A COMPARISON OF EXTRACELLULAR PROTEASES FROM THREE PSYCHROTROPHIC STRAINS OF *PSEUDOMONAS FLUORESCENS*

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Extracellular proteases from three psychrotrophic strains of *Pseudomonas fluorescens* were purified and characterized. The cultivation temperature decisively influenced the excretion of protease into the medium. Highest enzyme production occurred at 10°C (one strain) or 20°C (two strains). The purified proteases showed a molecular weight between 47 kDa and 51 kDa, the *pI* values ranged from 7.8 to 7.4. All three proteases were characterized by several closely focused bands. Sensitivity to metalchelating agents indicates that *P. fluorescens* proteases are metalloproteases (EC 3.4.24). The pH optimum for azocaseinolytic activity was at pH 6–7, the temperature optimum was at 40–45°C. The apparent energies of activation were 36.9–38.0 kJ mol⁻¹. The proteases were thermolabile. By adding calcium ion an increasing thermostability was observed. SDS, urea and several metal ions inhibited proteolytic activities to different extents. Casein was the preferred substrate for the proteases. With casein concentrations up to 0.4–0.75% a Michaelis-Menten-type kinetics was observed, at higher casein concentrations a substrate inhibition occurred. None of the proteases showed aminopeptidase activity.

Extremophile organisms have adapted to their environment in such a way that metabolic processes, reproduction and survival strategies are optimal in their natural biotopes. One factor that requires particularly high standards of adaption is the temperature. The environment of psychrophilic microorganisms from glacial areas is characterized by permanently cold temperatures, a constant change from freezing to thawing, as well as by permanently fluctuating substrate conditions. Psychrophilic organisms are able to grow and to multiply at 0°C, and are thus adapted to these conditions. Obligate psychrophiles have an optimum growth

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temperature at about 15°C or lower, whereas the optimum growth temperature of facultative psychrophiles (psychrotrophs) is higher than 15°C (1).

While mesophilic organisms form enzymes with temperature optima of around 60°C, enzymes from psychrophiles are characterized by a low optimum temperature, a high activity at low temperatures, and a low activation energy (2–4). Therefore, enzymes from cold-adapted organisms could be useful for practical application (e.g., proteases for fining beer, leather industry, additives to detergents).

Psychrophilic microorganisms with proteolytic activities are ecologically highly important because they play an important role in the nitrogen cycle in cold biotopes. They gain further significance as spoilers of refrigerated food (5, 6). Most proteolytic psychrophilic microorganisms have been isolated from soil (7, 8), aquatic environments (7, 9, 10) and mainly from dairy products (4, 6, 11–13), but little is known about this group of organisms from alpine and nival environments (14, 15).

The purpose of this work was the isolation and characterization of the proteases produced by three psychrotrophic *P. fluorescens* from alpine environment. By establishing the biochemical and the physicochemical properties of the enzymes we wanted to determine to what extent these properties were strain-specific or species-specific. The effect of temperature on growth and protease excretion was also investigated.

MATERIALS AND METHODS

Abbreviations. *P.*, *Pseudomonas*; ACU, azocasein digestion unit; CU, casein digestion unit; Mes, morpholinoethane sulfonic acid; pNA, *p*-nitroanilides; PMSF, phenylmethylsulfonyl fluoride.

Chemicals. Azocasein, azoalbumin, casein *N,N*-dimethylated, bovine serum albumin, hemoglobin, ribonuclease, lysozyme, *p*-nitroanilides and the SDS molecular mass markers (SDS-6L) were from Sigma. Molecular mass markers for gel filtration were from Serva. Gel support film and Coomassie brilliant blue R250 were obtained from BioRad. S-Sepharose Fast Flow, Sephadex G-75, polyacrylamide gel plates for IEF and isoelectric focusing calibration kits were from Pharmacia LKB. Casein (Hammarsten), gelatine and Mes were obtained from Merck. Soybean meal and corn steep liquor were gifts from Biochemie Kundl (A). API 20 NE was obtained from API System, La Balme les Grottes, France. All other chemicals were of analytical grade.

Organisms. The strains used throughout this work were isolated from cryoconite (black organic accumulations) of glaciers (strains 164/03 and 177/30) and soil materials (strain 165/14) in the European Alps at altitudes between 2,900 m and 4,000 m. These strains were selected among 25 isolates as potent producers of protease. The characteristics of strains were determined microscopically, by gram-staining and by using API 20 NE strips which were incubated at 10°C for

72 h.

Cultivation. The strains were grown in the following medium: lactose, 2.5%; glycerol, 2.5%; corn steep liquor, 2%; sodium caseinate, 1%; soybean meal, 0.5%; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1%; KH_2PO_4 , 0.025%; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.25%; in distilled water. The pH of the medium was 6.9. Cultures of 50 ml in 500-ml flasks were inoculated with 1% of a subculture and were incubated at 10°C (strain 164/03) or 20°C (strains 165/14 and 177/30) on a rotary shaker with 180 rpm for 72 h. Each culture was incubated in triplicate. For determining the effect of the temperature on growth and protease excretion, strains were incubated at 10, 20 and 30°C. Growth was monitored by measuring the optical density at 660 nm.

Purification of proteases. All operations were carried out at 4°C. The cultures were centrifuged at 8,000 rpm for 15 min. The supernatant fluids of parallel cultures were pooled and 50 ml were dialyzed against 2,000 ml of 20 mM Mes-NaOH buffer (pH 6.0 at 4°C) for 15 h. All aliquot (25 ml) of the dialyzate was passed through an S-Sepharose Fast Flow column (1 × 10 cm) which had been equilibrated with 20 mM Mes-NaOH buffer, pH 6.0. The adsorbed proteins were eluted with an increasing NaCl gradient (0 to 0.5 M) in the same buffer. The flow rate was 1 ml min⁻¹; fractions of 5 ml were collected. Fractions with the same specific activity were pooled, dialyzed against 1,000 ml of 20 mM Mes-NaOH buffer (pH 6.7) for 4 h, and stored at -20°C.

Electrophoresis. SDS-PAGE according to Laemmli (16) was performed with 1.5 mm gels with an acrylamide concentration of 12%. Electrophoresis was carried out at pH 8.3 at a constant voltage of 200 V for 40 min. The gels were stained for 30 min with 0.1% Coomassie brilliant blue R250 dissolved in methanol-acetic acid-water (40:10:50) and destained overnight in methanol-acetic acid-water (40:10:50).

Isoelectric focusing was performed at 8°C and a pH gradient from 3.5 to 9.5 in thin-layer (1 mm) polyacrylamide gel plates. Samples were applied to the gels 30 mm from the anode. The gels were focused at 30 W, 1500 V, 50 mA with 585 Vh (ca. 90 min). A standard mixture of 12 proteins (pH 3.5 to 9.3) was used to indicate the pH gradient in the gel. Gels were fixed and stained as recommended by LKB instructions.

Detection of proteases in IEF gels. A solution of 1% purified agar, 0.5% casein (Hammarsten) and 0.2% NaN_3 in 67 mM phosphate buffer, pH 7.0, was boiled, cooled to 50°C and poured onto a gel support film (100 × 125 mm). An IEF gel was applied to the casein gel; air bubbles were removed by gentle pressure. After an incubation period of 60 min at 30°C in a humid chamber the casein overlay was removed, rinsed with distilled water, fixed and stained as described for the IEF gels.

Determination of the molecular mass. The molecular mass of proteases was determined under native conditions by gel filtration and under denaturing conditions by SDS-PAGE.

Gel filtration according to Andrews (17) was performed at 4°C. A Sephadex G-75 column (1.5 × 50 cm) was equilibrated with 67 mM phosphate buffer, pH 7.0.

The flow rate was $4.84 \text{ ml h}^{-1} \text{ cm}^2$, fractions of 1.43 ml were collected. The void volume was determined with blue dextran 2000. Cytochrome *c* (12.3 kDa), myoglobin equine (17.8 kDa), ovalbumin (45 kDa) and bovine serum albumin (67 kDa) were used for calibration.

For SDS-PAGE the protein standards lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa) and bovine serum albumin (67 kDa) were used.

Protein determination. Protein concentration was measured as described by Bradford (18). Bovine serum albumin was used as standard. Protein content in gel filtration fractions was monitored by its absorbance at 280 nm.

Protease assay. Proteolytic activity was measured by the digestion of azocasein (19). Enzyme solution (50 μl) containing 0.002% protein was incubated with 300 μl of 1% azocasein in 67 mM phosphate buffer, pH 7.0, at 30°C for 30 min. The reaction was stopped by adding 1 ml of 6% trichloroacetic acid. After a further 15 min the mixture was centrifuged and the absorbance of the supernatant was measured at 340 nm. The activity was expressed in relative azocasein digestion units (ACU). One ACU was defined as the increase of 0.001 per min in absorbance under the described conditions. Specific activity was expressed in ACU per mg protein.

Aminopeptidase assay. L-Leucine-pNA, L-alanine-pNA, L-lysine-pNA, L-phenylalanine-pNA, L-glycine-pNA, L-arginine-pNA were dissolved in dimethylsulfoxide and diluted with 67 mM phosphate buffer, pH 7.0, to a concentration of 1.1 mM. For each assay, 600 μl of substrate solution was mixed with 100 μl of enzyme solution and incubated at 30°C for 30 min. The reaction was stopped on ice and the absorbance was measured at 410 nm. One unit of aminopeptidase activity was expressed as μmol of *p*-nitroaniline formed per min.

Substrate specificity. Digestion of azoproteins (azocasein and azoalbumin) was measured with the standard protease assay.

Digestion of proteins was determined as previously described (19). Solutions (1%) of casein, casein *N,N*-dimethylated, soybean meal, gelatine, bovine serum albumin, hemoglobin, lysozyme and ribonuclease were prepared in 67 mM phosphate buffer, pH 7.0. Enzyme solution (0.1 ml) containing 0.004% protein was incubated with 0.6 ml of each of the preheated substrate solutions at 30°C for 30 min. The reaction was stopped by addition of 1 ml of 7.5% trichloroacetic acid. After 30 min the mixtures were centrifuged and the absorbance of the supernatants was measured at 275 nm. One casein digestion unit (CU) of the enzyme activity was expressed as μmol tyrosine liberated per min under the assay conditions.

Kinetics of casein utilization. Casein (Hammarsten) concentrations in the range from 0.05% to 1% were prepared in 67 mM phosphate buffer, pH 7.0. Proteolytic activities of 0.004% protein solutions were measured with the method described in the section dealing with substrate specificity.

Effect of pH and temperature on activity and stability of proteases. These effects

were determined using the standard protease assay with enzyme solutions containing 0.002% protein. Determination of the pH optimum was performed at 30°C with the following buffer systems (67 mM each): sodium acetate-acetic acid (pH 5), KH_2PO_4 - Na_2HPO_4 (pH 6–7), $\text{Na}_2\text{B}_4\text{O}_7$ -HCl (pH 8–9), $\text{Na}_2\text{B}_4\text{O}_7$ -NaOH (pH 10), Na_2HPO_4 -NaOH (pH 11–12). The pH values were adjusted at 30°C.

pH stability of the proteases was investigated in buffer solutions at various pH values. The following buffer systems were used (67 mM each): sodium acetate-acetic acid (pH 4–5), KH_2PO_4 - Na_2HPO_4 (pH 6–8), glycine-NaOH (pH 9–10), Na_2HPO_4 -NaOH (pH 11–12). Enzyme solutions (50 μl) were incubated with 450 μl of the buffer solutions at 20°C for 1 h. After neutralization of the mixtures by adding 0.5 ml of 200 mM phosphate buffer pH 7.0, the residual activities were measured.

For determination of the temperature optimum the reaction was carried out at various temperatures and at pH 7.0 for 30 min.

For determination of the effect of temperature on enzyme stability the proteases were incubated at pH 7.0 (67 mM Tris-HCl buffer) in the presence or absence of 10 mM CaCl_2 at temperatures ranging from 0 to 60°C for 10 min. In addition, the proteases were incubated at various temperatures for various periods. Afterwards the mixtures were rapidly cooled and the residual activities were determined.

Effect of various substances. The effect of several metal ions as well as of protease inhibitor was investigated. The proteases were also incubated with different concentrations of urea and SDS. For these experiments the enzymes (0.002% protein) were incubated with each of the reagents at 20°C and at pH 7.0 for 1 h. The residual activities were determined by the standard protease assay.

RESULTS

Characterization of strains

The strains were aerobic, gram-negative, motile, yellow-pigmented and rod-shaped bacteria. They were oxidase-positive, arginine dihydrolase-positive and liquefied gelatine, but showed no activities of β -glucosidase, β -galactosidase and nitrate reductase. The strains hydrolyzed glucose, arabinose, mannose, mannitol, *N*-acetyl glucosamine, gluconate, caprate, *L*-malate and citrate. Maltose, adipate and phenylacetate were not utilized. According to the APILAB computer program (version 3.1) the strains were identified as *P. fluorescens* with a probability of 99%. This result was confirmed by reference to Bergey's Manual of Systematic Bacteriology (20).

Effect of temperature on growth and protease excretion

Strain 164/03 showed a similar cell density (1×10^{10} cells per ml) at all three cultivation temperatures, whereas strains 165/14 and 177/30 grew better at 10°C and 20°C than at 30°C. The optimal growth temperature for strain 165/14 was 10°C (2×10^{10} cells per ml); strain 177/30 grew equally well at 10°C and 20°C (2

$\times 10^{10}$ cells per ml). None of the strains formed protease at 30°C. The strains 165/14 and 177/30 formed a higher amount of protease at 20°C than at 10°C, strain 164/03 exhibited the highest protease excretion at 10°C. The highest proteolytic activities were observed in the stationary growth phase. Protease excretion still increased even after 72 h. Growth and protease excretion at 10°C were delayed by 24 h in comparison to the ones at 20°C.

Purification of proteases

The specific activity values of the dialyzates of protease-containing samples were 3.4×10^3 (sample 164/03), 8.5×10^3 (sample 165/14), and 6.7×10^3 (sample 177/30) ACU mg^{-1} protein. The proteases were eluted at NaCl concentrations of 0.15 M (protease 165/14), 0.2 M (protease 164/03) and 0.25 M (protease 177/30) as single protein peaks. In each of the elution profiles the peak of protein coincided with the peak of protease activity (Fig. 1). Recoveries of 46% (protease 164/03), 51% (protease 165/14) and 75% (protease 177/30) were obtained. The specific activities of the proteases were 21×10^3 , 39×10^3 and 38×10^3 ACU mg^{-1} protein respectively. The protein contents of the purified enzyme solutions were 266, 334 and 293 $\mu\text{g ml}^{-1}$ (Table 4).

Homogeneity

Each of the purified enzymes gave one single protein band on SDS-PAGE (Fig. 2). UV-absorption spectra showed a maximum absorbance at 275 nm and a minimum absorbance at 250 nm.

Molecular mass

Estimations of molecular mass by gel filtration and by SDS-PAGE had the same results. Elution profiles of gel filtration showed one single protein peak coinciding with the peak of activity. The molecular mass of protease 164/03 was 49 ± 2 kDa, the one of both the proteases 165/14 and 177/30 was 47 ± 1 kDa (Table 4). Determination by SDS-PAGE showed molecular masses of 50.6 ± 0.5 kDa (protease 164/03), 48.6 ± 1 kDa (protease 165/14) and 49 ± 1 kDa (protease 177/30) respectively (Fig. 2, Table 4).

Isoelectric point

P. fluorescens proteases were characterized by several closely focused bands of proteins within a range of pI 7.5–6.8 (164/03), 7.9–7.5 (165/14) and 7.8–7.4 (177/30). The zymograms showed that in each case the protein bands in the IEF gels were associated with protease activity. The first 2–3 bands close to the cathode with

Fig. 1. Chromatography profiles of three *P. fluorescens* proteases. 25 ml of dialyzed culture supernatant were applied to an S-Sepharose Fast Flow column (1×10 cm). The adsorbed proteins were eluted with a linear NaCl gradient in 20 mM Mes-NaOH buffer, pH 6.0, at a flow rate of 1 ml min^{-1} . (○) Proteolytic activity toward azocasein; (△) protein content; dotted line, NaCl gradient (M).

Fig. 2. SDS-PAGE of purified *P. fluorescens* proteases. (2), protease 164/03; (3), protease 165/14; (5), protease 177/30; (4), standard proteins: lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), bovine serum albumin (67 kDa).

pI's of 7.5–7.4 (protease 164/03), 7.8 (protease 165/14), 7.8–7.7 (protease 177/30) had as well the highest protein content as the highest activity in the zymograms; a further 3–6 bands were less developed.

Effect of pH on activity

The proteases were found to have a neutral pH optimum. Enzymes 165/14 and 177/30 showed more than 80% of their maximum activity within the pH range of 6 to 9. Protease 164/03 lost 75% of activity at pH 9 and showed a higher activity at pH 5. The proteases lost their activities at pH 11 and 12 (Fig. 3).

Effect of pH on stability

Proteases 165/14 and 177/30 were stable from pH 4 to 9 at 20°C for 1 h, whereas protease 164/03 was stable from pH 6 to 9. None of the proteases showed a residual activity at pH 11 and 12 (Fig. 4).

Effect of temperature on activity

The proteases showed a maximum activity at 40–45°C. At temperatures above the optimum temperature proteases rapidly lost activity. At 20°C 40% of activity could still be detected (Fig. 5).

The Arrhenius law was found to be followed between 20°C and 40°C, and apparent activation energies in the range of 36.9–38.0 kJ mol⁻¹ for the hydrolysis of azocasein were calculated (Table 4). These activation energies were much lower than the values obtained from proteases of mesophilic origin. Subtilisin Carlsberg (Sigma) and Savinase^R (Novo) were tested under the same conditions and showed

Fig. 3. Effect of pH on activity of proteases. The reaction was carried out with azocasein at 30°C for 30 min. The buffer systems used (each 67 mM) were: sodium acetate-acetic acid (pH 5), KH_2PO_4 - Na_2HPO_4 (pH 6-7), $\text{Na}_2\text{B}_4\text{O}_7$ -HCl (pH 8-9), $\text{Na}_2\text{B}_4\text{O}_7$ -NaOH (pH 10), Na_2HPO_4 -NaOH (pH 11-12). (□) protease 164/03, (○) protease 165/14, (△) protease 177/30.

Fig. 4. Effect of pH on stability of proteases. Enzymes were incubated at 20°C for 1 h at varying pH. The buffers (67 mM) were: sodium acetate-acetic acid (pH 4-5), KH_2PO_4 - Na_2HPO_4 (pH 6-8), glycine-NaOH (pH 9-10), Na_2HPO_4 -NaOH (pH 11-12). After incubation the pH of the enzyme solutions was adjusted to pH 7.0 and the residual activities were determined at 30°C with azocasein. (□) protease 164/03, (○) protease 165/14, (△) protease 177/30.

Fig. 5. Effect of temperature on activity of proteases. The reaction was carried out with azocasein at various temperatures and at pH 7.0 for 30 min. (□) protease 164/03, (○) protease 165/14, (△) protease 177/30.

activation energies of 59.1 and 62.2 kJ mol⁻¹ respectively.

Effect of temperature on stability

All proteases were thermolabile. Among the enzymes, proteases 165/14 and 177/30 were somewhat more stable than protease 164/03. The activities of proteases were not affected after 1 h at 20°C, whereas after 1 h at 30°C protease 164/03 retained only 80% of activity. At 45°C and higher temperatures all three proteases were inactivated rapidly. By the addition of 10 mM calcium chloride the enzymes became more stable to temperature; the temperature inactivation profiles showed a shift of 5–10°C. The effect of temperature on stability of protease 177/30 is shown in Fig. 6.

Effect of inhibitors

Proteolytic activities of all three enzymes were not affected by sulfhydryl-reagents (iodoacetamide) or serine-active reagents (PMSF). All proteases were completely inactivated by 1 mM EDTA. *o*-Phenanthroline inhibited protease 164/03 only slightly. Citrate and oxalate, known to be Ca-specific, affected none of the proteases. Proteases 165/14 and 177/30 differed only with regards to their reaction to 2,2-bipyridyl (Table 1). These results indicate that the enzymes are metallo-proteases (EC 3.4.24). Regarding the stability against SDS and urea, protease 164/03 was most labile. Protease 165/14 showed the best stability against SDS, whereas protease 177/30 was more stable against urea (Table 1).

Fig. 6. Effect of temperature on stability of protease 177/30. A: The enzyme was incubated at pH 7.0 and at 20°C (○), 30°C (▽), 40°C (△), 45°C (□) and 50°C (×) for the indicated periods. B: The enzyme was incubated at pH 7.0 in the presence (□) or in the absence (○) of 10 mM CaCl₂ at temperatures ranging from 0°C to 60°C for 10 min. The residual activities were determined at 30°C and pH 7.0.

Effect of metal ions

Ag⁺, Cu²⁺, Hg²⁺ and Ni²⁺ affected in decreasing order the activities of the proteases. Fe²⁺, Mo⁵⁺, Co²⁺, Zn²⁺ and Pb²⁺ on average reduced proteolytic activity by 10%. The enzymes were unaffected by Mg²⁺ (Table 2).

Table 1. Effect of inhibitors on activity of proteases. The enzymes were preincubated with each inhibitor at the stated concentration at 20°C and pH 7.0 for 1 h. The residual activities were measured.

Inhibitor	Concentration	Residual activity (%)		
		164/03	165/14	177/30
None		100	100	100
Cysteine	10 mM	84	89	79
PMSF	10 mM	111	107	107
Iodoacetamide	10 mM	107	107	105
EDTA	10 mM	0	0	0
<i>o</i> -Phenanthroline	10 mM	93	57	80
2,2'-Bipyridyl	10 mM	35	94	31
KMnO ₄	10 mM	8	6	7
KCN	10 mM	87	100	100
Citrate	100 mM	101	100	97
Oxalate	100 mM	102	105	100
SDS	0.025%	4	99	57
	0.050%	0	82	40
	0.100%	0	47	4
	0.250%	0	6	0
	0.500%	0	0	0
Urea	1 M	102	100	104
	2 M	32	100	102
	3 M	1	57	79
	4 M	0	0	0

Table 2. Effect of metal ions on activity of proteases. The enzymes were preincubated with each metal ion (1 mM) at 20°C and pH 7.0 for 1 h. The residual activities were measured.

Salt	Residual activity (%)		
	164/03	165/14	177/30
None	100	100	100
AgNO ₃	4	69	25
CuSO ₄	13	41	25
HgSO ₄	69	40	46
NiSO ₄	69	79	79
FeSO ₄	91	96	93
MoCl ₅	90	96	93
CoSO ₄	93	96	94
ZnSO ₄	85	92	100
Pb(NO ₃) ₂	97	101	95
MgSO ₄	102	101	105

Substrate specificity

The proteases preferred high molecular and denatured substrates like dimethyl-casein and casein, whereas other proteins were hydrolyzed to a much lower extent.

Table 3. Hydrolysis of proteins and azoproteins. The reactions were carried out at 30°C and pH 7 for 30 min. Data are presented as percentages of the rates of hydrolysis of dimethylcasein and azocasein respectively.

Substrate (1%)	Rate of hydrolysis (%)		
	164/03	165/14	177/30
Dimethylcasein	100	100	100
Casein	89	88	80
Soybean meal	51	28	32
Gelatine	53	58	61
Hemoglobin	51	32	38
Bovine serum albumin	17	10	11
Lysozyme	26	14	17
Ribonuclease	0	0	0
Azocasein	100	100	100
Azoalbumin	21	25	16

Fig. 7. Lineweaver-Burk plot for the hydrolysis of casein by protease 164/03. The reaction was carried out with casein (Hammarsten), pH 7.0, at 30°C for 30 min. The dotted line is the extrapolation of the linear portion of the plot.

Ribonuclease was not utilized (Table 3).

Kinetics of casein utilization

The rate of casein hydrolysis by the proteases was found to be proportional to casein concentrations up to 0.4% (protease 164/03) and 0.75% (proteases 165/14 and 177/30) respectively. There was a marked substrate inhibition at higher casein concentrations (Fig. 7). K_m and V_{max} values were obtained by extrapolation of the linear portion of the Lineweaver-Burk plots and are shown in Table 4.

Table 4. Comparison of some properties of three *P. fluorescens* proteases.

Property	164/03	165/14	177/30
Producer	<i>P. fluorescens</i>	<i>P. fluorescens</i>	<i>P. fluorescens</i>
isolated from	cryoconite	soil	cryoconite
at altitude (m)	4,000	3,860	2,900
Cultivation	72 h	72 h	72 h
	10°C	20°C	20°C
Production in culture (ACU $\times 10^3$ ml ⁻¹)	4.7	11.6	9.7
Purified proteases:			
Recovery (%)	46	51	75
Specific activity (ACU $\times 10^3$ mg ⁻¹ protein)	21.3	39.1	38.1
Mol wt, Sephadex G-75 (kDa)	49	47	47
Mol wt, SDS-PAGE (kDa)	50.6	48.6	49
Isoelectric point (main bands)	7.5–7.4	7.8	7.8–7.7
Optimum pH	6–7	7	7
Optimum temperature (°C)	40	40–45	45
Activation energy (kJ mol ⁻¹)	37.5	38.0	36.9
Ca ²⁺ stabilization	+	+	+
K_m (% casein)	0.39	0.57	0.39
V_{max} (CU mg ⁻¹ protein)	8.9	19.4	15.6
Classification by inhibitors	metalloprotease	metalloprotease	metalloprotease

Aminopeptidase activity

None of the proteases utilized any of the *p*-nitroanilides tested.

DISCUSSION

The proteases formed by three psychrotrophic strains of *P. fluorescens* were purified after dialysis directly via ion chromatography. We did not use an ammonium sulfate precipitation as this is well known for its high enzyme losses. From preliminary investigations (IEF) it was known that interfering proteins in culture supernatants had to be attributed mainly to nutrient components with a *pI* of 3.9–3.7. The homogeneity of the purified proteases was tested via SDS-PAGE, via IEF and via UV-absorption spectra and could be verified. As a consequence of the considerably lower activity in the culture fluid in comparison to the proteases 165/14 and 177/30, protease 164/03 showed a low specific activity. Molecular mass of the proteases ranged from 49 to 51 kDa when determined with SDS-PAGE; the values were lower by ca. 2 kDa when determined with gel filtration. This close correspondence allows us to deduce that the proteases are monomers. Proteases from *P. fluorescens* are known to have a molecular mass of 35 kDa (21), 38.4 kDa (22), 45 kDa (6) and 47 kDa (13).

The results with IEF showed that all three purified proteases were characterized by several closely focused bands with decreasing concentrations of protein and activity respectively. As the *pI* values of the various bands showed only slight differences, we suppose that we are dealing with multiple forms of enzymes.

Multiple bands may arise from post-transcriptional modifications of one protease or from starting self-digestion. A similar pattern was observed with proteases from *Aeromonas* (23). Depending on the biotype, different *pI* values of 8.3–8.2 (biotype I) and 7.25 (biotype III) were determined for proteases from psychrotrophic *P. fluorescens* (11).

The further characterization of the enzymes showed that the proteases 165/14 and 177/30 were more closely related, whereas protease 164/03 exhibited various deviating properties.

The proteases belonged to the neutral proteases. With regards to the effect of pH on activity and stability, protease 164/03 showed a more narrow pH-tolerance range than the other two proteases. Proteases from pseudomonads are known to have optimum activity at pH 6.5 (22), 6.5–7.5 (6), 7.2–7.4 (12), 8 (24), but also at pH 10 (25). The pH stability of the investigated proteases (>85% residual activity after exposure to pH 5–9 at 20°C) was comparable to the one of a protease from a psychrotrophic *Pseudomonas* sp. (26).

Enzymes from psychrophiles and psychrotrophs differ from the ones from mesophiles mainly by their reaction to temperature. The excretion of enzymes seems to be more dependent on the low temperature than the growth of psychrophilic bacteria, thus reflecting the physiological adaptations to the extreme environments of the strains. For the formation of lipase (2) the same mechanisms were observed. The high excretion of protease in the stationary growth phase is thought to be controlled by nutritional conditions and catabolite repression.

Characteristics of the *P. fluorescens* proteases were temperature optima of 40–45°C, high activities at 20°C, low activation energies and asymmetrical slopes of the temperature profiles. An increase of 5°C above the optimum temperature caused a reduction of activity of 75%, thus indicating thermal denaturation (27). For proteases from psychrotrophic pseudomonads, temperature optima of 30°C (10), 37°C (22,28), 40°C (12) and 45–50°C (10,13) were observed. Yet the temperature optimum can be influenced by the pH of the reaction (8). A low temperature optimum is the result of a high thermosensitivity. As the protease producers are never confronted with high temperatures in their natural environment, there is no necessity for high thermostability. In the process of evolution the adaption to low temperatures led to a development of genetically determined cold-stable proteins. Enzymes of cold-adapted organisms may show a flexible conformation allowing the reduction of the energy barrier, which may be responsible for their thermosensitivity. These enzymes have higher catalytic efficiencies than those of warm-adapted species (29).

The stabilizing effect of calcium has repeatedly been observed (26,30). Metal ions such as calcium appear to be involved in the stabilization of the protein structure and are required for protection against heat inactivation (31).

Proteases formed by pseudomonads are mainly metalloproteases (6,9,24,30,31). Metalloproteases are widespread and exhibit a broad substrate specificity. The investigated proteases preferred denatured and high molecular substrates such as

casein and dimethylcasein. A comparable substrate specificity was determined with a protease from a psychrotrophic *P. fluorescens* (6). Yet the substrate utilization is dependent on the pH of the reaction (32). All three proteases showed a substrate inhibition from a certain casein concentration (0.4–0.75%) onwards. A similar pattern has already been observed with a protease from *P. fluorescens*, for which a casein content of 0.5% proved to be inhibiting (22). Interactions between enzyme and substrate at positions removed from the active site could be responsible for the substrate inhibition. On the other side, an increasing casein aggregation at high casein concentrations may cause a decrease of substrate availability (22).

The investigated proteases were typical metalloproteases and showed the same general properties as those shown by other proteases of psychrotrophic *P. fluorescens*. The enzymes were well adapted for growth at low temperatures. The proteases produced by various strains of the same species were very similar with respect to their activities and specificities. Yet they could be differentiated, especially regarding their stability. The differences between proteases formed by various strains of one species are seen in connection with the respective genetic and physiological adaption of the strains, and with environmental and substrate conditions of the sites the strains originated from.

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