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M. Poza · C. Sieiro · L. Carreira · J. Barros-Velázquez
T. G. Villa

Production and characterization of the milk-clotting protease of *Myxococcus xanthus* strain 422

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Abstract The cheese industry is seeking novel sources of enzymes for cheese production. Microbial rennets have several advantages over animal rennets. (1) They are easy to generate and purify and do not rely on the availability of animal material. (2) The production of microbial clotting enzymes may be improved by biotechnological techniques. In this work, the biochemical characterization of a novel milk-clotting extracellular enzyme from *Myxococcus xanthus* strain 422 and a preliminary evaluation of its cheese-producing ability are reported. Strain 422 was selected from four *M. xanthus* strains as the best producer of extracellular milk-clotting activity, based on both its enzyme yield and specific milk-clotting activity, which also afforded lower titration values than enzymes from the three other *M. xanthus* strains. The active milk-clotting enzyme from *M. xanthus* strain 422 is a true milk-clotting enzyme with a molecular mass of 40 kDa and a pI of 5.0. Highest milk-clotting activity was at pH 6 and 37 °C. The enzyme was completely inactivated by heating for 12 min at 65 °C. The crude enzyme preparation was resolved by anion-exchange chromatography into two active fractions that were tested in cheese production assays of compositional (dry matter, fat content, fat content/dry-matter ratio, and moisture-non-fat content) and physicochemical properties (firmness, tensile strength, pH and Aw) of the milk curds obtained. Purified protein fraction II exhibited a significantly higher milk-clotting ability than either protein fraction I or a total protein extract, underlining the potential usefulness of *M. xanthus* strain 422 as a source of rennet for cheese production.

Keywords *Myxococcus xanthus* · Milk-clotting proteases · Microbial coagulants · Industrial fermentations · Cheese making · Milk curds

M. Poza · C. Sieiro · T. G. Villa (✉)
Department of Microbiology and Parasitology,
University of Santiago de Compostela, Faculty of Pharmacy,
Campus Sur, 15782 Santiago de Compostela, Spain
E-mail: mpvilla@usc.es
Tel.: +34-981-592490
Fax: +34-981-594912

L. Carreira · J. Barros-Velázquez
Department of Analytical Chemistry, University of Santiago de Compostela, Faculty of Veterinary Sciences,
Nutrition and Food Science, Lugo, Spain

Introduction

Myxobacteria are gram-negative, strictly aerobic and have gliding motility. They are normally found in soil samples. Members of this microbial group exhibit a complex social behavior and a remarkably complex multicellular life, leading to the formation of fruiting bodies of different forms and sizes. Myxobacteria also biosynthesize and secrete large amounts of extracellular molecules, some of which are of considerable potential industrial interest, such as carotenoids, antibiotics, and a variety of hydrolytic enzymes [3]. The hydrolytic enzymes produced by myxobacteria also play a crucial role in a number of mechanisms involved in their vegetative growth and life cycle [5].

Proteases are lytic enzymes that catalyze the breakdown of peptide bonds of proteins. The potential applications of proteases in the food industry together with their high diversity and specific range of action have attracted the attention of biotechnologists worldwide. Although proteases are widely distributed in nature, microorganisms are the preferred source for fermentation bioprocesses, owing to their low cost and fast growth rate. In addition, they can be subjected to genetic engineering strategies to achieve either enzyme overproduction or modified enzymes with more desirable properties [11, 12].

Among the vast number of proteases with applications in the food industry, aspartic proteases, such as chymosin (EC.3.4.23.4) and related enzymes that specifically cleave the Phe-105–Met-106 bond in κ-casein, initiating milk coagulation, are the enzymes used for

cheese making. Several species of myxobacteria secrete a broad range of bacteriolytic enzymes, among them several proteases, during their vegetative growth, allowing them to feed on other bacteria and to survive under starvation conditions. During starvation, bacterial cells aggregate to form fruiting bodies. Thus, a proteolytic activity is associated with the A factor, the first extracellular signal involved in development [14]. Some of these proteases have been characterized regarding their caseinolytic or milk-clotting activity [1, 2, 9, 13].

The purpose of the present work was to evaluate the ability of several *Myxococcus xanthus* strains to produce milk-clotting enzymes with potential interest for the cheese industry. Thus, we report on the characterization of milk-clotting proteases from four *M. xanthus* strains. Among these, the best producer strain, which also showed the best ability to generate milk curds, was further characterized. Protein fractions containing clotting activity were purified from *M. xanthus* strain 422 and used for the preparation of milk curds. The results obtained in the physicochemical analysis of the milk curds were compared with milk curds prepared with conventional animal and microbial aspartic proteases. The potential usefulness of *M. xanthus* strain 422 as a source of microbial rennet for the cheese industry is discussed.

Materials and methods

Strains, media and culture conditions

Myxococcus xanthus strains CECT 422 (Spanish Type Culture Collection), DZF1 (University of Granada, Spain), DK1622 and DK10407 (S−) were used [4]. The strains were grown in CTT medium containing 8 mM $MgSO_4 \cdot 7 \cdot H_2O$, 1 mM KH_2PO_4 (pH 6.0), 10 mM Tris-HCl (pH 8.0), and 1% (w/v) pancreatic digest of casein (Biokar Diagnostics, Beauvais, France). The final pH of the medium was 7.6.

One-liter flasks containing 250 ml CTT medium were inoculated and incubated at 200 rpm for 48 h at 33 °C. Particular care was taken not to leave the cultures unshaken for more than 5 min in order to prevent cell clumping. The bacterial cells were removed by centrifugation (1,500 *g* for 15 min at 4 °C) and the supernatants were filtered through 0.22-μm pore size membranes (Millipore, Bedford, Mass., USA) and concentrated ten-fold by ultrafiltration using an Amicon device (Amicon, Oosterhout, Holland) equiped with a PM10 membrane (Amicon). The concentrated extracellular extracts were dialyzed overnight at 4 °C against 1 mM KH_2PO_4 (pH 6.0) and used for milk-clotting and caseinase assays.

Detection of protease activity and estimation of milk-clotting activity

Proteolytic activity in the concentrated extracellular extracts was initially detected by cup-plate Petri bioassays in a gel prepared by mixing 1.5% (w/v) agarose (Sigma, St. Louis, Mo., USA) and 2.5% (w/v) skimmed milk powder in 1 mM KH_2PO_4, pH 7.6. After solidification, 1.5-mm wells were filled with the concentrated extracellular extracts and the plates were incubated at 37 °C. Protease activity in the extracts was detected by clear hydrolysis halos surrounding the wells. Two microbial coagulants [microbial coagulant I (Arroyo S.A., Santander, Spain) and microbial coagulant II (Fromase TL, Gist Brocades, Delft, Holland)] were used as positive controls.

Milk-clotting activity in the enzyme extracts was investigated as follows. One ml of substrate [(26% (w/v) skimmed—or, when required, full-fat—milk powder in 10 mM KH_2PO_4, pH 6.0] was mixed with 1 ml of each concentrated extracellular extract and incubated at 37 °C until milk clots were visualized. One enzyme unit was defined as the amount of enzyme that coagulated 1 ml of substrate at 37 °C in 30 min. Reference assays were done with the two commercial microbial coagulants, as described above.

The milk-clotting properties of the *M. xanthus* proteases were compared with other commercial microbial coagulants by means of a standardized titration method. Briefly, 2 ml of each concentrated extracellular extract were diluted in 10 ml distilled water, and 2 ml of each diluted extract were added to 50 ml of a solution consisting of 13% (w/v) skimmed milk prepared in distilled water. The mixtures were then incubated at 37 °C, and the titers of each extract calculated by determining the time elapsed until the milk clots were visible. The milk-clotting times of titrated commercial microbial coagulants were also measured and used as references.

Investigation of caseinase activity

Caseinolytic activity in the concentrated extracellular extracts was measured in test-tube reactions according to the method of Kunitz [7]. The values obtained allowed estimation of the ratio of milk-clotting to protease (caseinase) activity.

The specific activity against κ-casein was determined by the method described by Lucas et al. [9]. Briefly, enzymatic reactions were carried out by mixing 100 μl of each concentrated extracellular extract with 900 μl of substrate [(0.25% κ-casein (Sigma) (w/v) in 10 mM KH_2PO_4 (pH 6.0) + 100 mM NaCl], and incubating the samples at 30 °C for 30–120 min. Optical density was followed at 550 nm. Reference assays were done with commercial microbial coagulants.

Proteins were measured by the method of Lowry [8].

The cellular location of the milk-clotting enzyme of *M. xanthus* strain 422

M. xanthus strain 422 was grown in shake-flasks (200 rpm) for at least 2 days in 2 l CTT at 33 °C, until clotting activity was detected in the culture medium. The cells were then harvested and divided into two identical portions as described by Villa et al. [16]. One portion was centrifuged, re-suspended in 30 ml 1 mM KH_2PO_4 (pH 7.6), and the cells were disrupted at 750 *g* for 3 min in a CO_2-cooled cell homogenizer (Braun MSK, Melsungen, Germany) in the presence of 0.1-mm glass beads. Cellular debris was removed by centrifugation at 1,500 *g* and the specific proteolytic activity in the supernatant was evaluated. Cells from the other portion were re-suspended in 0.1 M HCl and incubated for 1 h at 18 °C. Subsequently, the cells were washed and re-suspended in the same buffer and homogenized. Glyceraldehyde-3-phosphate dehydrogenase was used as the intracellular marker in both HCl-treated and non-treated portions.

Partial purification of the milk-clotting enzyme

Concentrated extracellular extract (4 ml) from *M. xanthus* strain 422 was applied to a Sephacryl S-200 column (80×3.5 cm) previously equilibrated with 10 mM KH_2PO_4, pH 6.0. Gel-exclusion chromatography was carried out with the same buffer and 4-ml fractions were collected at a flow rate of 0.5 ml min^{-1}. Absorbance of the fractions was measured at 280 nm. Aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), and lysozyme (14 kDa) were used as molecular mass standards. The molecular weight of the milk-clotting enzyme was estimated as described by Whitaker [19].

Anion-exchange chromatography was also used for partial purification of the milk-clotting enzyme and as a preparative method to obtain enough enzyme to prepare milk curds. Briefly, 4-ml aliquots of concentrated extracellular extracts were applied either to an analytical DEAE-Biogel A 38×3.5-cm column or to a preparative DEAE-Biogel A 120×20-cm column that had been previously equilibrated with 10 mM KH_2PO_4, pH 7.6. Proteins were eluted using a NaCl gradient (0–1 M) at a flow rate of 0.5 ml min^{-1} for the analytical column and 20 ml min^{-1} for the preparative column. Fractions of 4 or 200 ml, respectively, were collected, and those fractions showing protease activitywere concentrated six-fold in an Amicon device using a PM10 membrane.

Biochemical characterization of the milk-clotting enzyme

The optimum temperature determined in the purified fraction obtained by gel-exclusion chromatography was investigated at pH 6.0 in the 15–65 °C range; optimum pH was determined in the 5–10 range at 37 °C. Sodium acetate buffer (0.01 M) was used for the pH 5–6 range; 0.01 M KH_2PO_4 and 0.01 M Tris-HCl were used for pH range 6–8 and 8–10, respectively. The pI of the milk-clotting protease of *M. xanthus* strain 422 was determined by isoelectric focusing using ampholines in the range of pH 3–10 pH, as described elsewhere [15].

Production of the milk-clotting enzyme in a pilot plant fermentation unit

Fermentation was accomplished in a Biostat C (B. Braun Biotech International, Melsungen, Germany) 30-l fermenter using 20 l of CTT medium. This medium was sterilized in situ at 121 °C for 30 min with gentle stirring, following the manufacturer's instructions. After sterilization, an inoculum consisting of 3 l of a shake-flask culture was added to achieve an initial cellular density of 10^5 cells·ml^{-1}. Fermentation was started with an air flow of 15 l min^{-1}, at pH 7.6 and a stirring speed of 300 rpm and 33 °C. Addition of an antifoam product (Braun) was controlled automatically. The air flow was increased from 15 to 25 l min^{-1} as the biological oxygen demand increased. Samples were removed over 48 h in order to determine pH, cell density, and enzymatic activity. The cells were then harvested using a continuous-flow centrifuge.

Preparation of milk curds

To evaluate the potential usefulness of the extracellular protein extracts for the cheese industry, a preliminary investigation of the physicochemical properties of milk curds prepared with the purified protein fractions was carried out. The protein fractions used to induce milk coagulation were: (a) a total protein extract consisting of a concentrated extracellular extract, prepared as described above; (b) protein fraction I; and (c) protein fraction II. The latter two protein fractions corresponded to those fractions partially purified from cell-free supernatants of *M. xanthus* by means of anion-exchange chromatography, as described above.

Fifty ml of each protein fraction was added either to 400 ml reconstituted skimmed milk (13% w/v) or 400 ml whole milk (fat content: 3.2%). Two commercial cheese rennets, one of animal origin (Bescansa S.A., Santiago de Compostela, Spain) and the other of microbial origin (Arroyo, S.A., Santander, Spain) were used for the preparation of reference milk curds. In these two cases, 10 ml of each commercial rennet was employed for each assay. The mixtures were kept overnight at 37 °C to allow the milk to curdle. Thus, ten types of milk curd gels were obtained, five of them prepared in skimmed milk and the other five in whole milk. The physicochemical properties of the curds obtained with the *M. xanthus* 422 protein fractions were compared with those of the reference curds obtained with the commercial rennets.

Analysis of milk curds

The dry matter (DM) of the milk curds was measured by the gravimetric method, with a precision of ±0.1%. Thus, 3-g samples were heated at 102 °C to constant weight, which usually took 3 h. The results were expressed as percentages (w/w). The moisture contents were determined by the difference in the weights before and after drying.

The fat content (FC) was determined in 3-g samples. The samples were introduced in a butyrometer and covered with 62% sulfuric acid and incubated at 65 °C. After adding 1 ml isoamyl alcohol, the mixture was centrifuged for 5 min at 225 *g* in a heated Gerber centrifuge (Orto Alresa, Madrid, Spain). The FC was read directly from the butyrometer and the results were expressed as percentages, with a precision of ±0.2%. The ratio between FC and dry weight was also determined in the milk curds. The moisture-non-fat content (MNF) of the milk curds was calculated as a percentage according to the equation: % MNF = [(100−% DW)/(100−% FC)]×100

Physicochemical properties of the milk curds

The clotting ability of the protein fractions obtained from *M. xanthus* strain 422 was evaluated in the milk curds by penetrometry. Thus, the texture of the milk curds was assessed with an automatic Hounsfield equipment (HTE Hounsfield, Salfords, UK) equipped with a 500-N load cell. The milk curds were cut to obtain small rectangular prisms of 1 cm^2 in section and 3 cm length. The dimensions of the prisms were verified with a micrometric calibration system, allowing deviations of 0.05 cm for each dimension. Two to four prisms were assayed for each milk curd sample. The prisms were subjected to a vertical strength of 500 N and a cut cell with an opening of 50°. The strength was applied perpendicular to the sample, from a height of 20 mm, with a speed of 150 mm·min^{-1}. The resistance of the milk-curd prisms was estimated from the strength curves obtained; firmness (N) and tensile strength (N/area under curve) were determined with a precision of ±0.5%.

Milk curds were diluted at a 1:3 (w/v) ratio in Milli-Q water and the pH was determined using a 6-mm diameter electrode (Crison, Barcelona, Spain) with a precision of ±0.1 pH units. The water activity (Aw) of the milk curds was measured with an automatic device (Aqualab CX-2, GBX Sarl, Romans, France) with a precision of ±0.001 units.

Results

Production of milk-clotting enzymes

The ability of *M. xanthus* strains DK1622, DZF1, DK10407 and 422 to produce proteases was initially investigated in concentrated extracellular extracts by means of a plate bioassay using skimmed milk as substrate. After an incubation period of 50 h at 37 °C, all four strains hydrolyzed casein, as detected by unambiguous clearing zones surrounding the extracts.

The specific milk-clotting activities of all four *M. xanthus* strains increased during the exponential phase of growth, reaching a maximum at the beginning of the stationary phase (30–35 h). Table 1 compiles the milk-clotting specific activities of the concentrated extracellular extracts from *M. xanthus* compared with those of two commercial microbial coagulants. Although all *M. xanthus* strains exhibited milk-clotting activity, strain 422 was selected for further study because it exhibited a more intense milk-clotting activity

Table 1 Enzymatic assays carried out with the concentrated extracellular extracts of the *Myxococcus xanthus* strains and two commercial microbial coagulants

Milk-clotting enzyme source	Milk-clotting activity (U ml^{-1})	Protein concentration (mg ml^{-1})	Specific milk-clotting activity (U mg^{-1})	Titration (international units)	Concentration of tyrosine obtained from casein degradation (mM)	Turbidity obtained from κ-casein degradation (500 nm)
M. xanthus 422	1	0.99	1.01	1:125	0.55	0.25
M. xanthus DZF1	0.75	0.94	0.79	1:80	0.4	0.1
M. xanthus DK1622	0.75	1.03	0.73	1:80	0.34	0.08
M. xanthus DK10407	0.5	1.07	0.47	1:50	0.23	0.07
Microbial coagulant I	120	3.82	31.40	1:15000	7.6	0.26[a]
Microbial coagulant II	400	36.96	10.82	1:50000	15	0.85[a]

[a]Samples were diluted 1,000-fold

(1.01 U/mg protein) (Table 1). In addition, the extract from strain 422 showed the lowest titration values of the four *M. xanthus* strains tested. Interestingly, the higher milk-clotting activity detected in *M. xanthus* strain 422 did not derive from a higher protein concentration in the extract, as compared to the other strains, but rather from a more intense milk-clotting activity of its proteolytic enzyme.

The commercial microbial coagulants used as references exhibited higher milk-clotting specific activities than the *M. xanthus* extracts, a result that was expected due to their high protein concentration. For example, the commercial microbial coagulant II exhibited a titer that was 400 times lower than that of the extract of *M. xanthus* 422, but the latter extract contained 40 times less protein.

Besides this clotting ability, the *M. xanthus* strains were able to degrade casein and κ-casein (Table 1), indicating that all four strains produced both non-specific proteases and more specific proteases able to digest κ-casein. The caseinase activity of the *M. xanthus* extracts—estimated by the concentration of tyrosine obtained from casein degradation—was in the range of 0.23–0.55 mM; *M. xanthus* strain 422 had the highest activity (Table 1).

In order to determine whether the proteolytic enzymes of the *M. xanthus* strains were true milk-clotting enzymes rather than proteases, the ratio of clotting to protease (caseinase) activity was compared. Interestingly, similar milk-clotting to caseinase ratios (in the range of 1.83–2.14) were obtained for the *M. xanthus* strains, while ratios of 4.13 and 0.72 were obtained for the commercial microbial coagulants I and II, respectively. Thus, the proteolytic enzymes of the *M. xanthus* strains may be regarded as true milk-clotting enzymes.

Cellular location, purification, and biochemical characterization of the milk-clotting enzyme of *M. xanthus* strain 422

The milk-clotting activity occurred mainly in the extracellular medium, since no enzymatic activity was detected at either periplasmic or cytosolic levels in cultures subjected to cellular lysis after treatment with or without 0.1 M HCl. The mild-acid treatment [16] inactivated periplasmic enzymes but did not affect intracellular enzymes.

The milk-clotting enzyme was partially purified by molecular exclusion chromatography and the partially purified extract used to study its basic physicochemical parameters. The enzyme eluted as a single peak (Fig. 1a) with a V_e/V_0 value of 1.8, indicating a molecular mass of 40 kDa (Fig. 1b). Optimum pH and temperature were also determined for the partially purified enzyme (Fig. 2). Thus, while the milk-clotting enzyme produced by this strain was active in a pH range of 5–7, the highest clotting activity was at pH 6 (Fig. 2a). The enzyme was active in a temperature range of 5–60 °C, 37 °C being the optimum (Fig. 2b). The enzyme lost 55% of its activity after 5 min at 65 °C, and up to 88% of its activity after 10 min at 65 °C (Fig. 2b). Total inactivation of the enzyme was achieved after 12 min at 65 °C. The pI of the milk-clotting enzyme was 5.0.

Anion-exchange chromatography, carried out in parallel, was used to purify a portion of the concentrated

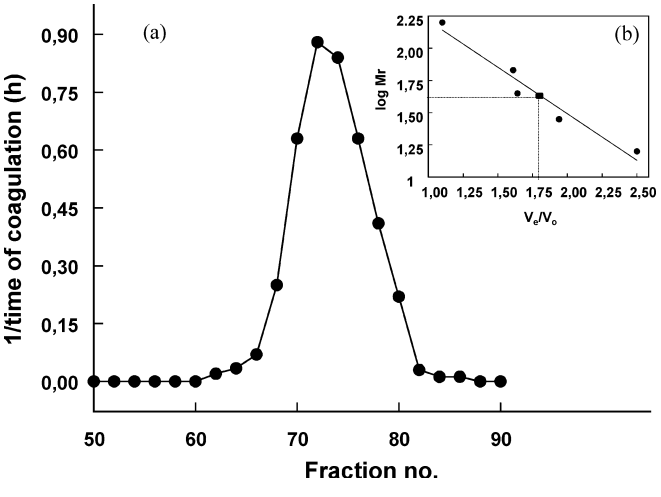

Fig. 1 **a** Gel-exclusion chromatography through Sephacryl S-200 of a concentrated extracellular extract of *Myxococcus xanthus* strain 422. **b** Molecular weight determination of the milk-clotting enzyme by the method of Whitaker [19]. *Circles* Molecular-weight standards, *square* enzyme

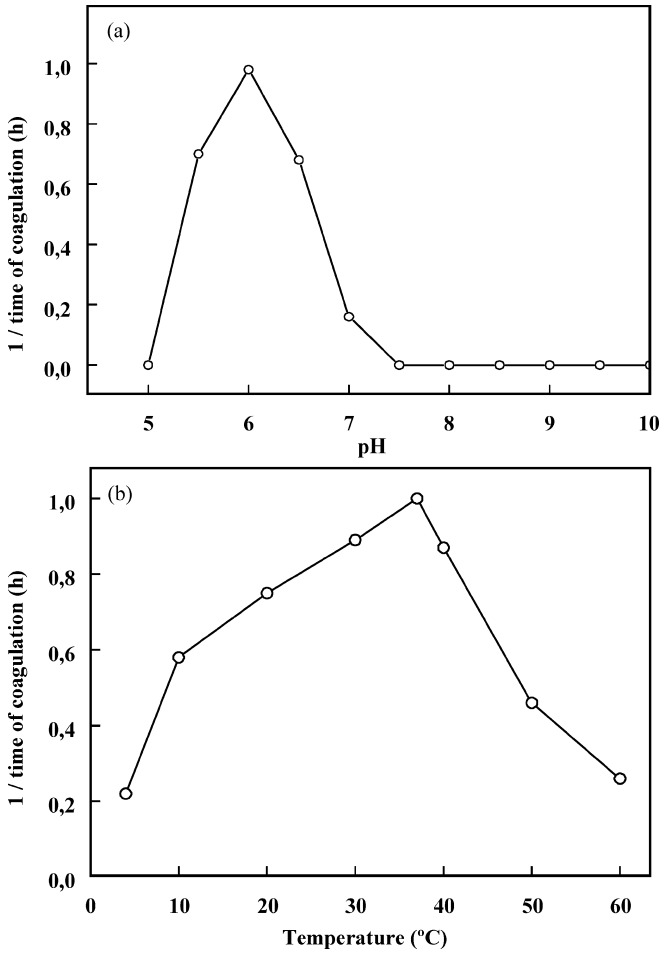

Fig. 2a, b Activity of the milk-clotting enzyme of *M. xanthus* strain 422 purified by gel-exclusion chromatography. Effect of **a** pH, **b** temperature

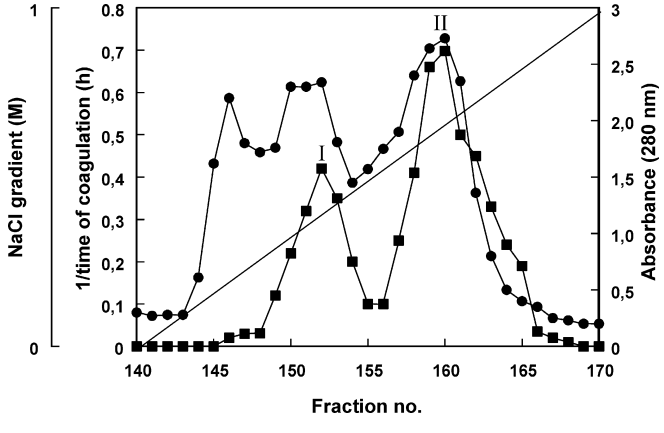

Fig. 3 Partial purification by anion-exchange chromatography on DEAE-Biogel A of the milk-clotting enzyme of *M. xanthus* strain 422 grown in a 30-l Biostat C fermentation unit. – NaCl gradient, ● absorbance at 280 nm, ■ enzyme activity

extracellular extract and revealed two active peaks (Fig. 3). These peaks showed milk-clotting activity with an optimum pH of 6.0, an optimum temperature of 37 °C, and a pI of 5.0, results that may be interpreted as

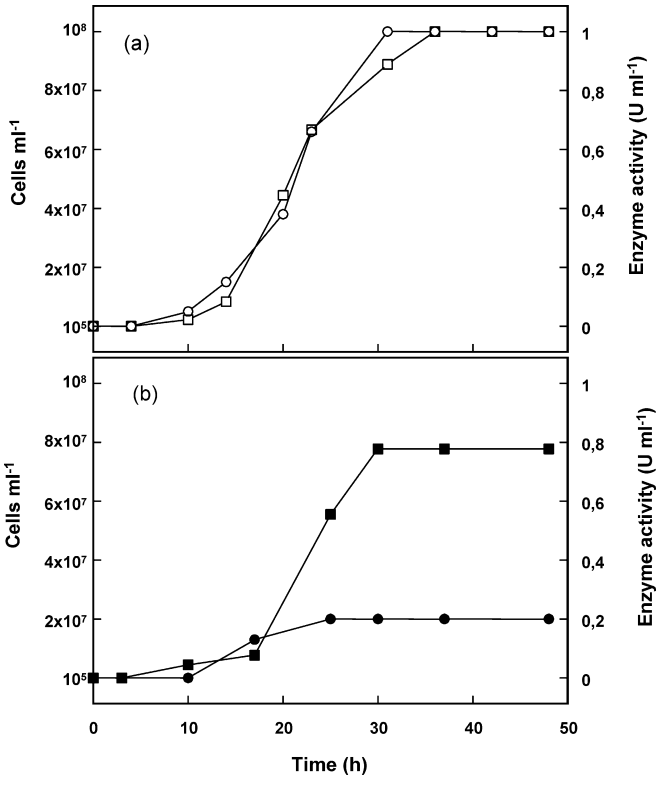

Fig. 4a, b Growth and milk-clotting activity of *M. xanthus* strain 422 in CTT medium. **a** Growth (□) and milk-clotting activity (○) in shake-flasks. **b** Growth (■) and milk-clotting activity (●) in the 30-l Biostat C fermentation unit

either (1) the production of more than one milk-clotting enzyme, or (2) the existence of two different isoforms of a single milk-clotting enzyme in *M. xanthus* strain 422. These possible explanations are discussed below.

Production of the milk-clotting enzyme of *M. xanthus* strain 422 in a pilot-plant fermentation unit

A preliminary experiment aimed at scaling-up production of the milk-clotting enzyme was carried out in a 30-l fermentor using CTT medium (Fig. 4). The growth rate of *M. xanthus* strain 422 in the fermentation unit (Fig. 4b) was slightly lower than that exhibited in shake-flasks (Fig. 4a). Moreover, the amount of milk-clotting enzyme produced in the fermentation unit (Fig. 4b) was about five times lower than produced in shake-flasks (Fig. 4a). Thus, while the milk-clotting enzyme can be produced using an inexpensive culture medium in a pilot-plant fermentation unit, optimization of the technical conditions of fermentation should be undertaken with a view to improving the levels of enzyme production.

Physicochemical properties of milk curds prepared with the purified milk-clotting enzyme

The 23 l of culture of *M. xanthus* strain 422 obtained in the fermentation unit were applied to a preparative

anion-exchange column. The two eluted fractions showing milk-clotting activity were concentrated to achieve an enzymatic activity of 6 $U \cdot ml^{-1}$ and then used to prepare milk curds.

In Tables 2 (whole milk) and 3 (skimmed milk), the results of the physicochemical analysis of milk curds prepared with the different protein fractions from *M. xanthus* and a comparison with their counterparts prepared with commercial rennets of microbial and animal origin are shown. The fat contents of milk curds 1 and 3, prepared with protein fractions I and II, respectively, purified from *M. xanthus* strain 422, were similar or even higher than those of milk curds prepared with commercial rennets (Table 2). Likewise, the values of both the fat content/dry-matter ratio and the moisture-non-fat content were higher in milk curds 1 and 3 than in curds prepared with commercial rennets. As expected, all of the milk curds prepared with whole milk had higher dry-matter values (Table 2) than their counterparts prepared in skimmed milk (Table 3).

An objective examination of texture was also carried out in the milk curds by measuring resistance to penetration and fragmentation. Tables 2 and 3 show the average values obtained for firmness and tensile strength in the curds prepared with whole milk and skimmed milk. Milk curds 7 and 9, prepared with commercial rennets and whole milk, were firmer than their counterparts prepared with the protein fractions of *M. xanthus* (Table 2). By contrast, milk curd 4, prepared with skimmed milk and protein fraction II from *M. xanthus*, exhibited a degree of firmness even higher than that determined for milk curds prepared with commercial rennets and skimmed milk (Table 3). Likewise, the tensile strength values determined were generally lower for the milk curds prepared with the protein fractions of *M. xanthus* than for the curds prepared with commercial rennets (Tables 2 and 3), with the exception of milk curd 4, prepared with skimmed milk and protein fraction II from *M. xanthus* (Table 3). Interestingly, protein fractions I and II, which were partially purified from *M. xanthus* strain 422 by anion-exchange chromatography, behaved differently with respect to their clotting activity, as determined by the properties of the milk curds obtained.

Table 3 Physicochemical properties of milk curds prepared with skimmed milk

Milk curd[a]	Aw	Firmness (N)	Tensile strength (J)	Dry matter (%)	pH
2	0.974	45.8	45.5	30.0	5.5
4	0.981	115.3	95.5	26.8	5.1
6	0.973	20.8	22.4	23.8	5.1
8	0.985	93.9	100.9	29.1	5.7
10	0.982	54.2	54.0	31.3	6.1

[a] *2* Protein fraction I from *M. xanthus*, *4* protein fraction II from *M. xanthus*, *6* total protein extract from *M. xanthus*, *8* commercial animal rennet, *10* commercial microbial rennet. The accuracy of the average results shown are: Aw (±0.001 units), firmness (±0.05%), tensile strength (±0.05%), dry matter (±0.1%), and pH (±0.1 units)

Discussion

The ability to produce extracellular proteolytic enzymes has been described for many *Myxobacteria* species [5]. The four *M. xanthus* strains tested in this study produced extracellular proteases that specifically acted on κ-casein, thus originating milk clotting. The highest milk-clotting activities of the four *M. xanthus* strains occurred at the beginning of the exponential phase of growth, strain 422 showing the highest specific activy and lower titers in liquid medium (Table 1). By contrast, the milk-clotting activity of *M. xanthus* strain DK10407 was remarkably lower than that of the other strains (Table 1). This strain harbors a mutation in the gene *pilA* which codes for pilus protein, implying that cells cannot aggregate and do not complete their developmental cycle.

The proteolytic enzymes of the *M. xanthus* strains tested proved to be true milk-clotting enzymes since the ratio of milk-clotting to protease (caseinase) activity was in the range of 1.83–2.14. Ratios of 4.13 and 0.72 were determined for the commercial microbial coagulants I and II evaluated in parallel in this work.

When purified by gel-exclusion chromatography, the crude enzyme preparation from *M. xanthus* strain 422 yielded a single active peak of milk-clotting activity (Fig. 1). By contrast, two fractions exhibiting milk-clotting activity were obtained when purification was carried out by means of either analytical or preparative anion-exchange chromatography (Fig. 3). Although these two

Table 2 Physicochemical properties of milk curds prepared with whole milk. *FC* Fat content, *DM* dry matter, *MNF* Moisture-non-fat content

Milk curd[a]	Aw	Firmness (N)	Tensile strength (J)	Fat content (%)	Dry matter (%)	FC/DM (%)	pH	MNF(%)
1	0.986	25.0	24.7	19.0	33.0	63.4	5.5	84.7
3	0.984	48.6	39.3	15.0	32.3	55.1	6.7	82.3
5	0.986	26.4	29.7	12.5	30.4	48.6	5.4	81.7
7	0.992	75.0	64.8	15.0	37.2	46.6	5.5	76
9	0.981	54.2	51.0	16.5	34.5	54.6	5.6	80.7

[a] *1* Protein fraction I from *M. xanthus*, *3* protein fraction II from *M. xanthus*, *5* total protein extract from *M. xanthus*; *7* commercial animal rennet, *9* commercial microbial rennet. The accuracy of the average results shown are: Aw (±0.001 units), firmness (±0.05%), tensile strength (±0.05%), fat content (±0.2%), dry matter (±0.1%), FC/DM (±0.3%), pH (±0.1 units), and MNF (±0.3%)

fractions exhibited similar biochemical properties, they produced milk curds with remarkably different physicochemical properties (Table 3). These results may indicate either that: (1) more than one milk-clotting enzyme is produced by *M. xanthus* strain 422, or (2) different complexes of a single milk-clotting enzyme are present in *M. xanthus* strain 422. The latter explanation may be more likely, and is more in accordance with studies by other authors suggesting that the proteolytic enzymes produced by other *M. xanthus* strains may form different complexes with lipopolysaccharides, which would explain the behavior of the enzyme during its purification [9]. These authors also suggested as very likely the interaction of the protease from *M. xanthus* with other compounds, which would imply significant changes in the properties of the enzyme [9]. The two protein fractions purified from *M. xanthus* strain 422 exhibited similar biochemical properties, but the milk curds prepared with them showed remarkable differences. Colleta and Miller [16] also proposed that the caseinolytic exoenzyme activities of *M. xanthus* exist as complexes that would show a different electrophoretic behavior.

In our hands, the enzyme lost 88% of activity after 10 min at 65 °C. This value is within the ranges described for other microbial rennets, such as those obtained from *Rhizomucor miehei* or *Cryphonectria parasitica* [17]. Different proteases that coagulate milk have been described in other *M. xanthus* strains. Thus, two milk-clotting enzymes of 45 kDa [1] and 12 kDa [9] have been identified in strain DK101. The latter has an optimum pH of 5.9, an isoelectric point of 4.5, and its sequence does not share any similarity with other known aspartic proteases involved in milk clotting. Moreover, a milk-coagulating enzyme with an optimum pH of 6 that is specifically inhibited by aspartic protease inhibitors has been described in strain DK1622 [13]. It seems clear that *M. xanthus* strains secrete acid proteases that can trigger milk coagulation by cleaving the Phe-105–Met-106 bond in κ-casein, in a similar manner to animal or fungal rennets, and can thus be considered of interest for preparing commercial curds for cheese making.

The industrial applicability of *M. xanthus* strain 422 will need an optimization study aimed at increasing enzyme production at the pilot-plant level. Growth of this strain in a 23 l-fermentation unit resulted in secretion of the milk-clotting enzyme into the extracellular medium, and no clotting activity was detected at either the periplasmic or intracellular level. However, the cell densities and, especially, the enzyme activities in the fermentation-unit culture were remarkably lower than those obtained in the shake-flask cultures.

In general, all the milk curds obtained with *M. xanthus* purified protein fractions had lower dry-matter values and a lower fat content than found in other fresh cheeses from our region (Galicia, Northwestern Spain), which unlike the milk curds prepared in this study are generally subjected to a short period of aging of 2–3 weeks. In a similar way, the moisture-non-fat content of all fresh cheeses obtained in the present study were significantly higher than those normally obtained in other Galician fresh cheeses after an aging period of several weeks. Guinee et al. [6] reported that reductions in the fat content of cheese were accompanied by both an increase in the moisture content—and consequently a decrease in dry matter—and a decrease in the moisture-non-fat content. These results are partially in agreement with those described here.

As expected, milk curds prepared in skimmed milk (Table 3) were firmer than their counterparts prepared in whole milk (Table 2). While in our work, the tensile strength values were similar (Tables 2 and 3), others have reported that low-fat cheeses exhibited firmer textures than full-fat cheeses [10]. Guinee et al. [6] also found a negative correlation between firmness and fat content. Other authors reported that the fat content of a cheese is the main factor responsible for texture, as determined by sensory and rheological analyses [18].

The pH values of all the milk curds prepared with whole milk or skimmed milk were in the range of 5.1 and 5.7, except milk curds 3 (prepared with protein fraction II from *M. xanthus* strain 422) and 10 (prepared with commercial microbial rennet), which exhibited significantly higher pH values. Additionally, no major differences were observed in the Aw values of curds prepared with the different protein fractions of *M. xanthus* strain 422 or with commercial rennets. The pH values obtained in the milk curds prepared in this work did not differ significantly from those of other Galician cheeses (unpublished data).

In summary, it can be concluded that extracellular protein fraction II, purified from *M. xanthus* strain 422 by anion-exchange chromatography, exhibits a significantly high milk-clotting ability. Strain 422 can thus be regarded as a novel potential source of milk-clotting enzyme with applications in the cheese industry. A preliminary study of the milk curds obtained with the protein fraction purified from *M. xanthus* strain 422 revealed that its physicochemical properties did not differ significantly from those of curds prepared with commercial rennets. Nevertheless, although the milk-clotting enzyme of *M. xanthus* strain 422 was obtained by culture in an inexpensive medium using a pilot-plant fermentation unit, scaling-up production of this enzyme in larger fermentation units requires additional efforts aimed at increasing the yield.

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