotometer. A unit of activity was defined as a difference of 1.0 in $A_{570\ m\mu}$ between 0 and 4 h.

Purification

Approx. 10 l of culture media containing a maximum of proteolytic activity (usually after 5 days of submerged growth) was filtered through gauze and finally through filter papter. The clear filtrate was dialyzed in 18/32 dialysis bags against 0.1 M acetic acid. This material could be lyophilized and stored without loss of activity. Aliquots corresponding to 100 g of dried material or 700 ml of non-diffusate were subjected to anion exchange chromatography on a DEAE-Sephadex column (A-25) with a total volume of about 2 l. The material eluted with the solute buffer was high in proteolytic activity but low in cellulase. After concentration with either dry Sephadex G-25 or vacuum dialysis, the eluate was subjected to electrophoresis on a large Porath electrophoresis column (LKB 5801A) packed with either G-25 or cellulose.

The applied volume for electrophoresis was 70 ml and the buffer system was 0.05

M sodium acetate–acetic acid (pH 4.5). The conditions of energy were: 500 V and 270 mA for 21 h followed by 800 V and 400 mA for 75 h. The column was eluted after electrophoresis and $A_{280\ m\mu}$ and proteolytic activity of the fractions were measured (see bottom, Fig. 1). The fractions which contained proteolytic activity were pooled and concentrated. This material could be frozen or lyophilized.

Determination of optimum pH

To 0.8-ml solutions of 1% bovine serum albumin at pH values from 2.6 to 8.8 was added 0.2 ml of the stock proteinase eluent from electrophoresis. The samples were

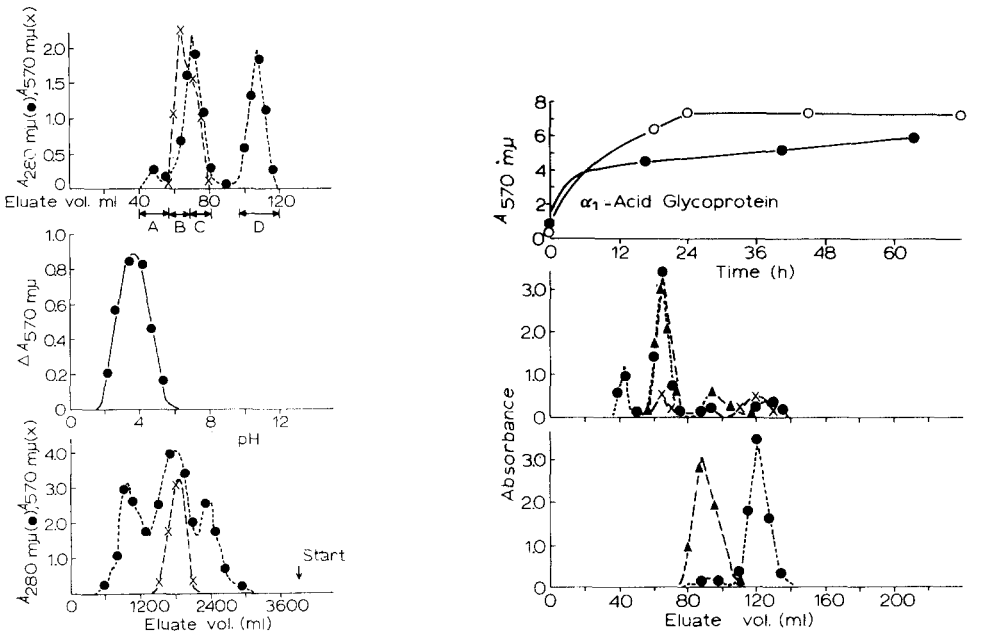


Fig. 1. Top: A gel filtration on G-100 of the electrophoretically obtained proteinase. See text for conditions; ●- - - -●, $A_{280\ m\mu}$; ×—·—×, proteolytic activity. Middle: The pH optimum of the proteinase as determined by the ninhydrin reagent. Bottom: An electropherogram of the anion-exchange column eluent of the culture medium. See text for conditions; ●— — —●, $A_{280\ m\mu}$; ×— · —×, proteolytic activity.

Fig. 2. Top: The rate of digestion of α_1-acid glycoprotein as measured by the ninhydrin reagent; ○— ○, by *P.notatum* proteinase; ●—●, by Pronase. Middle: A gel filtration on G-100 of the digestion of the glycoprotein with *S. griseus* Pronase; ×—·—×, $A_{570\ m\mu}$ as measured by the ninhydrin; ●— — —●, $A_{280\ m\mu}$; ▲— —▲, $A_{519\ m\mu}$ of bound sialic acid by the thiobarbituric acid reagent. Bottom: A gel filtration on G-100 of the digestion of the glycoprotein with *P. notatum* proteinase; same legend as that in the middle picture (see refs. 6 and 7 for greater detail).

incubated at 37° and the proteolytic activity was measured by the ninhydrin reagent at zero time and after 4 h (see middle, Fig. 1).

Estimation of molecular size and homogeneity

An aliquot of the stock solution resulting from electrophoresis was subjected to gel filtration in 0.07 M acetic acid—0.02 M pyridine (pH 4.5) on a column of Sephadex

G-100 having a void volume of 41 ml, total volume 125 ml (see top, Fig. 1). Fractions A, B, C and D as marked in Fig. 1 were concentrated and subjected to starch-gel electrophoresis at pH 8.6 according to POULIK[9].

Evaluation of stability

The activity of a concentrated aliquot of the column electrophoresis eluent containing 6 units/ml was measured before and after standing at 37°, pH 4.0 for 40 h. A similar aliquot was assayed before and after standing at 23°, pH 9.0 for 48 h.

Evaluation of digestion rate and substrate specificity

The rate of digestion was determined by mixing an aliquot of the enzyme with individual purified proteins at pH 4.0 and measuring the appearance of primary amines by the ninhydrin reagent over a period of 60 h. In each case a comparable aliquot of *streptomyces griseus* Pronase (Calbiochem Corp., Los Angeles, Calif.) and the same protein substrate was run in parallel at its optimum pH of 7.3 (see top, Figs. 2–6). After 60 h of digestion at 37°, the solution was chromatographed on columns of Sephadex G-100 with the same conditions of size and buffer as mentioned above. Each fraction was analyzed by the ninhydrin reagent for free amines, $A_{280\ m\mu}$ for aromatic amino acids and the thiobarbituric acid reagent for sialic acid[10] whenever glycoproteins were digested (see middle and bottom, Figs. 2–6). The proteins used were: albumin, α_1-acid glycoprotein (orosomucoid), bacitracin, casein, ceruloplasmin, collagen, γ-globulin, a human serum macroglobulin, bovine ribonuclease, transferrin and total serum proteins. Fig. 7 shows a separation of the solubilized and hydrolyzed soy gluten with an aliquot of the proteinase.

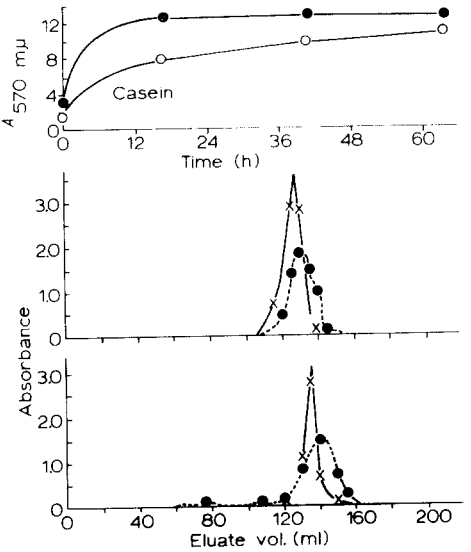
RESULTS

The highest levels of activity were found in the soybean submerged cultures of Strain 2A and Strain 9179. The highest levels of proteolytic activity consistently appeared after 90 h at about pH 6.0 in soybean cultures started at pH 6.5. Both of these strains were preferentially used in order to obtain purified quantities of the proteinase for studies of the oligosaccharide composition of the α_1-acid glycoprotein.

The bottom of Fig. 1 shows the electropherogram of the culture medium after the initial anion-exchange step. As can be seen, the proteolytic activity is eluted as one well-defined peak. The activity of each fraction was measured, however only 4 proteolytic values are represented in the figure. The gel filtration on G-100 of this material is represented on the top of Fig. 1 and shows a slightly askewed peak of activity corresponding to about 1.6 void volumes. The starch-gel electropherogram of Fractions B and C showed one strong band and three very faint bands with Amido black stain.

The optimum pH for proteolytic activity as shown in the middle of Fig. 1 is 3.8–4.2 with a working range from about 3.0–5.5.

As is shown in Fig. 2, the activity of the *P. notatum* proteinase against the α_1-acid glycoprotein of human serum is more rapid and more complete than the activity of Pronase. Of the 12 proteins used to determine the specificity of the proteinase, only casein was found to be more rapidly and fully digested by Pronase[11] (see Fig. 3). Fig. 4 shows the *P. notatum* proteinase to be capable of digesting soluble collagen to the extent of 50–75% as determined by the relative areas under the $A_{280\ m\mu}$ peaks at the



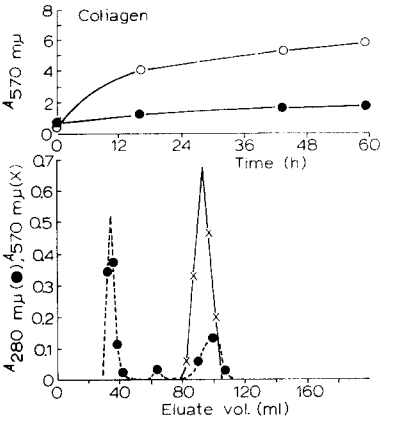
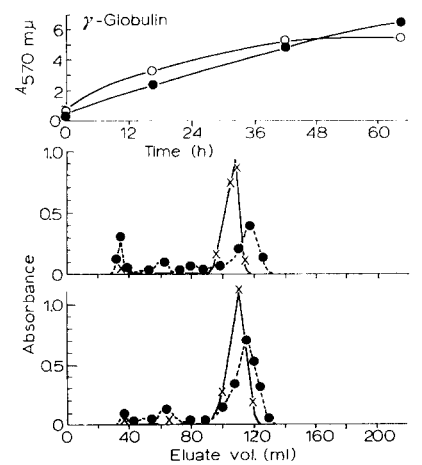


Fig. 3. A measure of the rate and extent of digestion of casein by Pronase and by *P. notatum* proteinase. Same legend as in Fig. 2.

Fig. 4. A measure of the rate and extent of digestion of collagen by the *P. notatum* proteinase. Same legend as in Fig. 2.

void volume and at the total volume. Very slight digestion was observed with Pronase.

The extent of digestion of γ-globulin was almost equal between the *P. notatum* proteinase and Pronase. In Fig. 5 the slightly greater rate and extent of digestion can be seen with the *P. notatum* enzyme. Similarly in the case of ribonuclease, the rate and extent of digestion were approximately equal between the two proteinases (see Fig. 6).



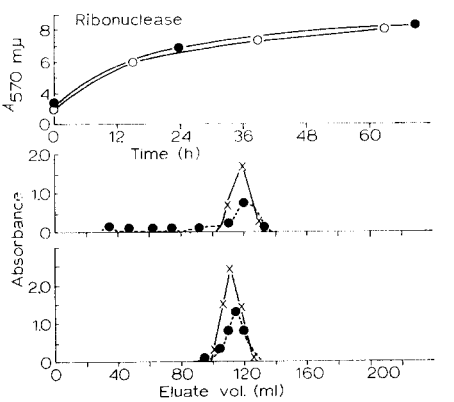


Fig. 5. A measure of the rate and extent of digestion of γ-globulin by Pronase and by the *P. notatum* proteinase. Same legend as in Fig. 2.

Fig. 6. A measure of the rate and extent of digestion of bovine ribonuclease by Pronase and by the *P. notatum* proteinase. Same legend as in Fig. 2.

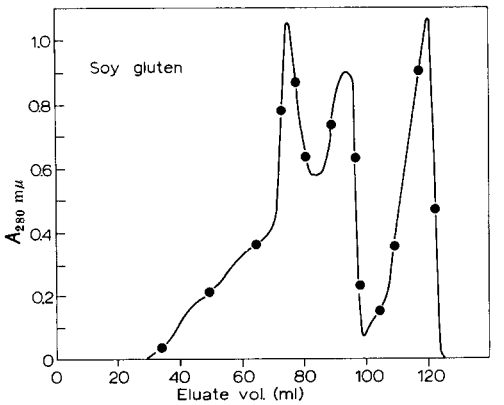


Fig. 7. Gel filtration on Sephadex G-100 of the soybean gluten solubilized by *P. notatum* proteinase; same conditions as in Figs. 2–6; ●—●, A_{280} mμ.

Fig. 7 shows the extent to which an insoluble soy protein is digested by the *P. notatum* proteinase. About 25% of the soy gluten was solubilized by the action of the proteinase. It can be seen that most of the solubilized material is of low molecular weight. Pronase was unable to solubilize the soy gluten to any appreciable extent.

A loss of 10% activity occurred due to autodigestion when a concentrated sample of the proteinase was stored over a period of 40 h at 37° at the pH optimum of 4.0. No loss in activity could be detected at pH 9.0 after 48 h or when the dried enzyme was stored at 10° for 3 years.

DISCUSSION

The soybean flour which yielded the highest levels of proteolytic activity is manufactured for microbial proteinase production by the Archer–Daniels–Midland Co. of Minneapolis, Minn.

Since the *P. notatum* proteinase does not contain appreciable amounts of carbohydrate, a partially purified preparation can be used to obtain glycopeptides from glycoproteins. Earlier work on the number and nature of the oligosaccharide side chains of four human serum glycoproteins made use of preparations of the enzyme purified only by gel filtration on G-100 of the culture medium or anion exchange chromatography of the culture medium[6]. Such a preparation does not release free sialic acid or give rise to the appearance of carbohydrate reducing groups when used in the digestion of a glycoprotein (see Fig. 2 of ref. 6). However, for more rapid digestion, a more highly purified preparation may be used[7].

Of the twelve protein preparations used to evaluate the ability of the proteinase to digest to the peptide level, only bacitracin was completely resistant to hydrolysis. With the single exception of casein, the *P. notatum* proteinase was capable of digesting all tested proteins more rapidly and more completely than Pronase. No proteins other than the twelve listed above were tested.

From the available experimental data, it cannot be said unequivocally that the proteinase is a single enzyme. However, if it is a mixture of proteinases, they are rather resistant to each other as evidenced by the stability experiments and possess very similar pH optima.

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