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ORIGINAL PAPER

Characterization of the purified actinidin as a plant coagulant of bovine milk

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Abstract In this work, actinidin was characterized in view of its possible suitability as a coagulant enzyme in the manufacturing process of cheese. The results show that actinidin does exhibit milk-clotting activity, which is correlated with the enzyme concentrations. The combined use of urea and SDS–PAGE led to the conclusion that the milk clot is clearly separated from the whey proteins and corresponds to casein coagulum. Moreover, both the enzyme dependence on pH and temperature and the stability profiles are fully suitable with the chemical–physical conditions adopted during the cheese-making procedure. The analysis of the kinetic constants as well as the electrophoretic pattern of the hydrolysis products suggests that β-casein is the preferred substrate of actinidin, whereas κ-casein seems to be hydrolyzed only in a few large fragments.

Keywords Actinidin · Plant coagulant · Milk-clotting · SDS–PAGE · Casein

Abbreviations

EDTA	Ethylenediaminetetraacetic acid
Tris	(hydroxymethyl)-aminomethane
DTT	Dithiothreitol
TCA	Trichloroacetic acid
CT	Clotting time

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Introduction

Proteolysis is a key biochemical event that occurs during both milk clotting and cheese ripening. It represents the most important factor for the development of the typical flavor and texture of cheeses. Calf rennet, which contains chymosin (E.C. 3.4.23.4) as a main enzymatic component, has been the most widely used milk-clotting enzyme preparation. Natural calf rennet is extracted from the inner mucosa of the fourth stomach chamber (the abomasum) of young, unweaned calves according to a complex and expensive procedure [1]. During the production of natural rennet, deep-frozen stomachs are milled and put into an enzyme-extracting solution. After the enzyme activation, the rennet extract is filtered in several stages and concentrated until reaching a typical potency of about 1:15,000. The increasing worldwide cheese production and the limited availability of proper stomachs for rennet production have led to a systematic search for new coagulant sources. The recombinant chymosin, expressed by *Aspergillus niger* var. *awamori*, *Kluyveromyces marxianus* var. *lactis* or *Escherichia coli*, is widely used in cheese-making in many countries [2]. Along with the recombinant chymosin as an alternative source of the natural calf rennet, fish [3]—and crustacean [4]—derived proteinases inducing milk clotting have been extensively studied. In particular, Rossano et al. [4] reported that the molecular mass of the peptides found in the cheese manufactured using a protease from the crustacean *Munida* was similar to that of the peptides commonly detected in cheeses obtained using chymosin as a coagulant, thus suggesting that both proteases raise a similar pattern of hydrolysis upon casein fractions. More recently, the *Munida* enzymes were found to degrade the chymosin-derived β-casein fragment f193–209, one of the peptide associated with bitterness in cheese, revealing their

possible application in cheese technology [5]. As the use of animal rennet may be limited for religious reasons (e.g. Judaism and Islam) or diet (vegetarianism), proteolytic enzymes extracted from plants, such as *Lactuca sativa* [6, 7], *Albizia lebbeck* and *Helianthus annuus* [8], *Streblus asper* [9] among others, are a subject of growing interest in dairy technology. Moreover, a lot of the applications of plant proteolytic enzymes on dairy products also refer to the use of the sulfhydryl protease ficin from fig latex, as reported in the traditional recipes of the Mediterranean area. Unfortunately, there is little published research on such use of this protease whose utilization has been limited to the Teleme and Gaziantep cheese production [10, 11]. Most of the above-mentioned coagulants were found unsuitable because they produced cheeses characterized by extremely bitter tastes. In fact, plant proteases exhibit high levels of proteolytic activity, leading to the production of short peptides that affect both the flavor, which results excessively acid and bitter, and the texture of the cheeses [11]. The aqueous extracts of *Cynara cardunculus* represent an exception to this general rule as the enzyme mixture extracted from cardoons is commonly utilized in Spain and Portugal for the manufacture of esteemed cheeses from ovine milk [12]. As regards fungi, Arima et al. [13] screened about 800 microorganisms and obtained a soil isolate of *Mucor pusillus* that produces a thermostable enzyme with a high level of milk-clotting activity, enabling the production of remarkable yields of curds. However, the high thermal stability of *Mucor* rennins turned out to be an undesirable property since residual enzyme activity, even after cooking, can spoil the flavor of cheese during the maturation process [14]. Recently, low thermostability as well as high milk-clotting activity was found in the enzymatic extract of *Thermomucor indicae-seudaticae* N31, encouraging future cheese production experiments to check its potential as a microbial rennin [15].

Actinidin (EC. 3.4.22.14) is a cysteine protease found in the fruit of Chinese gooseberry (*Actinidia chinensis*) or kiwi fruit, which catalyzes the hydrolysis of peptide bonds containing basic amino acids such as Lys and Arg in position P1 [16]. This enzyme consists of 220 amino acids with an apparent molecular weight of about 23.0 kDa [17]. Its complete amino acid sequence [18], the nucleotide sequence of the corresponding gene [19], and the three-dimensional structure [20] have been determined. Recently, Katsaros et al. [21] reported the application of either kiwifruit powder rich in actinidin or kiwi juice in the production of dairy products as well as the use of high pressure as regulator of enzymatic activity. In our work, we have investigated the suitability of the purified actinidin as a prospective substitute for chymosin during cheese-making procedures. In particular, we tweaked the original purification procedure of actinidin, making the protocol faster than others previously proposed [16, 22, 23]. Then, we characterized the actinidin proteolytic activity with respect to the cheese manufacture technology by determining both the milk-clotting activity of the enzyme and by the gel electrophoresis analysis of the hydrolysis products. Since the application of enzymes in biotechnological processes is frequently limited by protein denaturation, we also tested the compatibility of the enzyme with the parameters, such as pH and temperature, which might induce detrimental modifications in the enzyme structure and activity during milk processing.

Materials and methods

Enzyme preparation

Actinidin was purified by a slight modification of the method described in McDowall [16] and Boland et al. [24]. The fruits (100 g), obtained from the University farm located in Catania, were homogenized with buffer A (200 mM mannitol, 70 mM sucrose, 20 mM Hepes–KOH pH 7.5, 10 mM cysteine) in a 1: 1.25 (wt/vol) ratio. The homogenate was filtered and centrifuged at 24,400g × 20 min at 4 °C in a Beckman J2-HS centrifuge, rotor JA-20 (Beckman instruments, Fullerton, CA, USA). The resulting supernatant was adjusted to pH 4.4 and was again centrifuged at 150,000g × 60 min at 4 °C in a Sorvall ULTRA PRO 80 ultracentrifuge (Sorvall, Ramsey, USA). The pellet was discarded and the supernatant (crude extract) was applied onto a DEAE-Sepharose CL 6B column (Pharmacia Uppsala, Sweden) previously equilibrated with standard buffer B (25 mM CH_3COONa pH 4.4, 5 mM DTT, 1 mM EDTA). The first eluate was reloaded onto the column that was then developed with a linear gradient of 0–2 M NaCl. Fractions of 2 mL were collected and assayed for proteolytic activity using as substrate a 2% solution of total casein from bovine milk, technical grade (Sigma, St. Louis, MO, USA), dissolved by heating in 67 mM NaH_2PO_4 pH 7.2. The active proteolytic fractions were combined and precipitated with solid $(NH_4)_2SO_4$ at 70% of saturation. After centrifugation at 24,400g × 30 min at 4 °C, the precipitate was redissolved in standard buffer B and then dialyzed against 4 L of buffer B two times for 4 h at 4 °C. The dialyzed active proteolytic peak was used for enzyme characterization. The protein content of the enzyme preparations was routinely measured by the method of Bradford [25], using bovine serum albumin as standard.

Milk-clotting activity

Milk-clotting activity was measured by the method described in Uchikoba et al. [26] based on the visual evaluation of the

first clotting flakes' appearance. Different amounts of the enzyme (2, 5, 10, 20, and 40 μg) were added to a 10% solution of skimmed milk powder in 67 mM NaH_2PO_4 pH 6.8 at 30 °C both in the presence and in the absence of 10 mM $CaCl_2$ in a final volume of 3 mL. The time elapsing between the mixing of reagents and the first appearance of solid material against the background was measured (CT, clotting time). The specific milk-clotting activity (C) was expressed in MCU (milk-clotting units) defined as the amount of enzyme that clots 10 mL of the substrate within 40 min. The C/P value was calculated using an equal amount of purified actinidin (40 μg) both for the milk-clotting activity (C) and for the general protease activity (P) measurements. All the experiments were repeated four times on independent enzyme preparations. The whey was separated from the clot through four layers of cheesecloth and further centrifugation at 9,000*g* × 5 min in a benchtop centrifuge ALC PK 121 R (ALC, New Jersey, USA). The protein content of both clot and whey was determined according to the method described in Lowry et al. [27] and then analyzed by SDS–PAGE and urea–PAGE.

Proteolytic activity assay

The proteolytic activity was assayed using total casein, α-casein, β-casein, κ-casein (all from Sigma, St. Louis, MO, USA) and several types of milk as substrates as described in Lo Piero et al. [7]. The assay mixture (1 mL) contained 2% (wt/vol) of the substrates dissolved in 67 mM NaH_2PO_4 pH 7.2 and 2.5 mM DTT. The hydrolysis against different types of milk was measured by incubation of milk, in the amount corresponding to 20 mg of proteins as determined by the method described in Lowry et al. [27]. The milk types used in the experiments were namely pasteurized whole milk and pasteurized semi-skimmed milk. All the samples were incubated at 55 °C for 20 min, and then the reaction was stopped by adding 1.5 mL of 5% (wt/vol) TCA. After TCA precipitation, the supernatant was recovered by centrifugation at 9,000*g* × 10 min in a benchtop centrifuge. The absorbance of supernatant was measured at 280 nm using a Shimadzu UV–VIS 1240 spectrophotometer (Shimadzu Corporation, USA). Blank samples were performed by adding the enzyme at the end of the incubation time, just before TCA addition and precipitation. The enzyme activity was expressed in unit defined as the amount of enzyme that yields a 0.001 absorbance change per min at 280 nm. The kinetic constants of the enzyme, Kcat and Km, toward α-casein, β-casein, and κ-casein were measured by using substrate concentration values ranging between 0 and 6 mM and an enzyme concentration of 1.6 nM. The kinetic parameters were derived using nonlinear regression analysis of the Hyper32 program available at http://www.liv.ac.uk/~jse/software.html. The effect of $CaCl_2$ upon the enzyme activity was measured by adding the appropriate amount of salt to the standard assay mixture. All the experiments were repeated four times on independent enzyme preparations, and the standard deviation was calculated by the average of the four experiments.

Optimum pH and temperature

The optimum pH value was determined by monitoring the proteolytic activity of the enzyme against total casein at pH values ranging between 5.5 and 9.0. After incubation at 55 °C for 20 min, the reaction was stopped by adding 1 mL of 20% (wt/vol) TCA. The assay mixture (0.5 mL) contained 12 μg of the enzyme, 2% casein, 25 mM of the suitable buffer. The buffers used were MES (2-(N-morpholino) ethanesulfonic acid) pHs 5.5 and 6.0, bis–tris-propane pH 6.5, HEPES pHs 7.0 and 7.5, Tris pHs 8.0, 8.5, and 9.0. The optimum temperature of the enzyme was determined by carrying out standard assays at temperatures ranging from 5 to 55 °C at pH 6.5 and pH 7.5, respectively. All the experiments were repeated four times on independent enzyme preparations. Standard deviation was calculated by the average of four experiments.

Enzyme stability

The enzyme stability with respect to temperature and pH was determined by measuring the remaining caseinolytic activity after preincubation of the enzyme (10 μg) for 3 h either at different temperatures, ranging from 5 to 55 °C, or at 45 °C at different pH values included between 5.5 and 9.0. All the experiments were repeated four times on independent enzyme preparations. Standard deviation was calculated by the average of four experiments.

Electrophoretic analysis of the actinidin action upon casein and milk

Total casein, α-casein, β-casein, and κ-casein (10 mg) were incubated in 67 mM NaH_2PO_4 pH 7.2 with the enzyme (5 μg) for 20 min at 55 °C (final volume 0.3 mL). The hydrolysis of milk proteins was achieved by incubation of different commercial milk types (10 mg of proteins) with the enzyme (11.5 μg) for 30 min at 50 °C (final volume 0.3 mL) in 67 mM NaH_2PO_4 pH 7.2. The milk types used in the experiments were namely pasteurized whole milk and pasteurized semi-skimmed milk. After incubation, the samples were prepared for SDS–PAGE by adding an equal volume of double-concentrated loading buffer (4% SDS, 30% sucrose, 0.12% Tris pH 8, 0.042 mM DTT, 2% bromophenol blue) and for urea–PAGE by adding two volumes of loading buffer (8 M urea, 10 mM DTT, 2% bromophenol blue). Urea–PAGE was performed according

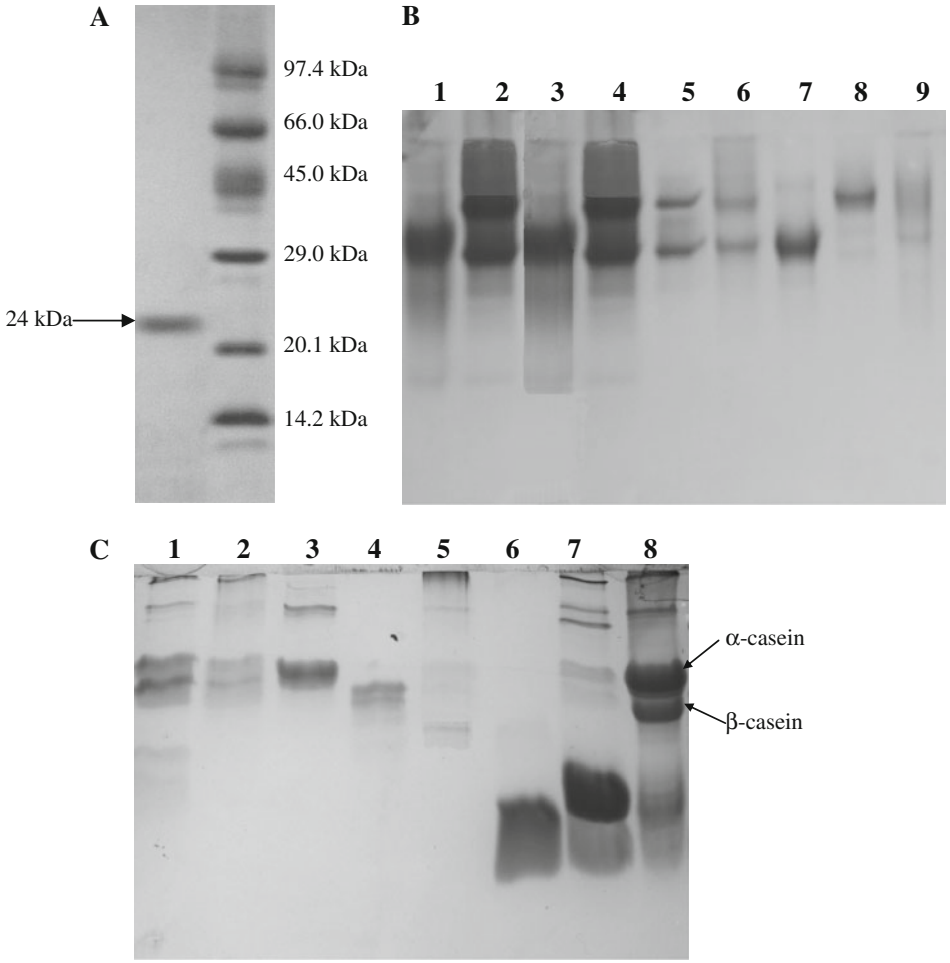

Fig. 1 **a** SDS–PAGE of the purified actinidin (24 μg); **b** urea–PAGE pattern of both the milk clot and the whey produced by actinidin in the absence (*lanes 1* and *2*) and in the presence (*lanes 3* and *4*) of 10 mM $CaCl_2$; *lanes 1* and *3* whey separated from clot; *lanes 2* and *4* milk clot; *lane 5* whole bovine milk; *lane 6* total casein; *lane 7* α-casein; *lane 8* β-casein; *lane 9* κ-casein. **c** SDS–PAGE pattern of the milk clot produced by actinidin treatment. *Lane 1* whole bovine milk; *lane 2* total casein; *lane 3* α-casein; *lane 4* β-casein; *lane 5* κ-casein; *lane 6* α-lactalbumin; *lane 7* whey separated from clot; *lane 8* milk clot

to Andrews [28]. SDS–PAGE (12.5% slab gels) was performed according to the method of Laemmli [29]. The gels were stained with Coomassie Blue R-250 and destained by repeated washing in a methanol/acetic acid/water (2:1:10) solution. Staining was for 8 h in 0.25% (w/v) Coomassie Blue G-250 in 50% methanol containing 7.5% acetic acid. This was followed by destaining in 15% methanol, 7.5% acetic acid.

Results and discussion

Purification of actinidin

A rapid method to isolate the fully active actinidin was performed based on a slight modification of the protocol described in McDowall [16] and Boland et al. [24]. The purified enzyme shows a specific activity against total casein of 129.6 U mg^{-1} with 8.7% recovery of the starting material with a purification fold of 2.4 (data cited but not shown). Although other purification strategies have been proposed by different laboratories [18, 22, 23], our procedure proved to be faster and leads to an enzyme preparation with a remarkably high specific activity. It also yields a single band upon SDS–PAGE showing an apparent molecular mass of about 24.0 kDa (Fig. 1a) in agreement with the primary structure of the protein [18].

Milk-clotting activity

The purified actinidin was tested for milk-clotting activity, and the results of this experiment are summarized in Table 1. Actinidin shows milk-clotting activity, which is correlated with enzyme concentration. The first appearance of solid material in the milk is observed just in 18–24 min using 0.49 nM (40 μg) of purified actinidin. As previously reported for calf rennet [30], the milk-clotting time measured in the presence of $CaCl_2$ is shorter (18 min) than in the absence of this salt (24 min; Table 1). The specific milk-clotting activity (C) seems to be slightly higher than the general protease activity (P) as the C/P value calculated for actinidin is 1.1 (data cited but not shown). This value, similar to that reported for calf rennet (1.0) and higher than that referred for microbial rennet (0.22) [31], is crucial in

Table 1 Milk-clotting activity of the purified actinidin performed both in the presence and in the absence of 10 mM $CaCl_2$ and expressed as clotting time (CT)

Enzyme concentration (nM)	Clotting time (CT) (min)	
	$-CaCl_2$	$+CaCl_2$
0.49	24	18
0.24	39	30
0.12	60	45
0.061	n.d.	67
0.024	n.d.	n.d.

Standard error of means ($N = 4$) is omitted as it is lower than 30 s

n.d. not detectable after 2 h of incubation

view of the likely application of actinidin in the cheese manufacture.

During the primary phase of the milk-clotting process, κ-casein is the unique casein fraction affected by chymosin. At the beginning of the enzyme reaction, a Phe–Met linkage (Phe^{105}–Met^{106}) is specifically split in cow as well as in sheep κ-casein [32]. Although actinidin cleaves bonds with basic amino acids in the P1 position, it does show milk-clotting activity, suggesting that others rather than Phe_{105}–Met_{106} bond of the κ-casein might be cleaved still inducing milk clotting. The comparison of actinidin specificity with other calf rennet substitutes revealed that most of them behave like chymosin cleaving the Phe_{105}–Met_{106} bond, although it has been reported that the *Endothia parasitica* protease hydrolyzes the preceding bond (Ser_{104}–Phe_{105}) without affecting the clotting [33]. More recently, lettucine, a serine protease purified from lettuce leaves [6, 7], and a protease from glutinous rice wine [34] have also been identified as milk-clotting enzymes hydrolyzing other peptide bonds rather than the calf rennet typical target site Phe_{105}–Met_{106}. The analysis of κ-casein primary structure suggests that either Arg_{97}–His_{98} or Lys_{111}–Lys_{112} bond might be a putative target site for actinidin action (data not shown, GenBank accession number AAQ87922.1).

In order to demonstrate that the clot observed by treating bovine milk with actinidin corresponds to casein coagulum, its composition was analyzed by urea–PAGE (Fig. 1b). The clot patterns (Fig. 1b, lanes 2 and 4) show that the totality of caseins occurs in the precipitate; however, the whey proteins cannot be clearly demarcated from the caseins (Fig. 1b, lanes 1 and 3). Therefore, the same samples were also analyzed by SDS–PAGE. As shown in Fig. 1c, the SDS–PAGE seems to be more suitable to separate the whey proteins from the casein fractions as well as from the proteolytic fragments obtained by the actinidin action, also allowing the determination of their molecular weights. In particular, the SDS–PAGE pattern of the whey proteins separated from the precipitate shows that almost the totality of caseins occurs in the coagulum (Fig. 1c, lane 7 and 8). The clot patterns obtained both in the presence (Fig. 1b, lane 2) and in the absence (Fig. 1b, lane 4) of 10 mM $CaCl_2$ show that there is no difference in the composition of the coagulums.

Characterization of actinidin

It is well known that coagulation of milk begins at a lower degree of hydrolysis of κ-casein if the temperature is progressively increased from 20 to 40–42 °C and pH is reduced. Normally, this last condition is achieved by the addition of different starter cultures to the milk, which produces changes in pH ranging between 5.2 and 6.6 during cheese manufacture [35]. The decrease in the net charge of casein molecules, which occurs in response to pH lowering, also induces their dissociation from micelles [36]. Moreover, also the addition of Ca^{2+} has become a common practice in the cheese-making procedure in order to accelerate the milk enzymatic gelation [37]. The observed acceleration of the clotting activity in Ca-supplemented milk is likely related to an increase in the rate of casein micelle aggregation mainly caused by the neutralization of the negative charges at the micellar surface [30]. Moreover, the addition of Ca^{2+} also lowers the pH of the milk, thus accelerating the clotting time [36]. Consequently, we analyzed the compatibility of actinidin with those thermochemical conditions usually applied during cheese manufacture. Firstly, the pH dependence of actinidin activity was studied against casein as substrate (Fig. 2a). The enzyme shows a maximum of activity at pH 6.5, which is retained for more than the 80% up to pH 8.0. As shown in Fig. 2a, the caseinolytic activity of actinidin is nearly stable after 3 h of preincubation in the pH range under investigation. The proteolytic activity of actinidin increases along with temperature and reaches a maximum at 55 °C (Fig. 2b). Moreover, the enzyme is totally stable at temperatures up to 45 °C. Only after 3 h of incubation at 55 °C, the enzyme activity abruptly decreases to 30% of the maximum value (Fig. 2b). The analysis of both pH and temperature dependence and stability of actinidin (Fig. 2a, b) suggests that the enzyme might be highly consistent with the physical–chemical conditions used during the industrial cheese-making process (40–42 °C, sub-acid pH values) as well as with chymosin action. Furthermore, the sharp decrease in the enzyme activity observed after 3 h of incubation at temperatures higher than 50 °C might turn out to be a positive feature. It ensures a lowering of the proteolysis rate during the ripening process, thus limiting the amount of short peptides that might be responsible for the development of bitterness. As shown in Fig. 2c, $CaCl_2$ does not affect the catalytic activity of the enzyme measured against κ-casein in the concentration range tested (0–10 mM); however, at 10 mM, a 40% reduction of the original activity was observed with

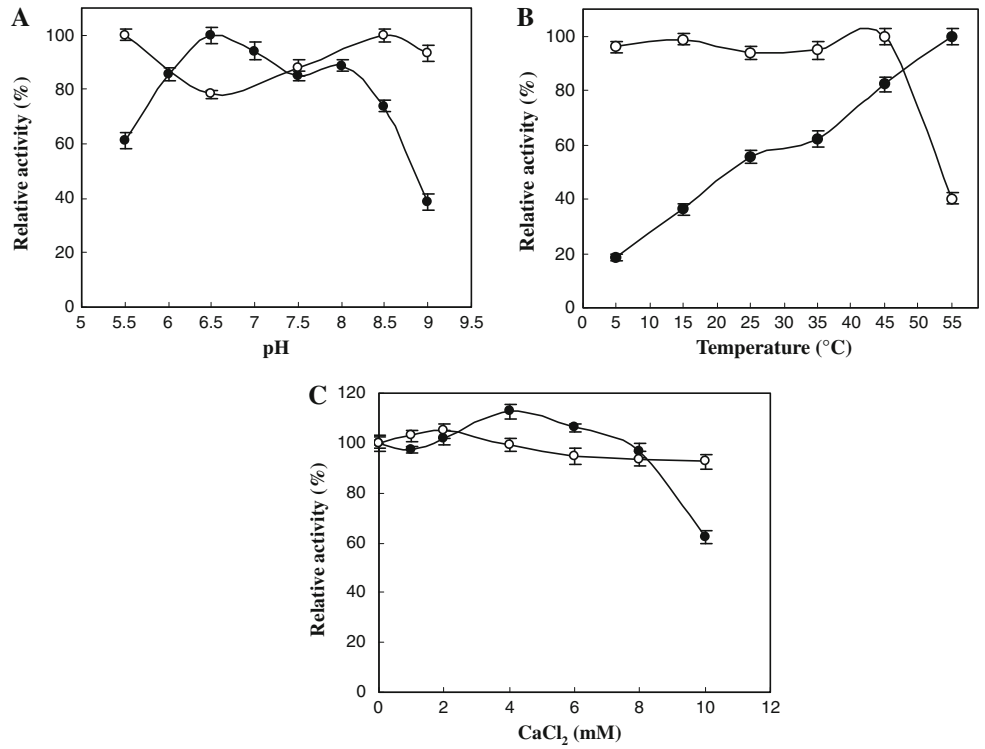

Fig. 2 **a** Effect of pH upon the activity and stability profiles of actinidin. Aliquots of the purified enzyme (12 μg) were used to measure the proteolytic activity toward casein (*filled circle*) at pH values ranging between 5.5 and 9.0. The pH stability of the actinidin (12 μg) was determined by monitoring the caseinolytic activity (*open circle*) after 3 h of incubation at different pH values ranging between 5.5 and 9.0 at 45 °C. **b** Effect of temperature upon activity and stability of actinidin. The caseinolytic activity (*filled circle*) of the enzyme (10 μg) was assayed at different temperatures ranging from 5 to 55 °C, whereas the thermal stability profile of actinidin was determined by assaying the remaining caseinolytic activity (*open circle*) after 3 h of preincubation at different temperatures ranging from 5 to 55 °C. **c** Effect of calcium chloride upon actinidin activity. The activity of the purified enzyme (10 μg) against total casein and κ-casein was assayed in standard buffer (pH 6.5) supplemented with increasing concentrations of $CaCl_2$ ranging between 0 and 10 mM. Casein (*filled circle*) and κ-casein (*open circle*) as substrate incubated with $CaCl_2$. The *error bars* represent the standard deviation ($N = 4$)

total casein as substrate. In this respect, it should be emphasized that calcium concentration during cheese manufacturing does not exceed 2 mM [1]. Furthermore, the inhibitory effect of 10 mM $CaCl_2$ upon the total casein hydrolysis does not negatively influence the initial stage of clotting, which begins with the cleavage of κ-casein in the region close to the Phe_{105}–Met_{106} bond.

The values of the kinetic constants for α-casein, β-casein, and κ-casein were also calculated (Table 2). The Km value for actinidin against α-casein and β-casein is similar, but the Kcat value is higher for β-casein, suggesting a higher catalytic efficiency (Kcat/Km value of 1.86×10^3 $s^{-1}\,M^{-1}$) of actinidin for β-casein; the Kcat/Km value for actinidin against κ-casein is considerably lower than that observed in the cases of α-casein and β-casein. These findings suggest that, since the number of basic residues in all three proteins are similar (data not shown), β-casein represents the best substrate for the enzyme probably because of a higher availability of the target residues in β-casein toward the active site of the enzyme. The analysis of kinetic parameters highlights the different specificity of actinidin toward the casein fractions in comparison with that of the proteases extracted from *Cynara cardunculus* showing higher specificity for κ-casein [33]. This difference might be crucial to the type of proteolysis produced.

Table 2 Kinetic parameters of purified actinidin using α-casein, β-casein, and κ-casein as substrates

Substrates	Km (M)	Kcat (s^{-1})	Kcat/Km ($s^{-1}\,M^{-1}$)
α-Casein	$0.11 \pm 0.01 \times 10^{-3}$	0.15 ± 0.01	$1.36 \pm 0.08 \times 10^3$
β-Casein	$0.15 \pm 0.01 \times 10^{-3}$	0.28 ± 0.02	$1.86 \pm 0.25 \times 10^3$
κ-Casein	$1.32 \pm 0.02 \times 10^{-3}$	0.12 ± 0.01	$0.09 \pm 0.008 \times 10^3$

The kinetic constants were determined using the substrates in the concentration range of 0–6 mM. The values are means ± standard deviations (SD) of data from four experiments ($N = 4$)

Hydrolysis of casein fractions and commercial milk by actinidin treatment

The SDS–PAGE pattern of the proteolytic fragments obtained by actinidin treatment against total casein, as well

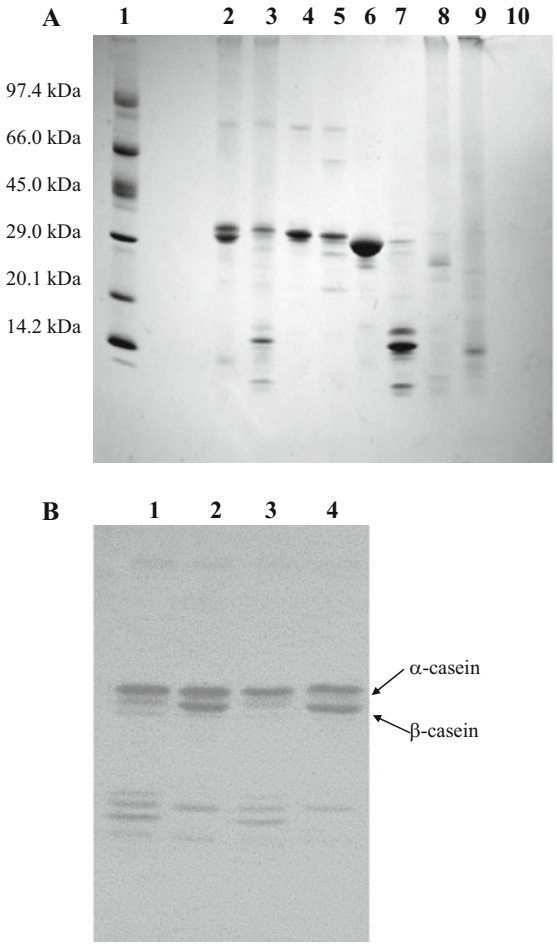

Fig. 3 SDS–PAGE patterns of the casein fractions (**a**) and of milk (**b**) subjected to actinidin treatment. **a** *Lane 1* molecular weight standard; *lane 2* control total casein; *lane 3* treated total casein; *lane 4* control α-casein; *lane 5* treated α-casein; *lane 6* control *β*-casein; *lane 7* treated *β*-casein; *lane 8* control κ-casein; *lane 9* treated κ-casein; *lane 10* actinidin (2 μg). **b** *Lane 1* treated semi-skimmed milk; *lane 2* control semi-skimmed; *lane 3* treated whole milk; *lane 4* control whole milk

as α, *β*, and κ fractions, is shown in Fig. 3a. The estimated molecular weights for α-, *β*-, and κ-caseins are 32.9, 30.7, and 25.7 kDa, respectively. The treatment of total casein with actinidin shows that the *β*-casein is widely digested, yielding three additional bands whose apparent molecular weights are 14.7, 13.7, and 9.6 kDa (Fig. 3a, lane 3). In fact, the same pattern is also observed when *β*-casein alone is the substrate for the enzyme (Fig. 3a, lane 7). This SDS–PAGE pattern is in agreement with the kinetic studies showing that actinidin preferentially degrades the *β*-casein (Table 2). The hydrolysis of α-casein is only partial and produces two fragments whose molecular weights are calculated at around 27.6 and 20.8 kDa (Fig. 3a, lane 5). The treatment of κ-casein with the enzyme leads to its complete hydrolysis, leaving a major fragment of 12.3 kDa (Fig. 3a, lane 9). Regardless, actinidin exhibits a lower specificity for κ-casein than that showed for the other casein fractions (Kcat/Km, Table 2), and the electrophoretogram of κ-casein treated with actinidin, supplied either as a single fraction or as a total casein, reveals its complete digestion mainly into the aforesaid 12.3-kDa fragment (Fig. 3a). These results suggest that κ-casein might be hydrolyzed by actinidin into a few large fragments, limiting the formation of oligopeptides but still inducing milk clotting (Table 1).

In order to understand whether the actinidin action toward total casein or the single casein fractions could also be observed in commercial milk, we measured the enzyme activity against both pasteurized whole milk and pasteurized semi-skimmed milk and analyzed the SDS–PAGE pattern obtained as a consequence of the hydrolysis. The whole milk is the preferred substrate, showing a value of specific activity equal to 318 U/mg, whereas the enzyme activity is lower with semi-skimmed milk (220 U/mg, data not shown).

The SDS–PAGE pattern obtained after actinidin treatment of milk containing different fat content (whole milk and semi-skimmed milk) is shown in Fig. 3b. It confirms that, regardless of the milk types used, the enzyme cleaves preferentially the *β*-casein and secondly the κ-casein fraction. In fact, the major digestion products detectable in all samples are four main bands, probably corresponding to *β*-casein hydrolysates (14.7, 13.7, and 9.6 kDa) and to the κ-casein degradation product (12.3 kDa; Fig. 3b, lanes 1 and 3). Therefore, these data suggest that minimal competition effects occur during the hydrolysis of caseins as identical degradation products have been obtained using either milk or single casein fractions as substrates.

Conclusion

In the present study, we evaluate the applicability of the purified actinidin as plant coagulant. The purification procedure is faster than others already available and allows the production of an enzyme preparation with high specific activity and with a C/P value similar to that reported for calf chymosin [31]. The data show that actinidin exhibits the ability to form milk clots in which the casein coagulum is separated away from the whey proteins. The results also show that the enzyme is fully compatible with the physical–chemical conditions utilized during cheese manufacture (40–42 °C, sub-acid pH values). The kinetic parameters in association with the electrophoretic analysis of the hydrolysis products show that *β*-casein represents the preferred substrate for the enzyme followed by κ-casein that seems to be hydrolyzed into few large fragments. Therefore, actinidin might be considered a promising alternative of natural calf rennet for the coagulation of milk leading to new dairy products.

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