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Original article

Extracellular proteinases from *Micrococcus* GF : II. Isolation and characterization

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Summary — *Micrococcus* sp GF, a microorganism isolated from a farmhouse blue cheese, produced 2 extracellular proteinases, I and II, which were purified ≈ 4 000 times to homogeneity with a yield of ≈ 10% by ultrafiltration, dialysis, freeze-drying and chromatography on DEAE–cellulose and Sephadex G-150. The optimum temperature for activity was around 45 °C for both enzymes. Proteinase I exhibited a pH optimum at 8.5 while proteinase II had a very broad optimum at pH 9.0 to 11.0. Both proteinases were activated by low concentrations of NaCl. They were considered to be metalloproteinases since they were inhibited by EDTA. Proteinase I was irreversibly inactivated by EDTA while the activity of apo-proteinase II was restored by treatment with Ca^{2+}, Ba^{2+}, Mg^{2+}, Sr^{2+} or Zn^{2+}. Both proteinases were inhibited by several heavy metals. Proteinase II was stimulated by some ions, especially Mg^{2+}, while proteinase I was not. Molecular weights were estimated by SDS–PAGE to be around 23.5 and 42.5 kDa for proteinases I and II, respectively. Proteinase I hydrolyzed β-casein preferentially to α_s-casein, while proteinase II hydrolyzed both caseins at approximately the same rate.

extracellular proteinase / *Micrococcus*

Résumé — Protéinase extracellulaire de *Micrococcus* GF: II. Isolement et caractérisation. Micrococcus *sp GF, un microorganisme isolé à partir d'un fromage bleu fermier, produit deux protéinases extracellulaires, protéinases I et II. Elles ont été purifiées ≈ 4000 fois, avec un rendement de ≈ 10%, jusqu'à homogénéité, par ultrafiltration, dialyse, lyophilisation et chromatographie sur DEAE-cellulose et Sephadex G-150.*

La température optimale d'activité pour les deux enzymes est d'environ 45 °C. Le pH optimum d'activité de l'enzyme I est de 8,5 tandis que celui de l'enzyme II s'étend de 9 à 11. Les 2 protéinases sont activées par de faibles concentrations en NaCl. Elles sont considérées comme des métalloprotéinases car elles sont inhibées par l'EDTA. L'EDTA a inactivé irréversiblement la protéinase I tandis que l'activité de l'apo-protéinase II a été restaurée par des traitements au Ca^{2+}, Ba^{2+}, Mg^{2+}, Sr^{2+} ou Zn^{2+}. Plusieurs métaux lourds inhibent les deux protéinases. Certains ions, Mg^{2+} en particulier, stimulent l'activité de la protéinase II mais pas celle de la protéinase I.

Les poids moléculaires, estimés pour les 2 protéinases I et II par électrophorèse SDS-PAGE, étaient respectivement, voisins de 23,5 et de 42,5 kDa. La protéinase I hydrolyse préférentiellement la caséine β à la caséine α_s; tandis que la protéinase II hydrolyse les 2 caséines avec approximativement la même intensité.

***protéinase extracellulaire* / Micrococcus**

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INTRODUCTION

Micrococci have been reported as one of the components of cheese microflora (*eg* Irvine and Beach, 1962; Obradovic, 1978; Ordóñez *et al*, 1978; Bhowmik and Marth, 1990) and may contribute to cheese flavour through their lipolytic and/or proteolytic activities (Marth, 1963; Bhowmik and Marth, 1990) or through a symbiotic activation of lactobacilli (Nath and Ledford, 1971). Several attempts have been made to use micrococci to improve and enhance Cheddar flavour and to accelerate ripening (Deane, 1951; Robertson and Perry, 1961).

Intracellular proteolytic and peptidase activities from micrococci have been reported; *eg* Baribo and Foster (1952) reported that the intracellular proteinases and peptidases of *M freudenreichii* contributed to the proteolytic activity in cheese. Nath and Ledford (1972) and Bhowmik and Marth (1988) detected intracellular proteolytic activity in all strains studied.

It is known that some *Micrococcus* spp are endowed with extracellular proteolytic activity, *eg* Husain and McDonald (1958) reported the partial purification of an extracellular proteinase from *M freudenreichii*. Desmazeaud and Hermier (1968, 1971) isolated and characterized an extracellular proteinase produced by *M caseolyticus*. Nath and Ledford (1972) detected extracellular proteolytic activity in 3 of 18 *Micrococcus* strain isolated from Cheddar cheese. Prasad *et al* (1986) isolated a metalloproteinase from *Micrococcus* MCC-315.

The objective of the present study was to isolate and characterize the proteinases from *Micrococcus* GF, isolated from the surface of a farmhouse blue cheese. The influence of several factors in the growth of, and proteinase production by, this organism was reported by García de Fernando and Fox (1991).

MATERIALS AND METHODS

Culture conditions

Stock cultures of *Micrococcus* (Kocur, 1986) GF were maintained frozen in 2% phytone peptone broth (BBL Microbiology Systems, Cockeysville, MD, USA) until required. When required, 2-l flasks, each containing 700 ml of 2% phytone peptone (BBL), were inoculated with *Micrococcus* GF and incubated at 30 °C on a rotary shaker operating at 100 rpm for 40 h.

Purification

Cell-free supernatant (CFS) was prepared by centrifuging the grown culture in a Sorvall RC 5B centrifuge at 4 °C at 15 000 *g* for 10 min. CFS was ultrafiltered to ≈ 1/10 volume reduction in a Millipore Minetan (Massachusetts, USA) ultrafiltration system, fitted with polysulphone membranes with a nominal molecular weight cut-off of 10 kDa. The retentate was dialyzed against 10 l of 2 mM $CaCl_2$–20 mmol.l^{-1} Tris(hydroxymethyl)aminomethane (Tris)-maleate buffer, pH 8.5, at 4 °C overnight. The dialyzed CFS was chromatographed at 4 °C on a 70 x 2.5 cm column of DEAE–cellulose (Whatman DE 23), previously equilibrated in 2 mmol.l^{-1} $CaCl_2$–20 mmol.l^{-1} Tris–maleate, buffer, pH 8.5. Adsorbed proteins were eluted with a linear NaCl gradient, 0–0.75 mmol.l^{-1}. Fractions (10 ml) were collected in a LKB Redirac 2112 fraction collector. The fractions were analyzed for absorbance at 280 nm, proteolytic activity and NaCl (Fox, 1963).

Some of the proteolytic activity did not adsorb on DEAE–cellulose under the experimental conditions while the remainder adsorbed and was eluted in the NaCl gradient; the active fractions for each enzyme were pooled. The pooled fractions were dialyzed against 2 mmol.l^{-1} $CaCl_2$–20 mmol.l^{-1} Tris–maleate buffer, pH 8.5, to remove NaCl. Both enzyme preparations were freeze-dried and chromatographed at 4 °C

on a 76 x 2.5 cm column of Sephadex G-150 that had been previously equilibrated with 2 mmol.l^{-1} $CaCl_2$–20 mmol.l^{-1} Tris-maleate buffer, pH 8.5. Fractions (10 ml) were collected and analyzed for absorbance at 280 nm and proteolytic activity. Proteolytically active fractions were pooled, freeze-dried and rechromatographed on Sephadex G-150.

Protein measurement

Protein was calculated from absorbances at 280 nm, using $A^{1\%}_{280} = 10$.

Proteinase assay

Proteolytic activity was measured using azocasein as substrate (Sigma Chemical Co Ltd, Dorset, UK). Samples (1 ml) of enzyme were added to 1 ml of 0.8% azocasein solution in 0.2 mol.l^{-1} Tris–HCl buffer, pH 8.5 or 9.1 (for unadsorbed and adsorbed proteinases, respectively). The mixture was incubated at 45 °C for 2 h. The reaction was stopped by adding 1 ml of 6% trichloroacetic acid, the mixture filtered through Whatman No 42 paper and the absorbance of the filtrates measured at 440 nm. One proteolytic activity unit represented a ΔA_{440} of 1 at 45 °C in 2 h per ml of sample.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed as described by Greaser *et al* (1983) using Coomassie blue R-250 as dye. Electrophoretograms were run with standards (Sigma) of known molecular weight (lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), trypsinogen (24 kDa), pepsin (34.7 kDa), ovalbumin (45 kDa) and bovine serum albumin (66 kDa)).

Optimum temperature

Proteolytic activity was assayed at temperatures ranging from 19–74 °C; before addition of enzyme, the substrate was tempered at the appropriate temperature.

Optimum pH

Azocasein was dissolved in universal buffer (Dawson *et al*, 1969), which contained citric acid (6.008 g), KH_2PO_4 (3.893 g), H_3BO_3 (1.769 g), barbitone (5.266 g), and the pH adjusted to values in the range 4.0–13.0 with 0.2 M NaOH. Proteolytic activity was assayed at 45 °C for 2 h.

Heat stability

Enzyme preparations, dissolved in 2 mM $CaCl_2$–20 mM Tris–maleate, pH 8.5, were heated at temperatures in the range 60.0–69.5 °C. Aliquots were taken at intervals up to 2 h and transferred to tubes in a water/ice bath. Proteolytic activity was assayed at 45 °C for 2 h.

Effect of chemical reagents and metal ions

K^+, Cs^+, Zn^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Hg^{2+} (as chlorides or sulphates), EDTA, 1,10-phenanthroline and *N*-ethylmaleimide were added at different concentrations to aliquots of the enzyme preparations and maintained at room temperature for 1 h; remaining proteolytic activity was then assayed. The effect of NaCl at concentrations ranging from 0.0 to 3.0 M on proteolytic activity was assayed.

Reactivation of apo-enzyme

The enzymes were inactivated by making the solution to 2 mM EDTA at 20 °C for 1 h. The solutions were then dialyzed against distilled water overnight. Different concentrations of Ba^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+}, Co^{2+} and Zn^{2+} were added to the dialyzed preparation, maintained at room temperature for 1 h and then assayed for proteolytic activity.

Casein fractionation

Sodium caseinate (1 g) was dissolved in distilled water and applied to a 69 x 2.5 cm column of

DEAE–cellulose previously equilibrated at 4 °C with 0.02 mol.l^{-1} phosphate buffer, pH 6.5. Adsorbed caseins were eluted at 4 °C with a linear gradient of NaCl (0.0 to 0.5 mol.l^{-1}). Absorbance at 280 nm was measured and NaCl determined by titration with $AgNO_3$ (Fox, 1963). Protein-rich fractions showed 4 peaks which were pooled, dialyzed against distilled water for 36 h and lyophilized. These were shown by urea–PAGE (Andrews, 1983) to contain mainly γ, β, κ and α_s-caseins.

Caseinolytic activity of Micrococcus *GF*

Sodium caseinate or freeze-dried α_s and β-caseins were dissolved at 0.5% (w/v) in 0.2 M Tris–HCl buffer, pH 8.5, and enzyme preparations were added. The mixtures were incubated at 45 °C. Aliquots were taken at different times and added to equal volumes of sample buffer for electrophoresis (Andrews, 1983). Electrophoresis was performed according to Andrews (1983). Gels were stained by the method of Blakesley and Boezi (1977).

Milk clotting

Samples (10 ml) of a 10% solution of reconstituted milk powder in 3 mM $CaCl_2$ were incubated with 0.35 unit of proteinase I or 0.14 unit of proteinase II at 37 °C for 2 h.

RESULTS

Purification

A typical elution profile of concentrated CFS from DEAE–cellulose is shown in figure 1. Two peaks with proteolytic activity were detected; the more active peak (I)

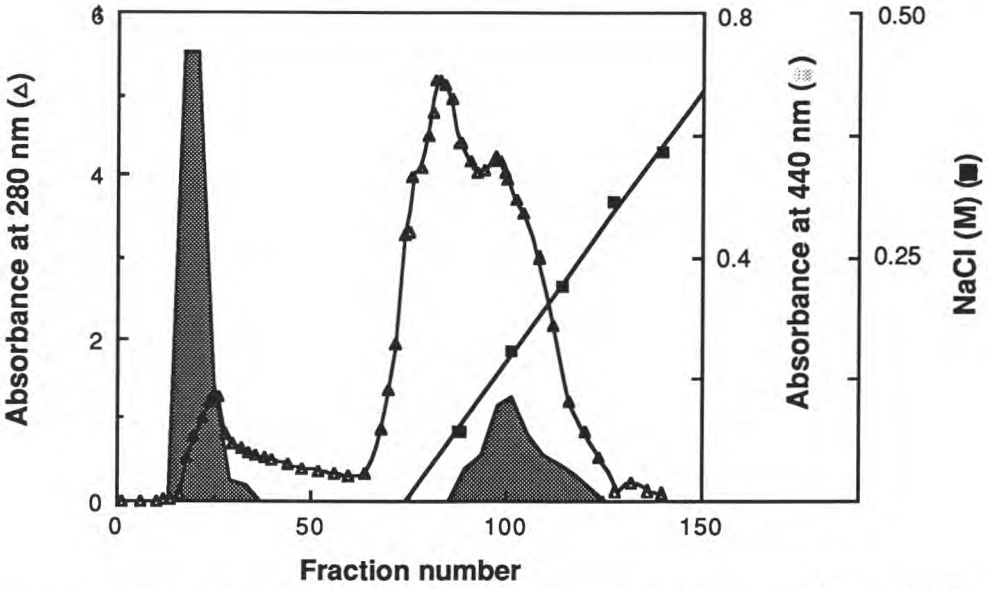

Fig 1. Elution profile of dialyzed, ultrafiltered, cell-free supernatant of *Micrococcus* GF from DEAE–cellulose equilibrated with 2 mM $CaCl_2$-20 mM Tris-maleate buffer, pH 8.5 using a linear NaCl gradient, 0–0.5 M, at 4 °C. Column size : 70 x 2.5 cm. Flow rate : 0.35 ml/min. Fraction size : 10 ml.
Chromatographie sur DEAE-cellulose équilibrée en tampon 2 mM $CaCl_2$-20 mM Tris–maléate, pH 8.5 du surnageant de Micrococcus *GF. Volume de la colonne : 70 x 2,5 cm. Débit : 0,35 ml/min. Volume des fractions : 10 ml.*

was not adsorbed on DEAE–cellulose, while the other (II) was adsorbed and eluted by ≈ 0.24 M NaCl. The proteolytically active fractions were pooled, dialyzed overnight against 10 l of 2 mM $CaCl_2$–20 mM Tris–maleate buffer, pH 8.5 and freeze-dried.

The dried preparations were chromatographed on a Sephadex G-150 column (76 x 2.5 cm) at 4°C. Proteolytic fractions were pooled, freeze-dried and rechromatographed on Sephadex G-150 (76 x 2.5 cm). The chromatograms of proteinase preparations I and II are shown in figure 2. A reduction of the absorbance at 280 nm was observed on rechromatography, with very little loss of activity. Table I shows the progress of purification and yields of the enzymes through the main stages. The final purification observed for both proteinases was > 4 000-fold with an activity yield of ≈ 10%.

Homogeneity of the purified enzymes was assessed by SDS–PAGE using ≈ 20 µg of protein per slot and staining with Coomassie brilliant blue R-250. Only one stained band was evident in each of the purified enzyme preparations. The molecular weights were estimated to be ≈ 23 500 and 42 500 Da for proteinases I and II, respectively.

Optimum temperature

Proteinases I and II from *Micrococcus* GF were optimally active at 45 °C. Below 20 °C, activity was < 20% of maximum and above 50 °C it decreased markedly.

Optimum pH

Proteinase I showed maximum activity at pH 8.5 and activity decreased markedly below pH 8.0, being totally lost at pH 4.0. However, activity decreased only slightly

Table I. Purification of the extracellular proteinases of *Micrococcus* GF.
Purification de l'activité protéolytique extracellulaire de Micrococcus *GF.*

	Volume (ml)	*Protein (mg)*	*Proteolytic activity (units)*	*Specific activity (units/mg protein)*	*Purification (fold)*	*Recovery (%)*
CFS[a]						
Proteinase I	700	11900	144.2[b]	0.0122		
Proteinase II	700	11900	61.8[b]	0.0052		
DEAE–cellulose						
Proteinase I	120	78	67.6	0.867	71	46.9
Proteinase II	270	901	26.8	0.030	6	43.4
1st Sephadex						
Proteinase I	70	19.0	36.6	1.93	158	25.4
Proteinase II	70	4.2	15.1	3.59	691	9.3
2nd Sephadex						
Proteinase I	70	0.4	22.4	56.0	4590	15.5
Proteinase II	70	0.6	12.6	21.0	4038	7.8

[a] Cell-free supernatant from centrifugation. [b] Values estimated from experimental data.

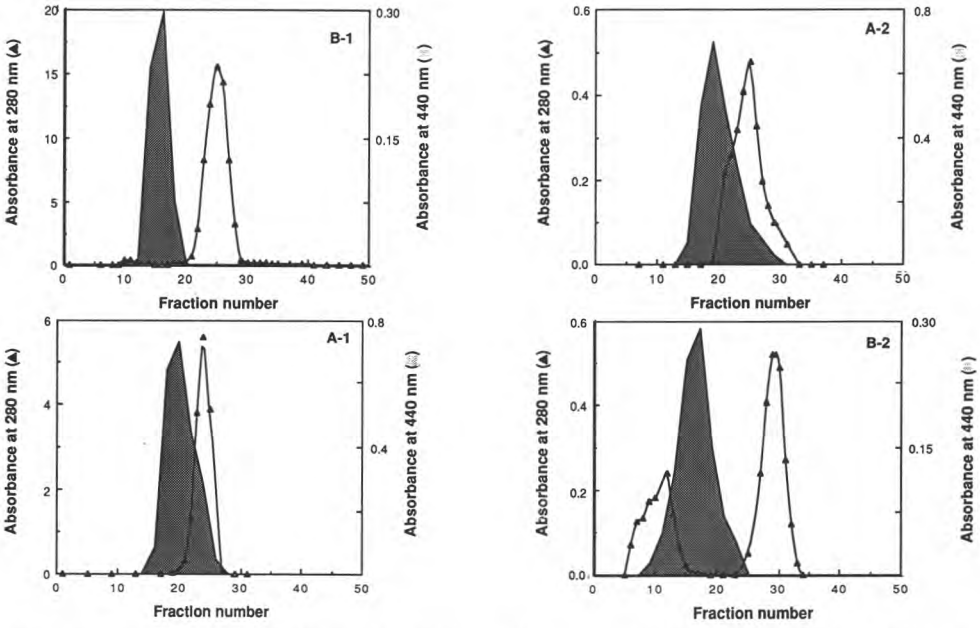

Fig 2. Elution profiles of peaks I (A) and II (B) from DEAE–cellulose chromatographed (A) and rechromatographed (2) on Sephadex G-150. Column size: 76 x 2.5 cm. Eluent: 2 mM $CaCl_2$–20 mM Tris–maleate buffer, pH 8.5. Flow rate: ≈ 0.3 ml/min. Fraction size: 10 ml. Temperature: 4 °C.
Chromatographie (1) et rechromatographie (2) sur Sephadex G-150 des pics I (A) et II (B) qui ont été séparés sur DEAE–cellulose. Volume de la colonne : 76 x 2,5 cm. Débit : ≈ 0,3 ml/min. Volume des fractions : 10 ml.

from pH 9.0 to 12.0 and sharply above this value. Proteinase II was inactive at pH 4.5, but activity increased markedly between pH 4.5 and 6.0 and was maximal in the range 9.0–11.0. Activity markedly decreased above the last value.

Effect of NaCl

The activity of proteinases I and II was increased by low NaCl concentrations; maximum activity was detected at 0.20 M NaCl for proteinase I and at 0.35 M for proteinase II. The activity of proteinase I decreased markedly with increasing NaCl concentration in the range 0.2–1.0 M and slightly thereafter, while the activity of proteinase II decreased linearly with increasing NaCl concentration > 0.35 M; neither enzyme was active in 4 M NaCl.

Effect of metal ions

After exposing the enzymes to different concentrations of several ions at room temperature for 1 h, the mixtures were assayed for proteolytic activity. The effect of the ions on proteinase activity is shown in figure 3. Enzyme I was strongly inhibited by Fe^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} and Hg^{2+}, mildly

by Fe^{3+}, Cu^{2+} and Mn^{2+}, while Mg^{2+}, Ca^{2+}, Ba^{2+}, Cs^{2+} and K^{+} did not affect activity. Enzyme II was strongly inhibited by Fe^{2+}, Fe^{3+}, Co^{2+} and Hg^{2+}, mildly by Cu^{2+}, Cd^{2+}, Mn^{2+} and Zn^{2+}, Cs^{+} and K^{+} had no effect while Ba^{2+}, Ca^{2+}, and especially Mg^{2+}, stimulated proteolytic activity.

Effect of N-*ethylmaleimide (NEM)*

Proteinase I was slightly inhibited above 1 mM NEM but retained ≈ 75% of its original activity in the presence of 20 mM NEM. NEM did not affect the activity of proteinase II in the range studied.

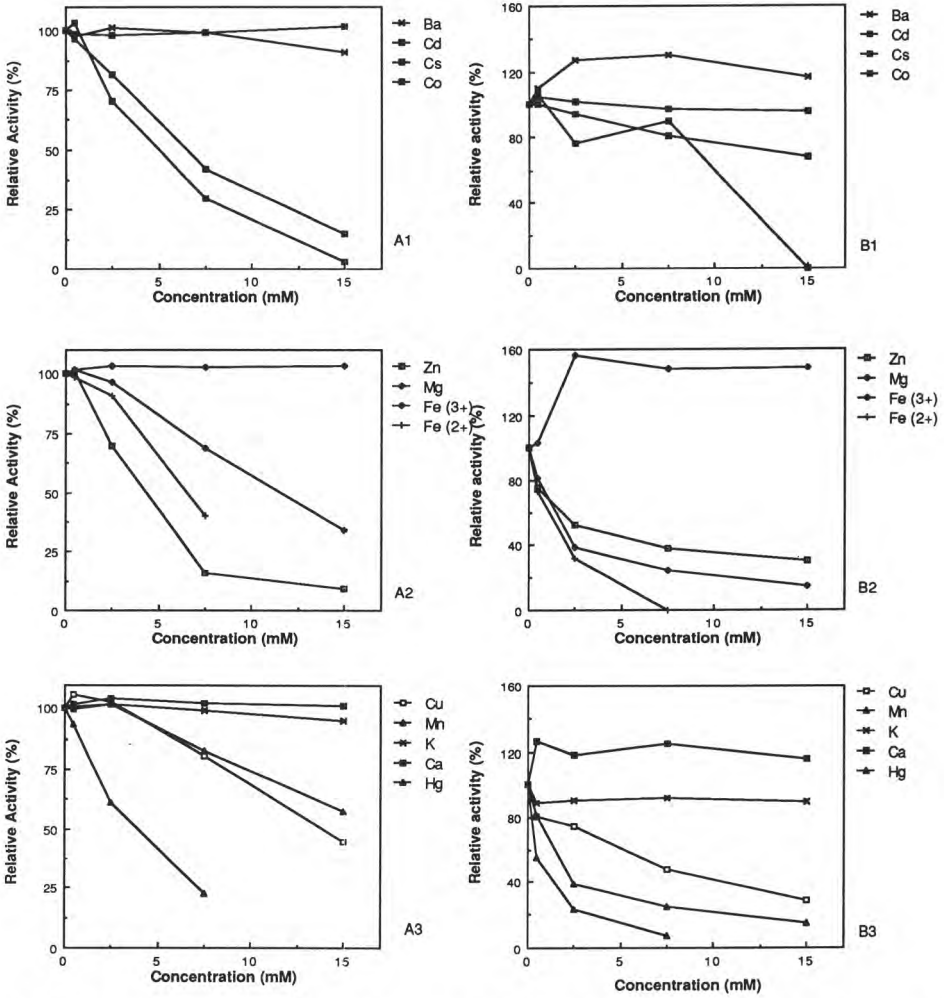

Fig 3. Effect of metals on the activity of the extracellular proteinases I (A) and II (B) of *Micrococcus* GF at pH 8.5 (proteinase I) or 9.1 (proteinase II) and 45 °C.
Effet des ions métaux sur l'activité des protéinases extracellulaires I (A) et II (B) de Micrococcus *GF à pH 8,5 (protéinase I) ou pH 9,1 (protéinase II) et à 45 °C.*

Effect of metal chelators

1,10-Phenanthroline, in the range 0–25 mM, did not affect the activity of the extracellular proteinases of *Micrococcus* GF. At 0.05 mM, EDTA did not affect proteinase activities. Above this concentration, the activity of both proteinases decreased markedly and they were completely inhibited at 1 mM EDTA.

Reactivation by metal ions

Samples of proteinases I and II were inactivated by treatment with EDTA (2 mM) and then dialyzed against distilled water. Proteinase I was not reactivated by any of the ions tested, while proteinase II was reactivated (fig 4) by Ca^{2+}, Ba^{2+}, Sr^{2+}, Mg^{2+} or Zn^{2+} but not by Co^{2+}. Mg^{2+} and Zn^{2+} reactivated the enzyme to ≈150% of the original activity, although above this concentration the extent of the reactivation decreased. Sr^{2+} gave maximum reactivation at 10 mM and its effectivness decreased slightly thereafter. Ba^{2+}, and especially Ca^{2+}, increased the activity of the enzyme up to 50 mM (the highest concentration tested) when the activity of the reactivated enzyme was 300% that of the control (without EDTA treatment).

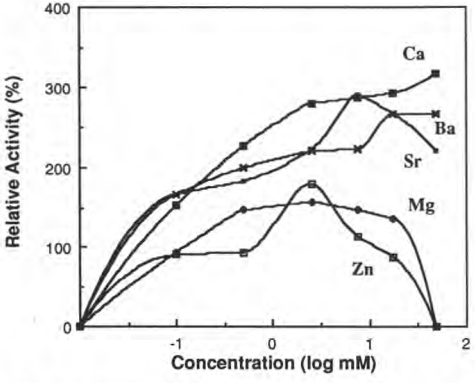

Fig 4. Reactivation of EDTA (2 mM)-treated extracellular proteinase II of *Micrococcus* GF by metal ions at pH 9.1 and 45 °C. Values are relative to the activity (100) of an enzyme sample that had not been treated with EDTA.
Réactivation de la protéinase II de Micrococcus *GF traitée avec l'EDTA (2 mM) par les ions métaux à pH 9,1 et à 45 °C. Les valeurs sont reportées à l'activité (100) d'une enzyme qui n'a pas été traitée avec l'EDTA.*

Thermostability

The thermostability of the proteinases was studied in 2 mM $CaCl_2$–20 mM Tris–maleate buffer, pH 8.5. The thermal inactivation plots are shown in figure 5. D-values were numerically calculated from the experimental data and are shown in table II. Likewise, Z-values were calculated from a graph (fig 6) which represents logarithms of D-values against temperature. Both enzymes were thermolabile; Z-values were 7.58 °C and 5.35 °C for proteinases I and II, respectively.

Caseinolytic properties

On 0.5% sodium caseinate at pH 8.5, proteinase I hydrolyzed β-casein more rapidly than α_s-casein but both caseins were completely hydrolyzed to a variety of peptides in ≈ 2h (fig 7). Proteinase II hydrolyzed both α_s- and β-caseins at approximately equal rates to a wide range of products (fig 8 A, B).

Solutions of α_s and β-caseins became turbid during incubation with the enzymes. The rate of increase and the intensity of the turbidity was greater when proteinase I was used. The turbidity disappeared after long incubation.

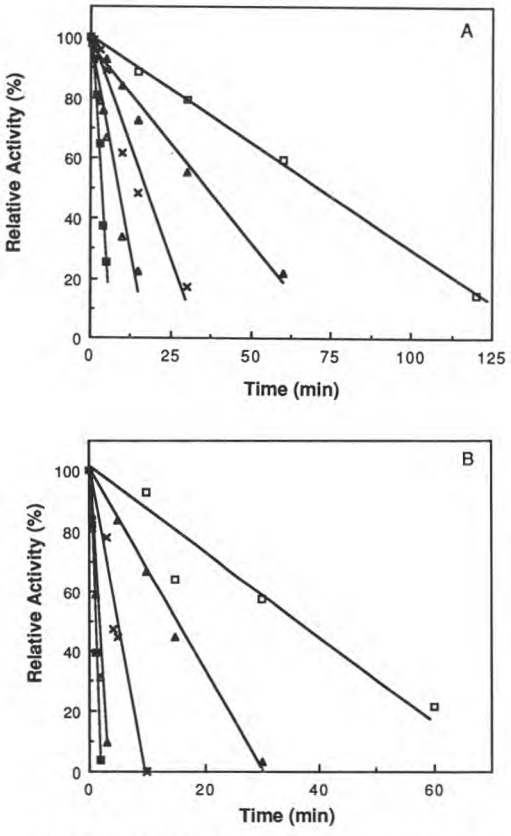

Fig 5. Residual activity of heat-treated extracellular proteinases I **(A)** and II **(B)** of *Micrococcus* GF in 2 mM $CaCl_2$–20 mM Tris, pH 8.5. Proteolytic activity was measured at pH 8.5 (proteinase I) or 9.1 (proteinase II) and 45 °C. ❑ 60.0 °C; ▲ 62.5 °C; X 65.0 °C; Δ 67.5 °C; ■ 69.5 °C.
Activité résiduelle des protéinases extracellulaires de Micrococcus *GF qui ont été soumises à des traitements thermiques. L'activité protéolytique était mesurée à pH 8,5 (protéinase I) ou pH 9,1 (protéinase II) et à 45 °C. ❑ 60.0 °C; ▲ 62.5 °C; X 65.0 °C; Δ 67.5 °C; ■ 69.5 °C.*

Milk clotting

Neither proteinases clotted milk under the experimental conditions, *ie* pH 6.6 at 37 °C for 2 h, even at the high enzyme levels used.

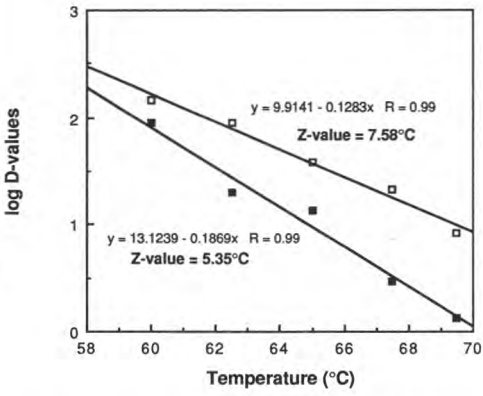

Fig 6. Z-values for the heat inactivation of extracellular proteinase I (❑) and II (■) of *Micrococcus* GF in 2 mM $CaCl_2$–20 mM Tris, pH 8.5.
Valeurs Z pour l'inactivation par la chaleur des protéinases I (❑) et II (■) de Micrococcus *GF.*

DISCUSSION

Micrococcus GF produced 2 extracellular proteinases which were purified to homogeneity ≈ 4 000 fold with a total yield of ≈ 10% by ultrafiltration, dialysis, freeze-drying and chromatography on DEAE–cellulose and Sephadex G-150. Prasad *et al* (1986) purified the extracellular proteinase from *Micrococcus* MCC-315 ≈ 80 fold with a yield > 50%. Desmazeaud and Hermier (1968) reported a final purification for the proteinase of *M caseolyticus* of ≈ 40 fold with a recovery of 7%. The purification method described here was similar to that used by Desmazeaud and Hermier (1968) and by Prasad *et al* (1986). The main difference was the first step: precipitation by $(NH_4)_2SO_4$ was used in the other studies while ultrafiltration was used in the present study. Precipitation with $(NH_4)_2SO_4$ frequently causes loss of activity, while ultrafiltration caused no loss of activity.

Fig 7. Urea–PAGE of 1 ml (5 mg/ml) of casein incubated with 0.07 units of proteinase I from *Micrococcus* GF at 45 °C and pH 8.5 for 5 (a), 15 (b), 30 (c), 60 (d), 120 (e) and 0 (f) min.
PAGE en présence d'urée de 1 ml (5 mg/ml) de caséine qui a été incubée avec 0,07 unité de protéinase I de Micrococcus *GF pendant 5 (a), 15 (b), 30 (c), 60 (d), 120 (e) et 0 (f) min à 45 °C et à pH 8,5.*

The optimum temperature for the activity of both extracellular proteinases of *Micrococcus* GF was 45 °C. The extracellular proteinases from *M freudenreichii* (Husain and McDonald, 1958) and *M caseolyticus* (Desmazeaud and Hermier, 1968) were optimally active at 50 °C. Prasad *et al* (1986) reported optimum temperatures of 37 and 60 °C, depending on the substrate used, for the extracellular proteinase of *Micrococcus* MCC-315. All these proteinases, including those from *Micrococcus* GF, retained less than 20% of maximum activity at 20 °C. In most cases, activity decreased markedly at temperatures >50 °C.

The pH optimum reported for the extracellular proteinases of *Micrococcus* spp varied widely: 5.7 at 35 or 45 °C and 6.7 at 20 °C for the extracellular proteinase of *M freudenreichii* (Husain and McDonald, 1958), 7.6 for the proteinase from *M caseolyticus* (Desmazeaud and Hermier, 1968), 10.6 for *Micrococcus* MCC-315 (Prasad *et al,* 1986) and 8.5 for proteinase I and 9.0 to 12.0 for proteinase II of *Micrococcus* GF. The extracellular proteinase from *M freudenreichii* can be considered as an acid proteinase, that from *M caseolyticus* as a neutral proteinase and those from *Micrococcus* MCC-315 and *Micrococcus* GF as alkaline proteinases.

Proteinases I and II of *Micrococcus* GF were optimally active in 0.20 and 0.35 M NaCl, respectively. McDonald (1961) reported that NaCl protected the extracellular proteinase of *M freudenreichii* against inactivation. However, Prasad *et al* (1986) re-

Table II. D-values (min) for the thermal inactivation of the extracellular proteinases of *Micrococcus* GF in 2 mM $CaCl_2$–20 mM Tris–maleate, pH 8.5.
Valeur D (min) pour l'inactivation thermique des protéinases extracellulaires de Micrococcus *GF dans 2 mM $CaCl_2$-20 mM Tris-maléate, pH 8,5.*

	Temperature (°C)				
	60.0	*62.5*	*65.0*	*67.5*	*69.5*
			D-values (min)		
Proteinase I	144.93	90.09	37.88	21.50	8.44
Proteinase II	89.28	19.84	13.78	2.95	1.32

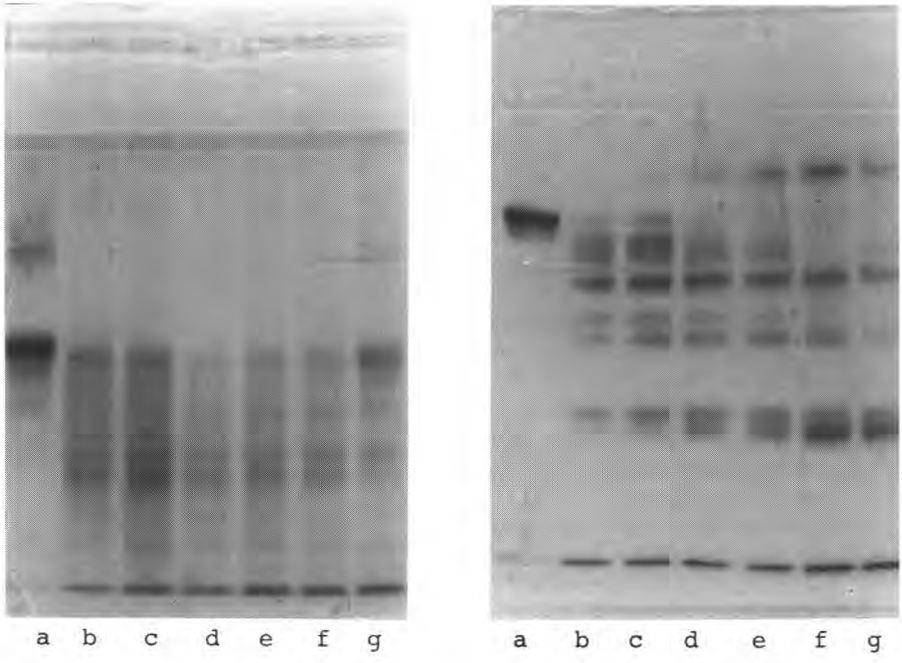

Fig 8. Urea–PAGE of 1 ml (5 mg/ml) of α_s- (A) and β-casein (B) incubated with 0.02 units of proteinase II from *Micrococcus* GF for 0 (a), 10 (b), 30 (c), 60 (d), 90 (e), 120 (f) and 180 (g) min at 45 °C and pH 9.1.
PAGE en présence d'urée de 1 ml (5 mg/ml) de αs-(A) et β-(B) caséines qui ont été incubées avec 0,02 unité de protéinase II de Micrococcus *GF pendant 0 (a), 10 (b), 30 (c), 60 (d), 90 (e), 120 (f) et 180 (g) min à 45 °C et à pH 9,1.*

ported that the activity of the extracellular proteinase of *Micrococcus* MCC-315 was reduced by 4–10% in the presence of 1.3 mmol.l^{-1} NaCl compared to the control without added salt.

Metal ions inhibit many enzymes but it is difficult to compare the results obtained by different authors since different salts at different concentrations or under different conditions have been used. Fe^{2+}, Co^{2+} and Hg^{2+} inhibited the proteinases of *Micrococcus* GF. Hg^{2+} is a common enzyme inhibitor; Co^{2+} and Fe^{2+} also completely or partially inhibited the proteinase from *M caseolyticus* (Desmazeaud and Hermier, 1968), but stimulated the extracellular proteinase of *Micrococcus* MCC-315 (Prasad *et al,* 1986). Zn^{2+} or Cd^{2+}, which partially inhibited the proteinases of *Micrococcus* GF, also partially inhibited the proteinase of *M caseolyticus* (Desmazeaud and Hermier, 1968). Mn^{2+} stimulated the extracellular proteinase of *Micrococcus* MCC-315 (Prasad *et al,* 1986) but inhibited the proteinase of *M caseolyticus* (Desmazeaud and Hermier, 1968) and inhibited the proteinases of *Micrococcus* GF. Cu^{2+} has been reported to inhibit all the proteinases discussed here. Ca^{2+}, Mg^{2+}, Sr^{2+} or K^{+} did not affect the activity of the extracellular proteinase of *M caseolyticus* (Desmazeaud and Hermier, 1968) while Ca^{2+} or Sr^{2+} stimulated the activity of the proteinase of *Micrococcus* MCC-315 (Prasad *et*

al, 1986). Proteinase I of *Micrococcus* GF was not affected by Ca^{2+}, Ba^{2+}, Mg^{2+} or K^{+}, but proteinase II was stimulated by Ba^{2+}, Ca^{2+} and especially by Mg^{2+}.

The failure of NEM to inhibit the proteinases of *Micrococcus* GF suggested that are not thiol-proteinases. The inhibitory effect of EDTA indicates that they are metalloenzymes, but neither molecule appears to contain Zn^{2+} since neither was inhibited by 1,10-phenanthroline. Many microbial proteinases are Zn-metalloenzymes (see McKellar, 1989), however, although some micrococcal proteinases are metalloenzymes, there is no evidence that the extracellular proteinases of *Micrococcus* spp contain Zn^{2+}. The extracellular proteinase from *M sodonensis* (now reclassified as *M luteus* (Baird-Parker, 1974)) is probably not a metalloproteinase (Mills and Campbell, 1974).

Proteinase II, after inhibition with EDTA, could be reactivated by Zn^{2+}, Mg^{2+}, Sr^{2+} and especially by Ba^{2+} or Ca^{2+}, while proteinase I was irreversibly inactivated by EDTA. The proteinase of *Micrococcus* MCC-315 was also irreversibly inactivated by EDTA (Prasad *et al,* 1986). The proteinase from *M caseolyticus* could be partially reactivated by Sr^{2+} and Ca^{2+} (Desmazeaud and Hermier, 1968).

Both proteinases from *Micrococcus* GF extensively degraded α_s- and β-caseins. Proteinase I degraded β-casein preferentially to α_s-casein (fig 7) but, proteinase II degraded these caseins at about the same rate (fig 8). Electrophoretic studies on the degradation of α_s- and β-caseins by cell free extracellular extracts from cultures of *M caseolyticus* ATCC 13458, *M freudenreichii* ATCC 407, *M luteus* 10240 and *Micrococcus* sp ATCC 8459 demonstrated that they preferentially hydrolysed β-casein but also hydrolysed α_{s1}-casein (Nath and Ledford, 1972; Bhowmik and Marth, 1988).

The use of β-caseinolytic micrococci in cheesemaking to accelerate ripening was suggested by Bohwmik and Marth (1988). If the hydrolysis of β-casein does improve cheese flavour or accelerate ripening, the use of *Micrococcus* GF as a starter, or the addition of a partially purified proteinase preparation to cheese curd may be interesting. β-casein is a source of hydrophobic peptides, which may be bitter (Visser *et al,* 1982). Therefore, if enzymes which preferentially degrade β-casein are used in cheesemaking careful regulation of the dose is essential.

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