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Purification and some properties of proteinase from *Pseudomonas fluorescens* No. 33

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SUMMARY. The extracellular proteinase from *Pseudomonas fluorescens* No. 33 was purified to electrophoretic homogeneity by a procedure including precipitation with HCl and $(NH_4)_2SO_4$, and column chromatography. The enzyme was purified 170-fold giving a yield of 7% of the original activity. The molecular mass of the purified enzyme was 48000 by SDS-PAGE. The optimum pH and temperature for the hydrolysis of casein were 8·0–9·8 and 30–35 °C respectively. The enzyme was more thermostable in synthetic milk salts solution than in 0·1 M-sodium phosphate buffer. but was heat-labile at 50 °C in both buffer systems. The activity was inhibited by *o*-phenanthroline, Hg^{2+}, Cu^{2+}, Fe^{2+} and, to a lesser extent, Ni^{2+}. Caseins were susceptible to the proteinase. but degradation patterns were dependent on the form of the casein.

Many species of psychrotrophic cells in raw milk produce heat-resistant proteinase and lipase. These enzymes can often continue their activity even after UHT sterilization (Burton, 1988: Adams. 1991). Proteinases induce bitterness and gelation of UHT sterilized milk (Fairbairn & Law 1986*a*). while lipases cause flavour defects due to fat breakdown in cream. butter. cheese and UHT products (Law. 1979: Cousin, 1982; Stead, 1986). Some workers noted that psychrotroph proteinase was more significant than lipase in milk flavour quality (Mottar. 1981: Christen & Wang 1985).

The aim of this study was to observe the nature of the proteinase from *Pseudomonas fluorescens* No. 33.

MATERIALS AND METHODS

Organism

Ps. fluorescens No. 33, previously reported as *Pseudomonas* sp. No. 33 (Kumura *et al.* 1991*a*, *b*) was originally isolated from pasteurized milk and stored on nutrient agar slopes at 2 °C.

Culture conditions

The organism was subcultured on a nutrient agar slope, then in skim milk and nutrient broth, at 17 °C for 24 h each. The broth culture was diluted $1:10^4$ with diluent (American Public Health Association, 1960) containing 0·85% NaCl and

2 mM-Mg^{2+}. and 150 μl portions were inoculated into 15 ml portions of sterile 10 %
reconstituted skim milk in 100 ml Erlenmeyer flasks. The skim milks were incubated
statically at 17 °C for 7 d. The culture was centrifuged at 10000 g and 17 °C for
30 min to remove precipitates. The supernatants were used as the crude enzyme.

Production and purification of the enzyme

The culture supernatant was acidified to pH 4·8 with M-HCl and centrifuged at
10000 g and 20 °C for 30 min to remove the precipitate. The supernatant was
immediately adjusted to pH 7·5 with aqueous NH_4OH. Wet octyl-Sepharose CL-4B
(Pharmacia, Uppsala, Sweden) was added to the supernatant and stirred gently for
20 h at 2–4 °C to entrap concomitant lipase. After removal of the gel on a glass filter,
solid $(NH_4)_2SO_4$ was added to the filtrate to 40 % saturation. The precipitate was
removed by centrifugation at 10000 g and 4 °C for 30 min. Additional solid
$(NH_4)_2SO_4$ was added to the supernatant to 55 % saturation. The resulting
precipitate was collected by centrifugation at 10000 g and 4 °C for 30 min and
dialysed against 0·01 M-Tris–HCl buffer, pH 7·5. The dialysed preparation was
applied to a column (22 × 212 mm) of DEAE-Sepharose CL-6B (Pharmacia) which
was pre-equilibrated with 0·01 M-Tris–HCl buffer, pH 7·5. The column was washed
thoroughly with the same buffer to remove unabsorbed materials and then the
enzyme was eluted by a linear gradient of 0·1–0·3 M-NaCl in the same buffer (360 ml)
at a flow rate of 45 ml/h. Proteinase-rich fractions were pooled and solid $(NH_4)_2SO_4$
was added to give 40 % saturation. The mixture was applied to a column
(10 × 450 mm) of Butyl-Toyopearl (Tosoh, Tokyo) which was pre-equilibrated with
40 % saturated $(NH_4)_2SO_4$ in 0·01 M-Tris–HCl buffer, pH 7·5. The enzyme was eluted
by a decreasing gradient of saturated $(NH_4)_2SO_4$ (25–7 % saturation) in the same
buffer (135 ml) at a flow rate of 45 ml/h. The proteinase-rich fractions were pooled
in a cellulose tube, and concentrated by burying in solid sucrose at 2 °C. The
concentrate was then applied to a column of Toyopearl HW-50S (Tosoh)
(10 × 970 mm) pre-equilibrated with 0·01 M-Tris–HCl buffer, pH 7·5 and was eluted
with the same buffer. The flow rate was ∼ 10 ml/h. Proteinase-containing fractions
were pooled and stored at 2 °C as the purified proteinase.

Measurement of proteinase activity

The proteolytic activity was determined as previously described (Kumura et al.
1991a) using 0·5 % acid casein in 0·05 M-glycine–NaOH buffer, pH 9·0 as substrate at
30 °C for 10 min. The acid casein was prepared by isoelectric precipitation of raw
skim milk at pH 4·6 with M-HCl. The enzyme was appropriately diluted with 0·01 M-
Tris–HCl buffer, pH 7·5. One proteinase unit (PU) of enzyme activity was defined as
the number of micrograms of tyrosine released per min at 30 °C (1 PU = 1 μg
tyrosine/min).

Protein determination

The protein concentration was determined by a modified Lowry method
(Bensadoun & Weinstein. 1976) using bovine serum albumin as a standard.

Characterization of the enzyme

The optimum pH of the proteinase was determined using 0·5 % casein dissolved
in Universal buffer (Dawson et al. 1969) or 0·05 M-glycine–NaOH buffer at 30 °C for
10 min.

The relationship between pH and stability was determined by measuring the residual enzyme activity after storage at 2 °C for 24 h at various pH values. The protein concentration was adjusted to 50 μg/ml with Universal buffer.

The optimum reaction temperature was determined using 0·5% acid casein buffered with 0·05 M-glycine–NaOH buffer, pH 9·0 at a defined temperature for 10 min.

Thermostability of the purified proteinase in 0·1 M-sodium phosphate buffer, pH 6·6 and synthetic milk salts solution, pH 6·6 (Jenness & Koops, 1962) was determined as previously described (Kumura *et al.* 1991*a*). The protein concentration was 50 μg/ml.

Effect of metal ions and inhibitors

The enzyme (50 μg/ml) was dissolved in 0·01 M-Tris–HCl buffer pH 7·5, containing metal ions (6 mM) or additives (1 mM or 0·01 mM) and incubated at 30 °C for 10 min with subsequent addition of 0·5% acid casein in 0·05 M-glycine–NaOH buffer, pH 9·0 containing the additive at the same concentration. The remaining activity was assayed and expressed as a percentage of the control to which only buffer was added. Metal ions were used as chloride compounds (Ca^{2+}, Mg^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+}) except sulphate for Fe^{2+}. Proteinase inhibitors used were EDTA (Nakarai Chemicals, Kyoto), *o*-phenanthroline (Dojindo Laboratories, Kumamoto), *p*-chloromercuribenzoate (PCMB, Nakarai), phenylmethylsulphonyl fluoride (PMSF, Sigma, St Louis, MO 63178, USA), soyabean trypsin inhibitor (Sigma) and bestatin (Sigma).

Molecular mass

The molecular mass of the purified proteinase was estimated by SDS-PAGE performed on a slab gel calibrated using phosphorylase b (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soyabean trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14400) as standards (Laemmli, 1970). To prevent autolysis, mM-*o*-phenanthroline was dissolved in the enzyme. Samples were loaded on a 10% polyacrylamide gel containing 0·1% SDS with a stacking gel.

Amino acid composition

A sample of the enzyme was hydrolysed in 6 M-HCl for 24 h at 110 °C, and amino acids were identified on an amino acid analyser. The tryptophan content of the enzyme was determined in 6 M-guanidine hydrochloride (GuHCl) by a spectrophotometric method (Edelhoch, 1967).

Analysis of milk protein hydrolysis

The degradation profile of milk proteins by the proteinase was investigated by electrophoresis using as substrates 0·5% acid casein, 0·5% micellar casein separated by centrifugation (at 30000 *g* for 90 min) from raw skim milk or 0·3% whey proteins, prepared by isoelectric precipitation of the casein from raw skim milk, dissolved in synthetic milk salts solution, pH 6·6. The proteinase (15 μl, 22·6 PU) was added to the substrate (10 ml), immediately divided into 1 ml per tube and incubated at 4 °C for up to 24 h. The reaction was terminated by addition of an equal volume of 0·4% mercaptoethanol in 9 M-urea for casein substrates or 0·05 M-citrate buffer, pH 5·0 containing 2 M-urea for whey proteins substrate and subjected to gel electrophoresis.

Urea-PAGE of casein was performed according to Aoki *et al.* (1986).

Table 1. *Purification† of proteinase from* Pseudomonas fluorescens *No. 33*

	Specific activity, PU‡/mg	Purity index	Recovery, %
Culture supernatant	14·3	1·0	100
HCl precipitation (pH 4·8)	43·6	3·1	93·5
(NH₄)₂SO₄ (40–55% saturation)	440·1	30·8	64·5
DEAE-Sepharose CL-6B	2029	142	39·9
Butyl-Toyopearl	1876	131	8·9
Toyopearl HW-50	2451	171	7·7

† Details are given in the text.
‡ For definition, see text.
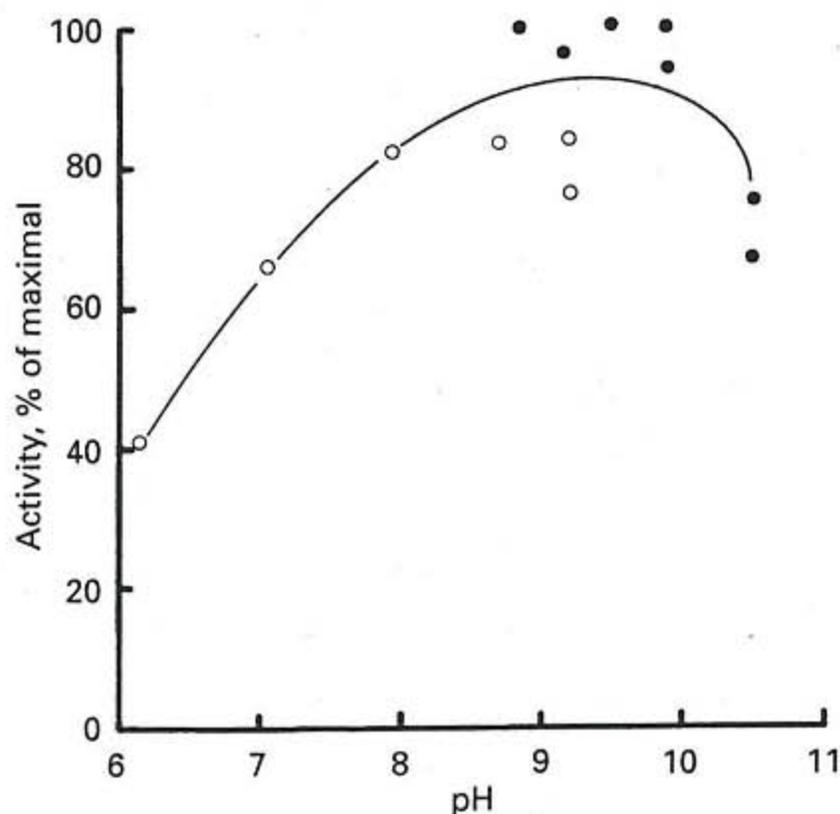
Fig. 1. Effect of pH on the activity of purified *Pseudomonas fluorescens* No. 33 proteinase on 0·5% casein at 30 °C. Buffer systems were Universal buffer (○) and 0·05 M-glycine–NaOH buffer (●).

PAGE of whey protein was performed in non-urea buffer by a modified Hillier (1976) method. The separating gel contained 11% acrylamide dissolved in buffer (46 g Tris + 60 ml M-HCl/l). The stacking gel was composed of 2·5% acrylamide in buffer (7·5 g Tris + 60 ml M-HCl/l). These gels were stained with Coomassie blue.

<center>RESULTS</center>

Purification of proteinase

A representative result of purification is summarized in Table 1. During each of the purification steps, all proteolytic activity was concentrated in a single chromatographic fraction.

Characterization

The optimum pH for action on casein at 30 °C was 8·0–9·8 (Fig. 1). The proteinase had a broad activity range with ∼ 50% of its maximal activity around the pH of bovine milk (pH 6·6). The enzyme was very stable at 2 °C for 24 h over the wide range of pH 4·5–10·0 (Fig. 2).
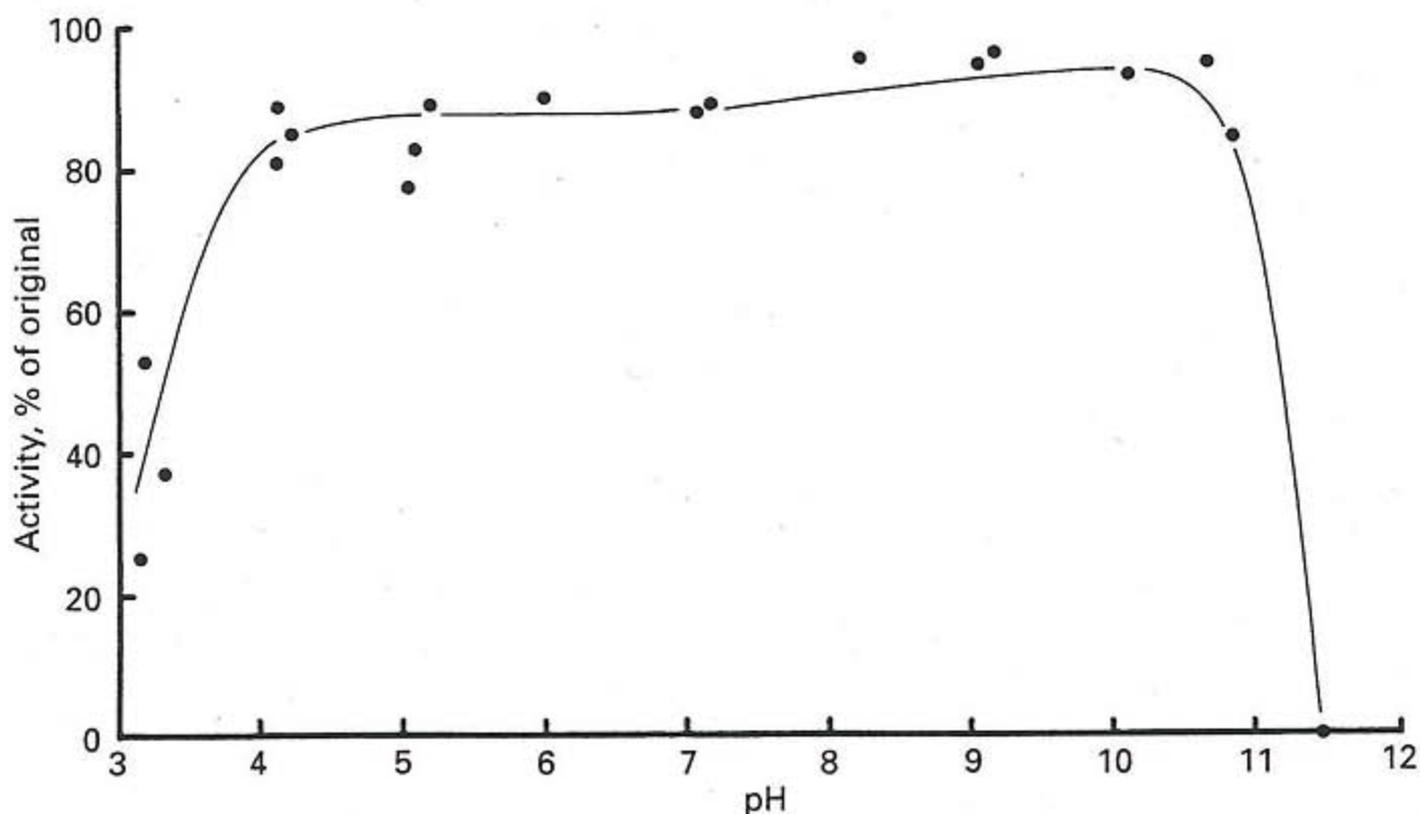
Fig. 2. Stability at different pH of purified *Pseudomonas fluorescens* No. 33 proteinase (50 μg/ml) at 2 °C for 24 h in Universal buffer. Enzymic activity was assayed as described in the text.
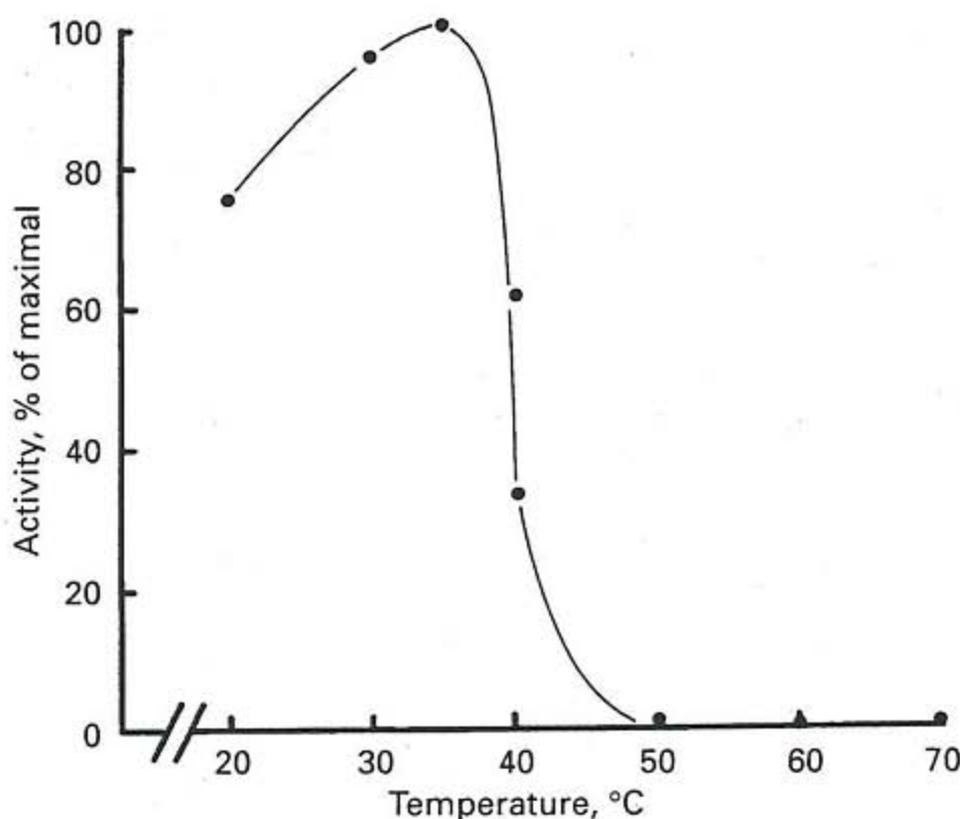

Fig. 3. Effect of temperature on the activity of purified *Pseudomonas fluorescens* No. 33 proteinase on 0·5% casein in 0·05 M-glycine–NaOH buffer. pH 9·0 determined as described in the text.

The optimum temperature for action on casein at pH 9·0 was 30–35 °C (Fig. 3). The enzyme was heat-labile at 50 °C in both 0·1 M-sodium phosphate buffer and synthetic milk salts solution (Fig. 4). Except at 50 °C, the proteinase was more stable in synthetic milk salts solution than in sodium phosphate buffer.

The molecular mass of the proteinase was 48000 by SDS–PAGE.

The effects of various additives are shown in Table 2. The metal-chelating reagent *o*-phenanthroline strongly inhibited the proteolytic activity. Partial inhibition was observed by EDTA. A serine proteinase inhibitor (PMSF), thiol proteinase inhibitor (PCMB) and exopeptidase inhibitor (bestatin) were ineffective.

Among the dibasic metal ions, Hg^{2+}, Cu^{2+}, Fe^{2+} and, to a lesser extent, Ni^{2+} inhibited the activity. No effect of Ca^{2+} and Mg^{2+} was observed in this study.
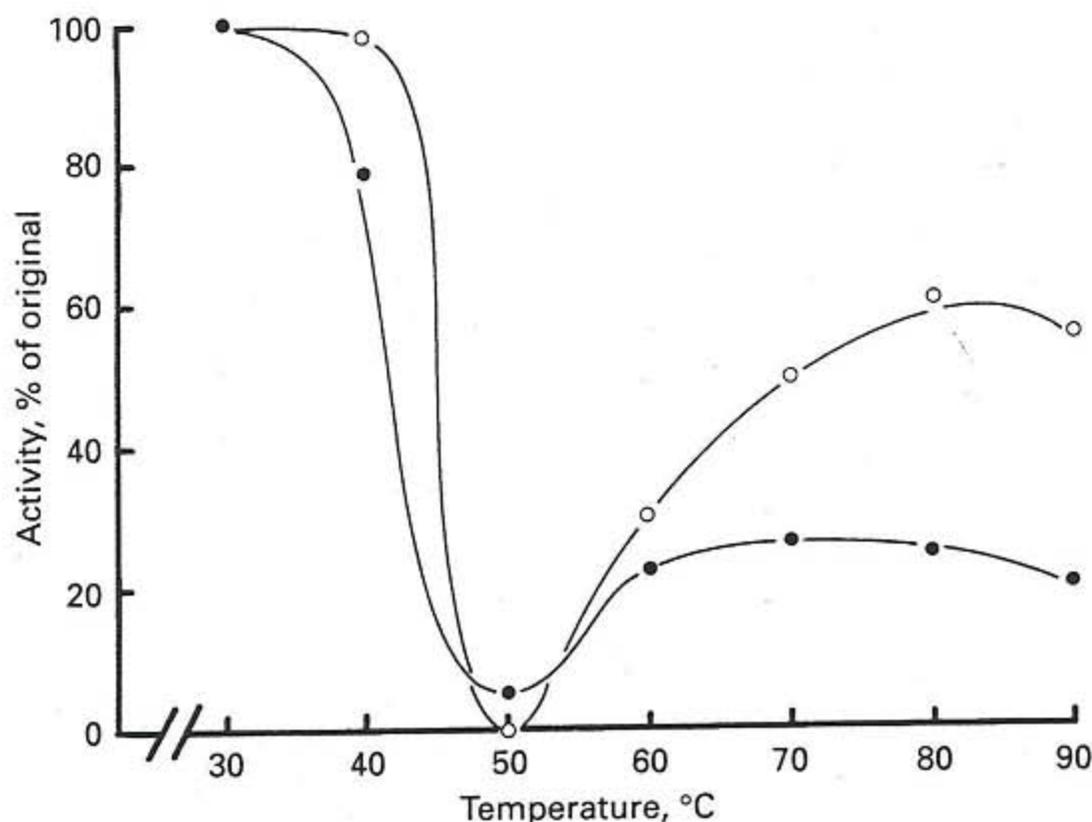
Fig. 4. Stability of purified *Ps. fluorescens* No. 33 proteinase in 0·1 M-sodium phosphate buffer, pH 6·6 (●) or in a synthetic milk salts solution (Jenness & Koops, 1962), pH 6·6 (○) to incubation for 10 min at different temperatures. The enzyme protein was adjusted to 50 μg/ml. Enzymic activity was assayed as described in the text.

Table 2. *Effects of additives on the activity† of purified proteinase from*
Pseudomonas fluorescens *No. 33*

Additive	Concn	Remaining activity, %
Buffer (control)		100
EDTA	1 mM	52
o-Phenanthroline	1 mM	4
p-Chloromercuribenzoate	1 mM	90
Phenylmethylsulphonyl fluoride	1 mM	97
Soyabean trypsin inhibitor	0·1 %	97
Bestatin	0·01 mM	99
Ca^{2+}	1 mM	97
Mg^{2+}	1 mM	104
Zn^{2+}	1 mM	72
Cu^{2+}	1 mM	8
Fe^{2+}	1 mM	2
Hg^{2+}	1 mM	2
Ni^{2+}	1 mM	30

† Enzyme (50 μg/ml) was pre-incubated with additive for 10 min at 30 °C before adding substrate, also containing additive. Details are given in the text.

The amino acid composition of the purified proteinase is given in Table 3. There were low levels of methionine, lysine and arginine, and high levels of acidic and small residues, such as glycine and alanine. No cysteine was detected in the proteinase.

Analysis of milk protein degradation

Qualitative PAGE analyses of milk protein hydrolysis are presented in Fig. 5. Different degradation patterns were observed for acid and micellar caseins. Acid casein was hydrolysed almost completely within 24 h at 4 °C (Fig. 5a). For micellar casein (Fig. 5b), the rate of hydrolysis was slow compared with that of acid casein. κ-Casein was the most readily hydrolysed, while α_s-casein was the least susceptible. Whey proteins were resistant to the action of No. 33 proteinase (Fig. 5c).

Table 3. *Amino acid composition*† *of purified proteinase from pseudomonads*

Amino acid	*Ps. fluorescens* No. 33		*Pseudomonas* sp. MC60‡
	Residues/mol	Residues/ 100 residues	Residues/ 100 residues
Asp	78	17·5	17·6
Thr	41	9·2	10·7
Ser	39	8·7	7·7
Glu	28	6·3	6·0
Pro	7	1·6	1·7
Gly	60	13·5	14·0
Ala	44	9·9	10·3
½-Cys	0	0·0	0·0
Val	22	4·9	4·5
Met	3	0·7	0·4
Ile	14	3·1	4·1
Leu	36	8·1	8·6
Tyr	18	4·0	4·5
Phe	22	4·9	4·5
Lys	15	3·3	2·8
His	7	1·6	1·1
Arg	6	1·3	0·6
Trp	6	1·3	0·9
Total	446		(466)

† Determined as described in the text.
‡ From Barach & Adams (1977).

DISCUSSION

Ps. fluorescens No. 33 produces a proteinase typical of its genus in many respects; the molecular mass was similar to that of other proteinases from *Ps. fluorescens* (Richardson, 1981; Stepaniak *et al.* 1982; Mitchell *et al.* 1986). From information in the review of Fox *et al.* (1989), the amino acid composition of the No. 33 proteinase was similar to that reported for other proteinases from pseudomonads, especially the thermostable proteinase of *Pseudomonas* sp. MC 60 (Barach & Adams, 1977). Fairbairn & Law (1986b) studied the similarities of amino acid composition of proteinases from several pseudomonads and suggested that the heat stability of a proteinase cannot be predicted from its overall amino acid composition. Richardson (1981) noted that a content of low molecular mass amino acids such as glycine may be a prerequisite for heat stability at high temperatures since small side chains would minimize steric hindrance and allow structural flexibility. Information on the relationship between amino acid sequence or higher order structure of protein and heat stability of the enzymes is of interest. The No. 33 proteinase showed low-temperature inactivation (LTI) (Barach *et al.* 1976). The cause of LTI is attributed to self-digestion (Stepaniak & Fox, 1983; Diermayr *et al.* 1987; Stepaniak *et al.* 1991). Higher thermostability (except in the LTI region) in synthetic milk salts solution than in sodium phosphate buffer was also observed by Stepaniak *et al.* (1982) and Stepaniak & Fox (1983), and attributed to the presence of Ca^{2+}.

Although most pseudomonad proteinases have maximum activity around neutral pH (Alichanidis & Andrews, 1977; Stepaniak *et al.* 1982; Patel *et al.* 1983), some were reported to be alkaline proteinases (Fairbairn & Law, 1986b; Mitchell *et al.* 1986). *Ps. fluorescens* No. 33 proteinase falls into the latter category. Furthermore, the pattern of inhibition of the No. 33 proteinase suggested its classification as a metallo-endopeptidase.
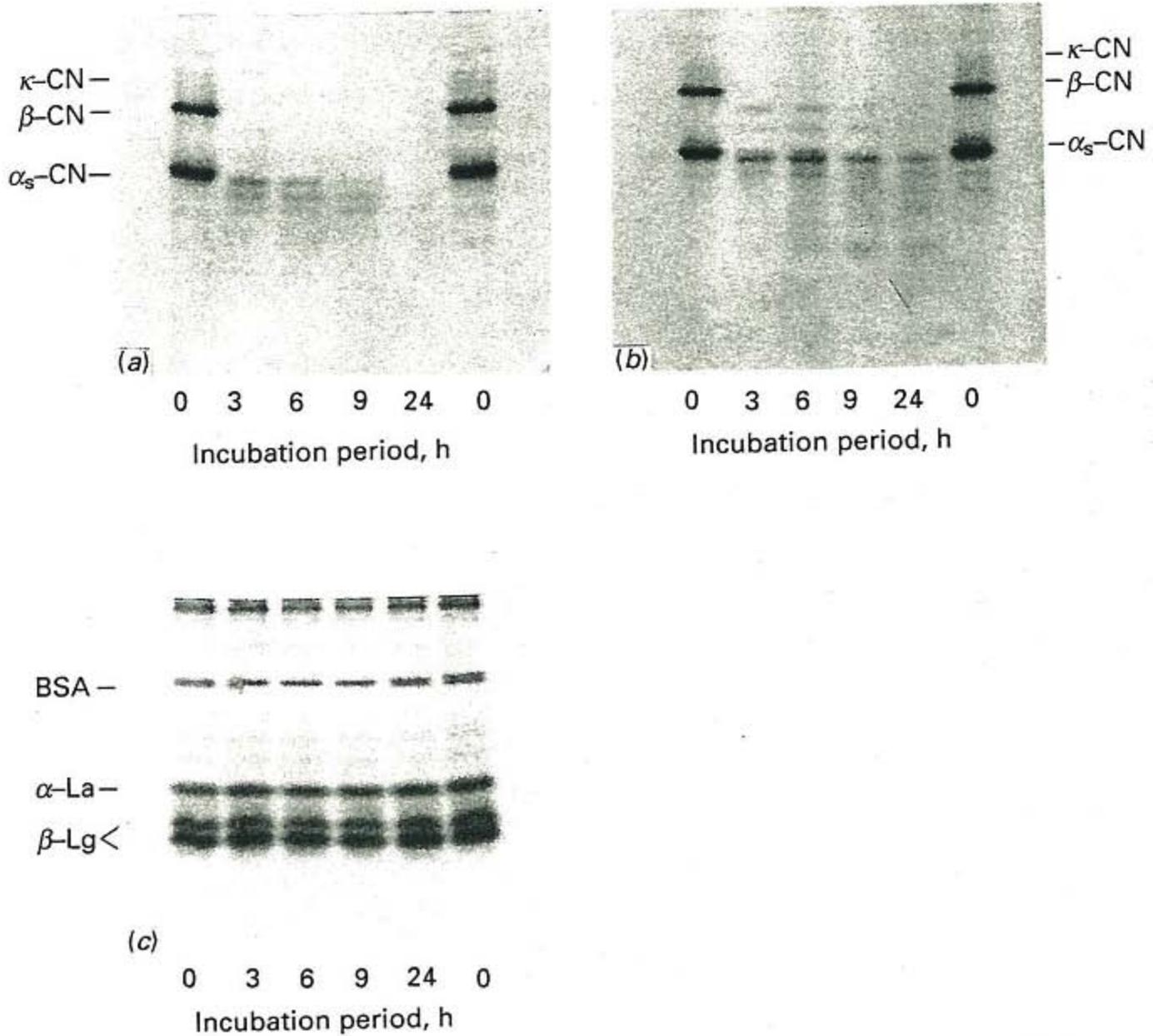
Fig. 5. Hydrolysis of milk proteins in synthetic milk salts solution, pH 6·6 at 4 °C by purified *Ps. fluorescens* No. 33 proteinase. Substrates: (*a*), 0·5 % acid casein; (*b*), 0·5 % micellar casein; (*c*), 0·3 % whey proteins, prepared as described in the text. CN, casein; α-La, α-lactalbumin; β-Lg, β-lactoglobulin, BSA, bovine serum albumin.

The degradation of caseins by the No. 33 proteinase depended on the state of aggregation and the position of the casein in the micelles. As β-casein dissociates from casein micelles on cooling, refrigeration would be expected to make it susceptible to proteolysis (Fox, 1989). Although preference towards caseins depends on the individual character of the proteinase, our results suggest that the substrate condition should also be taken into account; the association of each casein component may affect the rate of hydrolysis.

According to the review of Fairbairn & Law (1986*a*), whey proteins are resistant to psychrotroph proteinase, as confirmed for the No. 33 proteinase.

Not only the proteinase, but also the lipase from *Ps. fluorescens* No. 33 has been isolated from the culture (H. Kumura, unpublished results). The yield of the proteinase (3·7 mg protein/l culture) was much more than that of the lipase (0·7 mg protein/l culture) and the recovery of the lipolytic activity was 37 %. This implies that the culture supernatant contained much more proteinase than lipase.

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