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AN EXTRACELLULAR RENNIN-LIKE ENZYME PRODUCED BY *PHYSARUM POLYCEPHALUM*

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SUMMARY

Three new proteases have been detected in the culture fluid of the myxomycete *Physarum polycephalum*. Production of these enzymes reached a maximum after 120 h. The main protease has been isolated and purified by $(NH_4)_2SO_4$ fractionation and DEAE cellulose chromatography. The enzyme has a pH optimum of 4.5–5.0 and a temperature optimum of 35 °C. The enzyme has been shown to have a specificity similar to that of rennin (chymosin, EC 3.4.4.3) when acting upon cow κ-casein.

INTRODUCTION

The myxomycete *Physarum polycephalum* produces ribonucleases [1], one of which has been purified [2]. In addition, previously unreported extracellular proteases have been found in the culture supernatant of *P. polycephalum*, reaching a maximum activity after 120 h. One of the separated proteases had a specificity similar to that of rennin (chymosin, EC 3.4.4.3) when acting on κ-casein. Other microbial proteases have been shown to possess similar specific activity, as for instance the rennet substitutes from *Mucor pusillus* [3], *Mucor miehei* [4], and *Endothia parasitica* [5].

MATERIALS AND METHODS

The following chemicals were obtained commercially as indicated. α,α'-Dipyridyl and iodoacetamide from Fluka, Switzerland. Azocoll (50–100 mesh), and dithiothreitol from Calbiochem, U.S.A. Sephadex G-100 and Blue Dextran 2000 from Pharmacia, Sweden. Tryptone and yeast extract from Difco, Germany. Carboxypeptidase A (EC 3.4.2.1) from Worthington, U.S.A. Rennin and the oxidized B-chain of insulin from Schwarz-Mann, U.S.A. Bovine serum albumin from Behringwerke AG, Germany. DEAE-cellulose (DE 52) from Whatman Biochemicals Ltd, England. Ampholine (pH 3–10) from LKB, Sweden.

Cow κ-casein was prepared according to the procedure of McKenzie and Wake [6].

Organism and cultural conditions

P. polycephalum was kindly supplied by Dr R. Braun, I.S.R.E.C., Lausanne, Switzerland.

Microplasmodia were grown according to the method of Daniel and Baldwin [7], in a semi-defined medium containing tryptone and yeast extract. Cultures were maintained in 50 ml of medium in 500 ml conical flasks equipped with baffles. The flasks were shaken in the dark at 25 °C (120 rev./min). For the production of enzyme, 2-l flasks equipped with baffles and containing 500 ml of medium were used. The organism was also grown in a 15-l Chemap fermentor stirred at 150 rev./min and aerated at 10 l/min.

Enzyme assay

Protease activity was determined routinely using Azocoll as a substrate. Azocoll (25 mg) and a suitable aliquot of enzyme in 0.05 M acetate buffer, pH 4.5, (final volume 6 ml) were incubated at 37 °C for 30 min with stirring. The digest was filtered and the absorbance of the filtrate was measured at 520 nm. Blank digests were prepared by the same procedure except that they were filtered immediately after the addition of enzyme. One unit of enzyme activity was defined as the amount which produced an increase in absorbance of 1.0 in 30 min.

Protein was determined by the method of Lowry et al. [8] using crystalline bovine serum albumin as a standard.

Enzyme action on κ-casein

Enzymatic hydrolysis of κ-casein was carried out as follows. Rennin or *P. polycephalum* proteases were incubated at 37 °C for 1 h with κ-casein in 0.05 M phosphate buffer, pH 5.1 (enzyme/substrate ratio 1:100). Carboxypeptidase A (enzyme/substrate ratio 1:100) was then added after adjusting to pH 7.7 and the mixture was further incubated for 1 h at 37 °C.

The amino acids released by carboxypeptidase A were qualitatively and quantitatively determined in the enzymatic hydrolyzate with an autoanalyzer after removal by centrifugation of residual substrate (κ-casein) or degraded κ-casein (*para*-κ-casein).

Isoelectric focusing

An LKB 8100 electrofocusing apparatus was used with Ampholine pH 3–10 according to the manufacturer's instructions.

Gel electrophoresis

Dodecylsulfate-polyacrylamide gel electrophoresis was performed according to Weber and Osborn [9].

RESULTS

Milk clotting activity

The culture fluid of *P. polycephalum* (120 h growth) was incubated at 37 °C, with an equal volume of skim milk (12 and 24 % dry weight). A stable clot was obtained after 10 and 4 min, respectively.

Purification of the protease

Production of the protease was found to follow the growth curve and was maximal after 120 h (1.5 activity units/ml of culture supernatant).

10-l cultures were harvested by centrifugation at 1000 × *g* for 10 min at 4 °C. The supernatant was slowly frozen at −10 °C in a plastic container. The protein concentrated at the top into a narrow band which was removed by dissolving it in 0.05 M acetate buffer, pH 4.5 (1.5 l). This treatment helped to reduce the amount of extracellular polysaccharide which is produced by *P. polycephalum* [10]. Unless the amount of extracellular polysaccharide is reduced, difficulties are encountered in column chromatography. Not all the activity, however, is recovered. This extract is referred to as the crude enzyme.

$(NH_4)_2SO_4$ (860 g) was added to the crude enzyme solution (1.5 l). After 12 h at 4 °C the suspension was centrifuged at 30 000 × *g* for 30 min. The brown precipitate was dissolved in 0.05 M acetate buffer, pH 4.5, (150 ml) and dialyzed against the same buffer (5 l) at 4 °C with frequent changes for 24 h.

The dialyzed enzyme solution (200 ml) was made 0.1 M in NaCl and 50-ml portions were chromatographed on a DEAE-cellulose column at 4 °C using a NaCl gradient (0.1–0.3 M NaCl) (Fig. 1). Three proteolytic fractions (Protease I, II and III) were eluted.

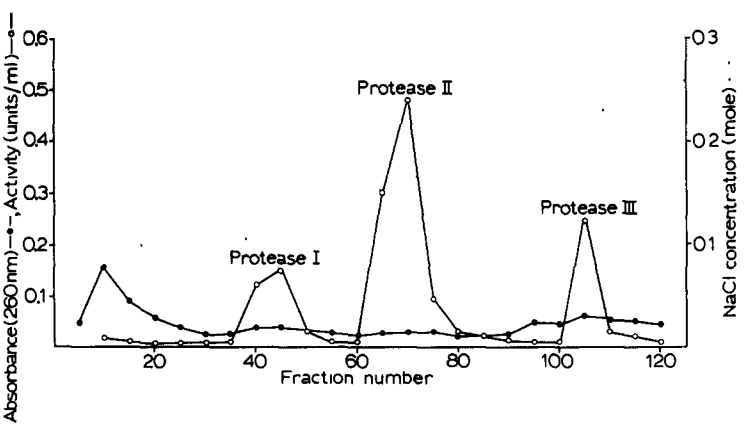

Fig. 1. Ion-exchange column chromatography of partially purified proteases of *P. polycephalum* on DEAE-cellulose (DE 52). The column (20 cm × 2.5 cm) was equilibrated with standard buffer containing 0.1 M NaCl and the sample was eluted with an NaCl gradient (0.1–0.3 M). 10-ml fractions were collected. ●, $A_{260\ nm}$; ○, proteolytic activity.

The Protease II fractions were pooled and concentrated to 20 ml by pressure dialysis at 4 °C against 0.05 M acetate buffer, pH 4.5, containing 0.1 M NaCl and rechromatographed on DEAE-cellulose and eluted at the same NaCl molarity as before. A single protein and activity peak, without a significant increase in specific activity, was obtained.

The enzyme was stable when stored at −20 °C. Protease II was thus purified 31-fold with 51% overall recovery (Table I).

TABLE I

PURIFICATION OF PROTEASE II FROM *P. POLYCEPHALUM*

Fraction	Total protein (mg)	Volume (ml)	Activity (units/ml)	Specific activity (units/mg)	Purification
Crude enzyme (freeze–thaw concentrated)	5250	1500	0.72	0.21	1
$(NH_4)_2SO_4$ precipitation	130	200	2.8	4.3	20.0
DEAE-chromatography	89	137	4.1	6.3	31.4

General properties of the enzyme

Molecular weight. An estimation of the molecular weight of Protease II was made by Sephadex G-100 column chromatography according to Andrews [11]. The apparent molecular weight was estimated to be 30 000.

Dodecylsulphate-polyacrylamide gel electrophoresis of Protease II indicated the presence of a single band. The molecular weight of Protease II was estimated to be 35 000 $\pm$ 10%.

Isoelectric point. The isoelectric point of Protease II was determined by isoelectric focusing and found to be pH 4.6.

pH optimum. The pH optimum of Protease II was assayed by the Azocoll method at 35 °C and found to be 4.5–5.0. Less than 15% of the maximal activity was exhibited at pH 7.0 (Fig. 2).

pH stability. Protease II was most stable between pH 3.5 and 5.0 and was rapidly inactivated above pH 6.0. When the enzyme was incubated at pH 4.5 for 20 h at 4 °C, 14% of the activity was lost.

Temperature optimum. Protease II assayed under standard conditions had a temperature optimum of 35 °C. At 50 °C the rate of proteolysis was approximately 50% of that observed at 35 °C.

Effect of metal ions, chelating and sulfhydryl reagents. The effect of various metal ions on the activity of Protease II was studied at a final concentration of 5.0 mM

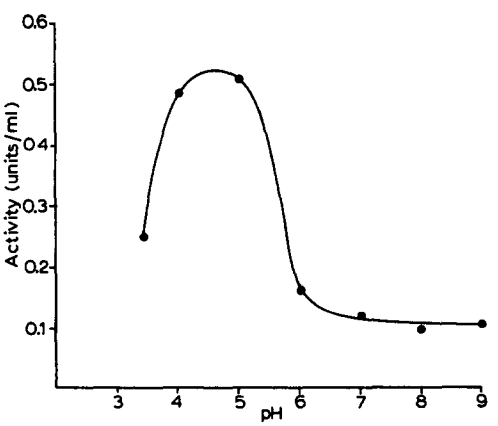

Fig. 2. pH optimum of Protease II assayed by the standard procedure. The buffers used were 0.1 M sodium acetate–acetic acid (pH 3.5–5.0), 0.1 M citric acid–sodium phosphate (pH 6.0) and 0.1 M Tris (pH 7.0–9.0).

under standard assay conditions. Ca^{2+} and Sr^{2+} enhanced enzyme activity (residual activity 145% and 110%, respectively) while Mg^{2+} (residual activity 88%), Mn^{2+} (87%), Zn^{2+} (77%), Hg^{2+} (74%), Fe^{2+} (35%), and Cu^{2+} (20%) reduced the activity.

At a concentration of 1 mM, EDTA strongly inhibited the enzyme while α,α'-dipyridyl affected only partially the activity (Table II) indicating that metal ions are required for enzyme activity.

TABLE II

THE EFFECT OF METAL CHELATORS AND THIOL REAGENTS ON PROTEASE II ACTIVITY

The enzyme (300 μg) was preincubated with the reagents for 20 min at 25 °C and the enzyme activity was assayed by the Azocoll procedure.

Reagent	Concentration (mmole)	Residual activity (%)
None		100
EDTA	1	9
α,α'-Dipyridyl	1	43
p-Hydroxymercuribenzoate	1	40
Iodoacetamide	50	31
Mercaptoethanol	1	37
Dithiothreitol	1	34

TABLE III

PROPERTIES OF *P POLYCEPHALUM* PROTEASE II AND CALF RENNIN

	P polycephalum protease	Calf rennin
Molecular weight		
Sedimentation/diffusion	—	40 000 [27]
Gel filtration	30 000	31 000–34 000 [12, 27, 28]
Gel electrophoresis	35 000	—
Amino acid composition	—	30 400 [28]
pH optimum	4.5–5 0	5 0–6 0
Temperature optimum	35 °C	35 °C
pH stability	3 5–5 0	5.3–6.3
Isoelectric point	4.6	4 6
Specificity on κ-casein	–Phe–↓–Met–	–Phe–↓–Met–

The enzyme was also partially inhibited by *p*-hydroxymercuribenzoate and iodoacetamide showing that free thiol groups participate in the catalysis. However preincubation of the enzyme with mercaptoethanol and dithiothreitol also caused a reduction in activity indicating that intact disulfide groups are essential for activity (Tables II and III).

Specificity of Protease II towards κ-casein. Carboxypeptidase A released 70 $\pm$ 5% of C-terminal valine of κ-casein and lesser amounts of alanine (40%), serine and threonine [12]. No release of phenylalanine or leucine was detected.

After the action of rennin, carboxypeptidase A released the same amino acids,

plus phenylalanine (30 ± 5%) and leucine (15 ± 3%). The amount of serine released was somewhat higher than that detected when rennin was omitted. This is in agreement with the fact that the C-terminal sequences of κ-casein and *para*-κ-casein contain this amino acid.

After the action of Protease II, carboxypeptidase A released the amino acids of the C-terminal moiety of κ-casein, plus phenylalanine (72%) and leucine (10%), indicating that the enzyme behaved like rennin. No release of tyrosine was detected. The presence of an enzyme having a specificity similar to that of chymotrypsin (EC 3.4.4.5) is therefore unlikely.

After the action of Protease I and III under the same conditions, carboxypeptidase A released only 37% and 12.8% of phenylalanine, respectively.

DISCUSSION

During the first phase of the clotting of milk by rennin, the only significant change at the molecular level occurs in one fraction of the complex called casein, i.e. the κ-casein fraction, the only fraction containing sugars [13]. This phenomenon has been studied in detail in the clotting of cow's milk, and Jollès et al. [14, 15] have shown that the linkage Phe–Met in κ-casein is broken.

This results in the splitting of κ-casein into a *para*-κ-casein part having phenylalanine as the C-terminal amino acid and a glycomacropeptide containing methionine as the N-terminal amino acid. The *para*-κ-casein contains all the aromatic amino acids, all the cystine and all the basic amino acids (except three lysine residues) of κ-casein [16]. The primary structure was established independently by two groups [17–19]. The soluble product which is split off during the action of rennin on κ-casein is called caseinoglycopeptide [20] or caseinopeptide [14, 21] when κ-casein is free of sugars. Methionine is the N-terminal amino acid, and Ser–Thr–Ala–Val the C-terminal sequence [12]. The sugars have been localized with a sequencer [17, 22]. The tryptic peptide containing the linkage Phe–Met was one of the first sequences of κ-casein to have been studied in detail, i.e. His–Pro–His–Pro–His–Leu–Ser–Phe–Met–Ala–Ile–Pro–Pro–Lys–Lys [18, 19, 23].

An enzyme having a specificity identical to rennin must theoretically break the Phe–Met bond and only this one, at least during the first phase, when κ-casein is the substrate. This specific breakage can be demonstrated when κ-casein is treated with the enzyme and then with carboxypeptidase A. The latter will release Val, Ala, Ser and Thr arising from the C-terminal moiety of the substrate. After the action of an enzyme supposedly having the specificity of rennin, Phe together with Ser and Leu arising from the C-terminal moiety of *para*-κ-casein, must be in turn released by carboxypeptidase A. The amino acids released can be then quantitatively determined.

The Protease II from *P. polycephalum*, which forms a stable clot when incubated with milk, shows a specificity similar to that of rennin when incubated with κ-casein. However, when the B-chain of oxidised insulin was hydrolyzed with rennin and Protease II at pH 4.5 and 3.6 according to the procedure of Bang-Jensen et al. [24], the resulting hydrolysates gave different peptide maps. Protease II produced many more peptide fragments.

The possible function of the extracellular proteases of *P. polycephalum* is of interest.

The failure to form heterocaryons between non-isogenic strains in fungi is due to protoplasmic disintegration following hyphal fusion. Begueret [25] has suggested that incompatibility in *Podospora anserina* is due to proteases directly involved in the incompatibility process. Incompatibility could be a consequence of a disorder in the regulation of proteolytic activity.

A lethal interaction can occur in *P. polycephalum* after fusion between plasmodia of some strains, one plasmodium completely eliminating the other. Carlile [26] has suggested that this type of lethal interaction is an elimination of alien nuclei by one of the plasmodia by a phagocytic reaction. The extracellular proteases may also be involved in such lethal interactions.

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