Biochimica et Biophysica Acta, 384 (1975) 235—241

BBA 67430

ISOLATION OF AN EXTRACELLULAR NEUTRAL PROTEINASE FROM *PSEUDOMONAS FRAGI*

M.A. PORZIO and A.M. PEARSON
Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Mich. 48824 (U.S.A.)
(Received September 23rd, 1974)

Summary

A single proteolytic enzyme (EC 3.4.4.-) was isolated from culture supernatants of *Pseudomonas fragi* with 20% yield and 60-fold purification by means of stepwise DEAE-Sephadex batch adsorption, ammonium sulfate precipitation, gel filtration and DEAE-cellulose chromatography. The enzyme was Zn^{2+} activated and Ca^{2+} stabilized, had optimum activity at pH 6.5—8.0 and 40°C. The molucular weight range was 40 000—50 000 as determined by dodecylsulfate gel electrophoresis, gel filtration and Zn assay. This proteinase has properties similar to other extracellular bacterial neutral proteinases.

Introduction

Psychrophilic bacteria of the genera *Achromobacter*, *Arthrobacter*, *Proteus* and *Pseudomonads* have been identified as the predominant flora in low temperature meat spoilage [1]. The Pseudomonas are the most ubiquitous and extensively studied of these organisms [2,3]. During the course of spoilage, breakdown of muscle structure and protein degradation have been observed and the presence of extracellular proteolytic enzymes demonstrated [4,5]. Nunokawa and McDonald [6] partially isolated several proteases from *Pseudomonas* species. More recently, Kato et al. [7] isolated several proteases from salt tolerant pyschrotrophic *Pseudomonas*.

P. fragi (ATCC 4973), which has been shown to produce an extracellular proteinase in protein and non-protein media [5,8], was used in this study. Isolation, purification and some properties of *P. fragi* extracellular proteinase are reported.

Materials and Methods

All reagents were of analytical grade. Materials were obtained from the

following commercial sources: DEAE-Sephadex A-50 (Pharmacia); Bio-gel P-100, Chelex 100 Resin (Bio-Rad); DEAE-cellulose (0.89 meq/g) (Sigma); APT Broth (BBL); Ampholine, pH 3—10 (LKB).

Bacteria and culture conditions. P. fragi (ATCC 4973) stock cultures were maintained in All Purpose Tween (APT) broth at 4°C and loop transfers made weekly. A culture medium consisting of 10.0 g/l hydrolyzed casein (salt-free), supplemented with 0.30 g/l L-tryptophan and 0.10 g/l cysteine · HCl; 0.20 g/l $MgSO_4$; 1.4 g/l $CaCl_2 \cdot 2H_2O$, 1.0 g/l KH_2PO_4; 0.50 g/l KNO_3 and NaCl; 4.0 mg/l Zn-EDTA was prepared, adjusted to pH 7.5 with 1 M NaOH and autoclaved at 120°C for 20 min. For enzyme production, 10 ml of innoculum was added to 225 ml of culture medium in a 1 liter Erlenmeyer flask and shaken at 10°C. The culture was collected after 50—60 h growth.

Enzyme assay. Proteolytic activity was determined according to the general procedure of Hagihara et al. [9] using 2% (w/v) casein made up with 0.10 M Tris · HCl (pH 7.2) in place of phosphate buffer. Absorbance was read at 660 nm and equivalents of tyrosine released determined from a standard curve. One proteolytic unit was defined as one μg of tyrosine equivalent released per ml of enzyme solution per min at 35°C. Specific activity was defined as proteolytic units per mg protein.

Protein determination. The protein content of samples from the purification procedure was estimated with Folin reagent according to Lowry et al. [10].

Polyacrylamide disc gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed with a 7.5% acrylamide/0.18% bis-acrylamide at pH 8.9 according to the procedure of Davis [11]. Protein was stained with 0.5% Coomassie Brilliant Blue in 10% (w/v) trichloroacetic acid. Gels were diffusion destained in 7.5% (v/v) acetic acid/5% (v/v) methanol.

Isoelectric focusing. Polyacrylamide gel isoelectric focusing was accomplished using the method of Wrigley [12]. The pH gradient was established with 1% Ampholine, pH 3—10 and the protein stained according to Vesterberg [13].

Zinc assay. The dithizone colorimetric method of Malstrom [14] using the procedure for protein preparation of McConn et al. [15] was employed.

Molecular weight determination. Molecular weight determination by dodecylsulfate polyacrylamide disc gel electrophoresis was performed according to Weber and Osborn [16]. Molecular weight was also determined by means of gel permeation chromatography with Sephadex G-200 (medium) according to Andrews [17].

Enzyme purification. Unless otherwise noted, all solutions were maintained at 5.0 mM $CaCl_2$ and 4°C. The culture was centrifuged at 5000 × *g* for 1 h, the cell-free supernatant brought to approx. 10 mM $CaCl_2$ by addition of 1.0 M $CaCl_2$ and adjusted to pH 8.5 with 1 M NaOH. The solution was recentrifuged at 18 000 × *g* for 1 h.

Step 1. DEAE-Sephadex A-50 equilibrated with 50 mM Tris · HCl (pH 8.5) was added to the supernatant on the basis of 30 g (wet weight) Sephadex/l of solution. The adsorbent was separated by filtration on a Büchner funnel fitted with a glass fiber pad, then washed with several hundred ml of 50 mM Tris · HCl (pH 8.5) buffer. Adsorbed protein was eluted with 1/10 original

culture volume of 0.20 M Tris/Maleate, 0.40 M NaCl, 10 mM $CaCl_2$ (pH 6.0) and the Sephadex removed by filtration.

Step 2. Solid $(NH_4)_2SO_4$ was added slowly with stirring until 50% saturation was reached, and the system was stirred overnight. The precipitate was centrifuged at 70 000 × *g* for 1 h and the pale yellow precipitate redissolved in 0.005 M calcium acetate (pH 7.2).

Step 3. The protein solution was loaded on a 2.5 × 40 cm column of Bio-Gel P-100 equilibrated to 0.050 M Tris · HCl (pH 7.5) and 10 ml fractions collected at the rate of 30 ml/h. The fractions were tested for activity and protein content, and absorbance was read at 280 nm. The pooled active fractions were dialyzed against 100-fold volume of 50 mM Tris · HCl buffer (pH 7.8).

Step 4. DEAE-cellulose chromatography was performed on 1.6 × 24 cm column, pre-equilibrated with dialysis buffer. After loading, the buffer was changed to 0.015 M NaCl, 0.050 M Tris · HCl (pH 7.8) and the inactive protein eluted in 2—3 bed vols. The salt concentration was increased to 0.050 M and a single active protein band collected.

Enzyme activity and stability. The effect of pH, temperature and ionic strength upon the relative activity of the proteinase was examined. The buffers employed were acetic acid/acetate (pH 5.0), Tris · Acetate (pH 6.0—7.0), Tris · HCl (pH 7.0—10.5) and borate (pH 11.0) made up to 0.10 M. The enzyme solutions were dialyzed 24 h at 25°C for stability studies and the sample readjusted to 0.10 M Tris · HCl (pH 7.2) for assay.

For activity studies, the enzyme was dialyzed for 1 h against 100 vols of the desired buffer and the assay performed with 2% (w/v) casein containing the respective buffers. Azocoll substrate, 5.0 mg/ml in 0.10 M Tris · HCl (pH 7.2) at a series of KCl concentrations (0—1.2 M), was prepared. The samples were incubated at 35°C, filtered and the increase in absorbance read at 520 nm.

Results

Enzyme purification. The proteolytic enzyme was obtained in 20% yield with a 60-fold purification of specific activity. During the initial two steps, a yellow pigment was present with the protein. Although the pigment does not effect either the stability or activity, it was removed during gel chromatography (Fig. 1). Specific activity could not be increased further by hydroxylapatite adsorption chromatography (employing phosphate, KCl or $CaCl_2$ elution), CM-cellulose or additional DEAE-cellulose chromatography. The results of purification are given in Table I. The values are those of a single preparation but are representative of all samples.

Enzyme homogeneity. The DEAE-cellulose eluant (800 units/mg) was analyzed with disc gel electrophoresis and dodecylsulfate gel electrophoresis. In each case, a single protein band was observed. With samples, which were stored at ambient temperature and low Ca^{2+} concentrations (< 1.0 mM), additional protein bands resulting from autodigestion were observed.

Molecular weight determination. The molecular weight was determined using dodecylsulfate gel electrophoresis and gave a minimum value of 50 000 for the proteinase. A molecular weight obtained from gel permeation chroma-

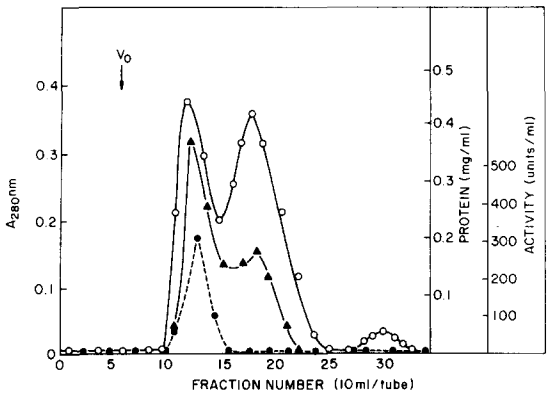

Fig. 1. Elution pattern of Bio-gel P-100 gel chromatography. The protein solution (6.5 ml of 3.6 mg/ml protein solution, total units 14 000) was loaded on 2.5 × 40 cm column equilibrated with 0.05 M Tris · HCl, $CaCl_2$ 5 mM (pH 7.50). The enzyme was eluted at 30 ml/h with 10 ml fractions per tube. ○———○, Absorbance at 280 nm; ▲———▲, protein concentration; ●———●, proteinase activity; V_0 designates the column void volume.

tography gave a value of 40 000. Zn microassay gives a value of 1.6 μg Zn/mg protein. By assuming a single Zn atom per active sight per molecule of proteinase, a molecular weight of 40 000 was calculated.

Isoelectric point. Gel isoelectric focusing gave a value of 5.2 for the isoelectric point and activity of the proteinase was congruent with the protein band.

Reagent inhibitor studies. P. fragi proteinase was reacted with 10^{-2} to 10^{-3} M solutions of the following reagents: L-cysteine · HCl, iodoacetic acid, *p*-chloromercuribenzenesulfonic acid (sodium salt), dithioerythriol, 5,5′-dithiobis-(2-nitrobenzoic acid) and soybean trypsin inhibitor (1.0 mg/ml). In each case, proteolytic activity was unchanged.

Inhibition of enzyme by metal chelating agents. P. fragi proteinase was treated with a series of metal complexing agents as described in Table II. A plot of % inactivation versus complexing agent concentration gave a concentration

TABLE I

PURIFICATION OF *P. FRAGI* NEUTRAL PROTEOLYTIC ENZYME

Step	Volume (ml)	Protein (mg)	Activity (total, units*)	Activity (unit/ml)	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Culture supernatant	1500	3230	42000	28	13	100	—
1. DEAE-Sephadex batch adsorption	147	129	29400	200	228	70	17
2. 50% $(NH_4)_2 SO_4$ precipitation	12	44	16092	1341	368	38	28
3. Bio-Gel P-100 gel filtration	60	31	14010	235	450	36	45
4. DEAE-Cellulose chromatography	10	9.3	6000	660	750	15	58

* 1 Unit equals release of 1 μg tyrosine equivalent per min at 35°C.

TABLE II

EFFECT OF METAL CHELATING AGENTS ON *P. FRAGI* PROTEINASE

Samples were treated with chelating agent for 5 min at pH 7.0 and 25°C before assay.

Chelating agent	Concentration required for 50% inhibition (M)
1 : 10 Phenanthroline	$1.3 \cdot 10^{-4}$
Ethylenediaminetetraacetic acid (EDTA)	$3.4 \cdot 10^{-4}$
Ethyleneglycol-bis (β-aminoethylether) *N,N'*-tetraacetic acid (EGTA)	$6.0 \cdot 10^{-4}$
Dithizone*	$7.5 \cdot 10^{-3}$
8-Hydroxyquinoline	$7.4 \cdot 10^{-3}$
Cyanide	$1.8 \cdot 10^{-2}$

* As dithizonate, pH 7.5.

equivalent to 50% inhibition. The large inhibition of 1 : 10 phenanthroline is characteristic with enzymes containing Zn at the catalytic site [15].

Metal ion reactivation of apo-enzyme. Table III lists the results of reactivation studies of apo-enzyme with selected metal cations. The two methods gave similar results. The strong activating effect of Co and Mn follows a similar pattern with other neutral proteinases [18].

Enzyme stability. The stability of the proteinase in the presence and absence of Ca^{2+} was investigated. At temperatures up to 40°C, the lack of Ca^{2+} does not markedly effect the stability of the enzyme. With increased temperature, a rapid loss of activity is observed (Fig. 2). However, with Ca^{2+} present, residual activity remained up to 60°C.

In an alternate study, the enzyme was equilibrated with solutions containing 0—100 mM $CaCl_2$ for 24 h at 25°C and the relative activity determined. A minimum concentration of 2—5 mM $CaCl_2$ was necessary for maximum reten-

TABLE III

METAL REACTIVATION OF THE METAL-FREE ENZYME

Enzyme treated with $1 \cdot 10^{-3}$ M metal* for 10 min at 25°C before assay.

Metal	Activation (EDTA treated) % relative activity	Activation (Chelex-100 treated) % relative activity
none (EDTA 10^{-3} M)	0	—
Ca^{2+}	11	8
Zn^{2+}	88	78
Zn^{2+}/Ca^{2+} (**)	55	50
Co^{2+}	63	49
Mn^{2+}	57	—
Fe^{3+}	5	—
Fe^{2+}	3	—
Cr^{3+}	0	—

* Metal salts used in chloride form except for zinc, as $ZnSO_4 \cdot 7H_2O$.
** Concentration Zn and Ca each 0.5×10^{-3} M.

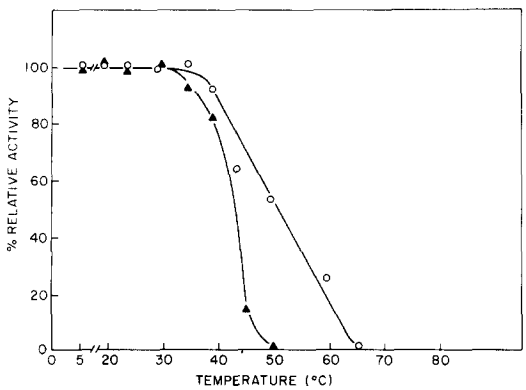

Fig. 2. Thermal stability of *P. fragi* proteinase. The samples were incubated 10 min at the designated temperatures in 0.05 M Tris · HCl buffer (pH 7.2); ▲, no calcium; ○, 5 mM $CaCl_2$.

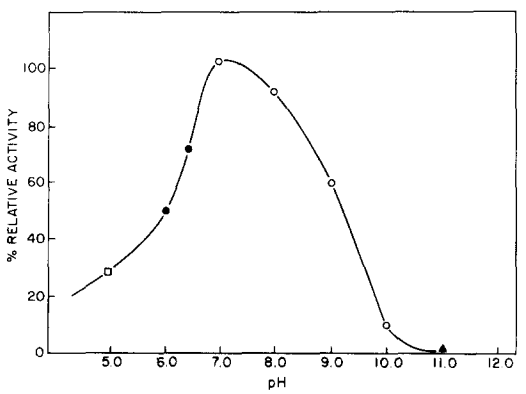

Fig. 3. Influence of pH upon the activity of *P. fragi* casein hydrolysis. The enzyme was brought to respective pH using 0.10 M buffers containing 5 mM $CaCl_2$. ▲ Borate (pH 11.0); ○———○, Tris · HCl (pH 10.0—7.0); ●———●, Tris/acetate (pH 7.0—6.0); □, Acetic Acid/Acetate (pH 5.0). The casein solutions were also made 0.10 M in respective buffers.

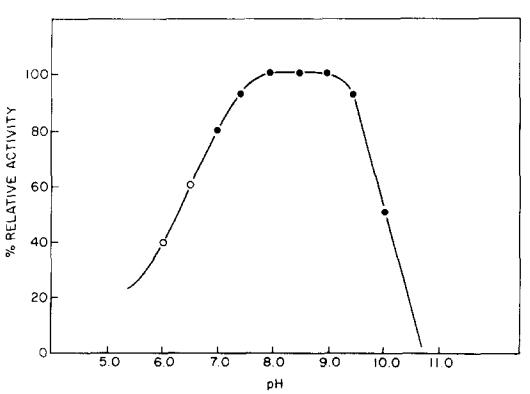

Fig. 4. Influence of pH upon the stability of *P. fragi* at 25°C in 5 mM $CaCl_2$. The enzyme was dialyzed for 24 h in 0.10 M buffers of different pH. ●———●, Tris · HCl (pH 7.0—10.0); ○———○, Tris/Maleate (pH 6.0—7.0). The assay was performed in 0.10 M Tris · HCl (pH 7.2) at 35°C.

tion of activity. The effect of pH upon enzyme activity was evaluated and the results depicted in Fig. 3. The proteinase is stable at the pH range of 7.0—9.5, with rapid inactivation occurring at more extreme values.

Enzyme activity. The effect of ionic strength upon proteolytic activity was determined. Maximum activity was found in the range of 0—0.50 M KCl and decreased to 50% relative activity at 0.20 M KCl. Further increases of salt to 1.2 M KCl had little additional effect.

The maximum proteolytic activity occurred in the pH range 6.5—8.0, but outside those values, activity decreased rapidly (Fig. 4).

Discussion

The extracellular proteolytic activity produced by *P. fragi* is due wholly to a metalloenzyme. Treatment of the cell-free culture with complexing agent results in a complete loss of activity, in contrast to other psychrophilic bacterial extracellular enzymes [6,7].

The pH optimum, molecular weight and metal requirements are consistent with those obtained from other microbial extracellular proteases. A notable difference is the rather low specific activity and sensitivity to thermal inactivation. This thermal sensitivity is not unexpected since *P. fragi* is a psychrophile and does not grow at temperatures approaching 35° C.

Acknowledgement

Michigan Agricultural Experiment Station Journal Article No. 6971. This investigation was supported in part by research grant FD-00097 from the Food and Drug Administration of the U.S. Department of Health, Education and Welfare.

References

1 Ayres, J.C. (1960) Food Res. 25, 1—18
2 Farrell, J. and Rose, A. (1967) Bacteriol. Rev. 21, 101—120
3 Green, B.W. and Jezeski, J.J. (1954) Appl. Microbiol. 2, 110—111
4 Hasagawa, T., Pearson, A.M., Price, J.F. and Lechowich, R.V. (1970) Appl. Microbiol. 20, 117—122
5 Tarrant, P.J.V., Pearson, A.M., Price, J.F. and Lechowich, R.V. (1971) Appl. Microbiol. 22, 224—228
6 Nunokawa, Y. and McDonald, I.J. (1968) Can. J. Microbiol. 14, 215—231
7 Kato, N., Nagasawa, T., Adachi, S., Tani, T. and Ogata, K. (1971) Agr. Biol. Chem. 36, 1185—1192
8 Tarrant, P.J.V., Jenkins, N., Pearson, A.M. and Dutson, T.R., (1973) Appl. Microbiol. 25, 996—1005
9 Hagihara, B., Matsubara, H., Nakai, M. and Okunuki, H. (1958) J. Biochem. Tokyo 45, 185—194
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
11 Davis, B. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
12 Wrigley, C.W. (1971) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.D., eds) Vol. XXII, pp. 449—564, Academic Press, New York
13 Vesterberg, O. (1971) Biochim. Biophys. Acta 243, 345—348
14 Malstrom, B. (1955) in Methods of Biochemical Analysis (D. Glick, ed.), Vol. 3, pp. 327—353, Interscience, New York
15 McConn, J.D., Tsuru, D. and Yasunobu, K. (1964) J. Biol. Chem. 239, 3706—3715
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
17 Andrews, B.P. (1965) Biochem J. 96, 595—606
18 Matsubara, H. and Feder, J. (1971) in the Enzymes (P. Boyer, ed., 3rd edn) Vol. III, pp. 721—795, Academic Press, New York