.

Actinidin: A Promising Milk Coagulating Enzyme

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Research Article

ABSTRACT

The aim of this work was to study proteolytic activity of actinidin in comparison with chymosin and ficin on bovine milk substrate. The specific activities of purified ficin and actinidin were 7.9 and 8.3 unit/mg protein, respectively. The optimum clotting activity of both actinidin and ficin was at 45°C, although chymosin was relatively less sensitive to temperature. Increasing $CaCl_2$ concentration resulted in an enhancement of the clotting activities of all coagulating enzymes, this effect noticeable for ficin. In ficin treated sample significant decrease of bands intensity in the range 25-30 KD and appearance of some of κ-casein in 20 KD regions was observed by using SDS-PAGE. In conclusion, the chymosin and actinidin gave similar relative activity at different temperatures, pH values and $CaCl_2$ concentrations for bovine milk substrate. Comparable electrophoresis profile of actinidin, ficin and chymosin by analysis of the whey with SDS-PAGE indicates that actinidin could be a potential alternative for chymosin.

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1. INTRODUCTION

It is well known that the coagulation of milk is a combination of two phases: initial enzymatic hydrolysis reaction and a subsequent enzyme-independent protein aggregation reaction (Hooydonk and Walstra, 1987; Nájera et al., 2003). Although primary proteolysis is an essential phase in the development of proper cheese texture, secondary proteolysis is of great importance to assure a well-balanced breakdown of curd proteins in order to avoid formation of low viscosity and high bitterness that is related to cheese flavour (Silva and Malcata, 2000; Visser, 1993). The majority of cheeses produced around the world are manufactured using chymosin, a milk coagulant traditionally extracted from the fourth stomach (abomasums) of milk-fed calves, lambs, and kids (Rogelj et al., 2001). The decrease of calf abomasums has led to a search for other coagulating enzymes such as aspartic acid proteinases of microbial and cysteine proteinases from plants (Repelius, 1998; Rogelj et al., 2001). Plant coagulants share many biochemical features with chymosin. Both chymosin (EC 3.4.23.4) and plant coagulants cleave the Phel05-Metl06 peptide bond of κ-casein, but plant coagulants are more proteolytic and have broader specificity on αs_I- and β-caseins than chymosin (Esteves et al., 2003; Macedo, 1993).

Generally, excessive proteolysis by plant proteinases such as ficin (EC 3.4.22.3) can lead to a decrease in cheese yield due to excessive non-specific proteolysis in the cheese vat and loss of peptides in the whey, resulting in defects in the flavour and texture of ripened cheese (Fox and McSweeney, 1998; Low et al., 2006). Actinidin (EC 3.4.22.14) is a plant thiol proteinase which is present in the fruits of the Chinese gooseberry or kiwi fruit (*Actinidia Chinensis*). Actinidin and ficin have been demonstrated to have a striking homology in the primary structures but, important differences in proteolytic properties of actinidin and ficin do exist (Carne and Moore, 1978). Thus, for the first time we decided to examine proteolytic property of actinidin in compared with chymosin and ficin to introduce actinidin as a new alternative milk coagulating enzyme.

2. MATERIAL AND METHODS

2.1 FICIN PURIFICATION AND ASSAY

Ficin was purified as described by Kramer and Whitaker (1964) and Devaraj et al. (2008) with some modifications. In short, figs latex was obtained from the ends of the broken stems of green fruit of *Ficus carica* and clarified by centrifugation at 10,000 × g for 60 min. The clarified latex was saturated to 80% ammonium sulfate at 4°C to precipitate proteins. Chromatography was performed on 20 × 5.0 cm column of CM-Cellulose run at 4°C, in 0.05 M sodium phosphate buffer pH 7.0 and flow rate 0.5ml/min. Elution of the components from the column was made by a linear gradient of 0.0-1.0 M NaCl in the same buffer. Purified enzyme confirmed by SDS-PAGE in the presence of 5-20% gradient gel using Fermentas marker (Fermentas, Burlington, Ontario, Canada). Activity of enzyme was determined by Kunitz method (1947). A unit of activity is defined as amount of enzyme that increases one unit absorbance at 280 nm in 1 minute at 35°C and pH 7.0.

2.2 ACTINIDIN PURIFICATION AND ASSAY

Howard variety of kiwi fruit (*Actinida Chinesis)* was used. Approximately one kg un-peeled kiwi fruit was homogenized and the homogenate was passed through cheese cloth to remove insoluble materials. The filtrate was brought to 0.05 M sodium citrate buffer, pH 5.5.

The proteins of the mixture were precipitated by adding ammonium sulfate to 65% saturation followed by centrifugation at 15000 × g at 4°C for 10 min. The precipitate was dissolved in 20 mL 0.05 M sodium citrate buffer , pH 5.0 and dialyzed against same buffer at 4°C overnight (McDowall, 1970). Enzyme was partially purified by the method of Arcus (1959) with some modifications (Aminlari et al., 2009). In brief, anion-exchange chromatography was performed on a 22 × 4.5 cm column of DEAE-Sephadex A-25 at 4°C, in 0.05 M sodium citrate buffer pH 5.5 and at a flow rate of 1.0 ml/min. The column was washed with above buffer and enzyme eluted using a linear gradient of 0.0-1.0 M NaCl in the same buffer. Purified enzyme confirmed by SDS-PAGE in the presence of 5-20% gradient gel using Fermentas marker (Fermentas, Burlington, Ontario, Canada). Activity of enzyme was determined by digestion of 1% hemoglobin substrate in 50 mM formate buffer, pH 3.5 at 35°C for 20 minute (Matulis et al., 1999). Unit activity was defined as amount of enzyme that increases one unit absorbance at 280 nm in 1 min at 35°C and pH 3.5.

2.3 CHYMOSIN

The chymosin used in this study was a commercial powder; CHY-MAX[®] (Naturen, CHR Hansen, Hersholm, Denmark) consisted of 80% (w/w) bovine chymosin and 20% (w/w) pepsin. The rennet strength was 1400 I M C U/g of coagulant.

2.4 CHEESE MAKING

The pasteurized milk containing 2.5% fat and 3.2% protein used in all experiments was obtained from a local dairy firm. Coagulating enzymes were added at similar levels of unit activities and then mixtures were heat-treated at different temperatures (30 to 55°C), in the presence of 0.0 to 25.0 mM $CaCl_2$ and pH values (5 to 7.5). The pH values of mixtures were measured with a Mettler Toledo MP 120 pH-meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland), and adjusted with 0.05 M sodium citrate buffer for pH 5, 5.5 and 6.0 and with 0.05 M sodium phosphate buffer for pH 6.5, 7.0 and 7.5. Coagulating features of actinidin, ficin and chymosin at specific times were determined by visual observation of the proper firmness. Activities of coagulating enzymes were detected by visual observation of the curd at 30 minute intervals, and samples were prepared in triplicate. The mean of time required to observe the proper firmness was defined as relative activity and chymosin relative activity selected as a standard (Low et al., 2006).

2.5 ELECTROPHORESIS PROFILING OF WHEY

Whey was analyzed by SDS-PAGE in the presence of β-mercaptoethanol using 5.0 to 20% polyacrylamide gradient gels (Laemmli, 1970).The running gel was a 5.0 to 20 gr/dl gradient polyacrylamide gel in 1.2 M Tris-HCl buffer (pH 6.8) and 3 gr/l SDS. The stacking gel contained 3 gr/l acrylamide in 0.25 M Tris-HCl (pH 6.8) and 2 gr/l SDS. Whey of coagulants was added to the loading buffer to give a final concentration of 1 mg/ml protein, 0.01 M Tris-HCl, (pH 6.8), 0.4 % SDS, 100 gr/l glycerol and 0.04 gr/l bromophenol blue. The electrode buffer comprised 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% SDS, at pH 8.16. Electrophoresis was performed at constant 25 mA, and the gel was stained by Coomassie Brilliant Blue R250.

2.6 STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS for windows statistical package version 11.5. The relative activity (Mean of coagulation times) of chymosin used as standard coagulant value and coagulation times of actinidin and ficin were analyzed using One-Sample T-test for comparison of the mean values (P < 0.05).

3. RESULTS

3.1 SPECIFIC ACTIVITY OF PURIFIED ENZYMES

The specific activities of purified ficin and actinidin were 7.9 and 8.3 unit/mg protein respectively.

3.2 EFFECT OF pH, TEMPERATURE, AND $CaCl_2$ CONCENTRATION ON COAGULATION

The relative activities for chymosin, actinidin and ficin at different pH, temperatures and $CaCl_2$ concentrations are presented in Tables 1 to 3. Actinidin, chymosin, and ficin showed similar responses to pH in the pH range 5.0 to 6.5, with a sharp decrease of the relative activity at pH 7 to 7.5 for all enzymes.

Table 1. Effect of pH on coagulation times (Minute*) of cheeses that made with chymosin, actinidin and ficin

Enzyme	pH					
	5.0	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**
Chymosin	50[a]	53[a]	60[a]	96[a]	99 [a]	102[a]
Actinidin	51[a]	54[a]	63[a]	99[a]	108[b]	114[b]
Ficin	54[a]	57[a]	59[a]	102[a]	114[b]	126[b]

[a,b] *Means in the same column with different superscripts differ statistically (P<0.05).*
Values represent (Mean) at constant temperature 50 ºC and 15 mM $CaCl_2$ concentration.

Table 2 shows the effect of temperature on the relative activities of coagulating enzymes. The optimum clotting activity of both actinidin and ficin was at 45°C while, chymosin was relatively less sensitive to temperature (p<0.05).

Table 2. Effect of temperature on coagulation times (Minute*) of cheeses that made with chymosin, actinidin and ficin

Enzyme	Temperature(°C)					
	30	**35**	**40**	**45**	**50**	**55**
Chymosin	78 [a]	90 [a]	96 [a]	90[a]	66 [a]	60 [a]
Actinidin	71[a]	84 [a]	93 [a]	80[b]	63 [a]	59 [a]
Ficin	48[b]	66 [a]	84 [a]	75[b]	59 [a]	54 [a]

[a,b] *Means in the same column with different superscripts differ statistically (P<0.05).*
Values represent (Mean) at constant pH 6.5 and 15 mM $CaCl_2$ concentration

In Table 3 increasing CaCl$_2$ concentration resulted in an enhancement of the relative activities of all coagulating enzymes, this effect was more noticeable for ficin. Actinidin and chymosin in 0.0 and 5.0 mM CaCl$_2$ have been shown same relative activity (p<0.05).

Table 3. Effect of CaCl$_2$ concentration on coagulation times (Minute*) of cheeses that made with chymosin, actinidin and ficin

Enzyme	CaCl$_2$ (mM)					
	0.0	**5.0**	**10**	**15**	**20**	**25**
Chymosin	106[a]	96[a]	83[a]	74[a]	66[a]	60[a]
Actinidin	111[a]	104[a]	89[a]	83[a]	72[a]	62[a]
Ficin	144[b]	120[b]	93[a]	90[a]	81[a]	63[a]

[a,b] *Means in the same column with different superscripts differ statistically (P<0.05).*
* Values represent (Mean) at constant temperature 50 ºC and pH 6.5.

3.3 ELECTROPHORESIS PROFILING OF WHEY

Fig. 1 depicts typical SDS-PAGE results of whey proteins products by proteolytic activities of coagulating enzymes on bovine milk; the gel was loaded with 15 μg of each whey proteins. Extensive differences in the protein bands pattern is observed among samples treated with different coagulating enzymes. In ficin treated sample (Lane 2) significant decrease of bands intensity in the range 25-30 KD and appearance some of κ-casein in 20 KD region is observed and simultaneously several thin bands (15-5 KD) with different intensity exist in lanes 1, 2 and 3.
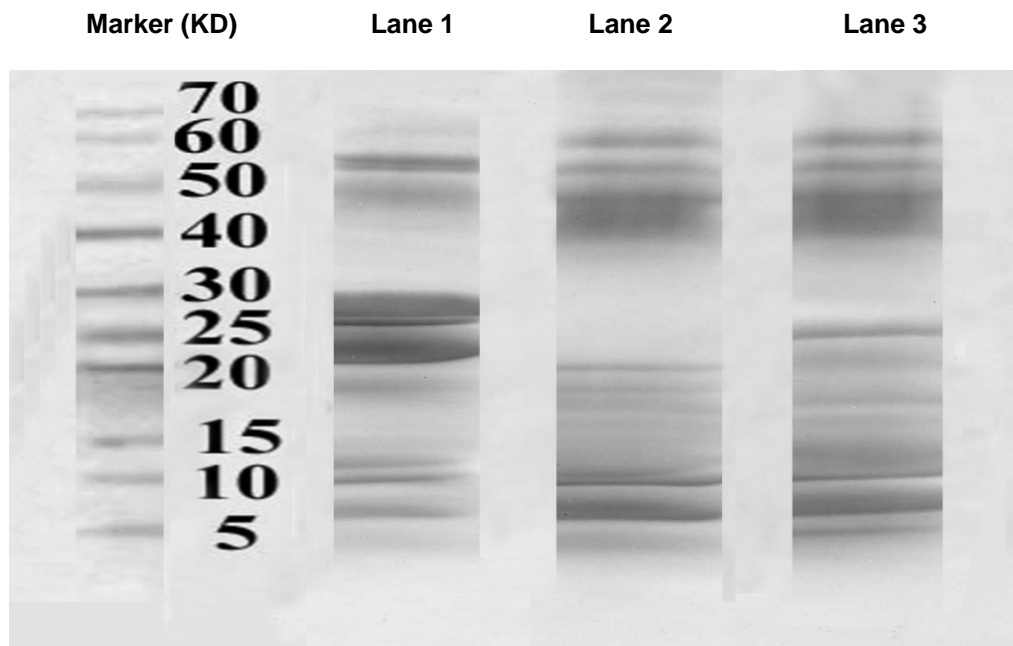

Fig.1. SDS-PAGE analysis of the whey proteins from coagulating enzymes, chymosin (lane 1), ficin (lane 2) and actinidin (lane 3).

Chymosin with β- and αs-caseins (25-30 KD region), ficin with the highest electrophoresis mobility is accounted for proteolysis of β- and αs-caseins and includes many different intensive thin bands (5-15 KD region) that related to small peptides in the whey. These peptides have less intensity in the whey from actinidin sample (lane 3).

4. DISCUSSION

In our recent study, the beneficial properties of purified actinidin successfully were employed to solubilize beef proteins, increase water capacity and attribute quality of sausage (Aminlari et al., 2009). In the present study, actinidin and ficin were purified and tested for their potential use in cheese production in comparison with chymosin. Actinidin, ficin and chymosin, showed similar responses to pH value in the pH range 5.0 to 7.5, with a sharp increase of the coagulation time at the pH value 7.0 to 7.5. The most important effects of lowering the pH of the milk are the solubilization of micellar calcium phosphate (Nájera et al., 2003; Visser, 1993). It has also been reported that lowering the pH causes an increase in the curd firming rate (Daviau et al., 2000). Higher activity of chymosin at lower pH values (from pH 5.0 to 6.0) in comparison with ficin and actinidin may be due to the activity of pepsin which has a lower pH optimum for general proteolysis, and existed in CHY-MAX$^®$ powder (Fox and McSweeney, 1998). However, the coagulation of rennetted milk is not very efficient at pH lower than 5.0 (Kowalchyk and Olson, 1977).

It is well known that the velocity of coagulum formation increases progressively from 20 to 40-45°C, but, at higher temperatures, the coagulation process slows down (Dybowska and Fujio, 1996; Nájera et al., 2003). This study indicated that the temperature of the milk affects protein aggregation rate to a large extent and that at higher temperatures decrease in coagulation time is observed. The optimum clotting activity was at 50°C for these coagulating enzymes. The less sensitivity of ficin and actinidin to higher temperature can not be explained at present, but could be an advantage in the production of hard type cheeses, where relatively high incubation temperatures are used at the time of the curd stirring before molding (Rogelj et al., 2001).

This study showed that the effect of concentration of $CaCl_2$ was significant for three enzymes used. It is known that addition of Ca^{2+} decreases the rennet clotting time (Montilla et al., 1995). However, at high concentrations of $CaCl_2$ (>25 mM), the clotting time may be increased (Patel and Reuter, 1986). At low concentration of $CaCl_2$ difference between ficin and other enzymes is noticeable. The influence of increasing the concentration of $CaCl_2$ reduces the pH, resulting in an increased proteolytic activity of ficin which is observed in fig.1 (Nájera et al., 2003). These results confirmed that the effect of Ca^{2+} on coagulation time was strongly dependent on the pH and agree with results found by other authors (Daviau et al., 2000).

The analysis of small peptides in the whey provided a direct estimation of the extent of general protein hydrolysis. This information is important as it provides an insight into the quality of cheese that could be made with this process (Maubois and Mocquot, 1975). A high degree of protein hydrolysis could cause the cheese to be too soft with a high concentration of peptides, including some bitter peptides; whereas a low degree of protein hydrolysis may result in a cheese with a hard texture and a lack of flavour development (Low et al., 2006; Shammet et al., 1992). Several analytical techniques have been extensively employed in the characterization of protein hydrolysates. Electrophoresis is one of the most accurate methods to monitor primary proteolysis (Silvestre, 1997).

Chymosin and plant coagulants have the ability to cleave some sites on αs_I- and β- caseins (Esteves et al., 2003; Silva et al., 2002). However, it has been shown that chymosin and plant coagulants had some differences in specificity and/or velocity of hydrolysis of casein bonds (Macedo, 1993). At the same experimental conditions at 30°C, plant coagulants and chymosin had similar specificity towards β-casein, but plant coagulants hydrolyzed peptide bonds on β-casein faster than chymosin; on αs_1-casein, plant coagulants attacked the C-terminal region but chymosin did not (Esteves et al., 2003; Macedo, 1993).

The electrophoretic profile of whey produced with chymosin, actinidin and ficin at pH 6.5, 50°C, and 15 mM $CaCl_2$, formed similar pattern with chymosin and actinidin and extensive proteolysis of β and αs caseins for ficin (Fig.1). The products of degradation of αs_1-casein, by both actinidin and ficin, yielded many thin bands placed after the 20 KD regions. Hence, plant coagulants may be useful in the production of cheeses where gelation proceeds at low temperature, while at higher temperatures, excessive proteolysis may occur impacting negatively on the texture and flavour of cheeses produced with plant coagulants (Esteves et al., 2003; Silva and Malcata, 2000).

5. CONCLUSION

Overall, chymosin and actinidin gave similar relative activity at different temperatures, pH values and $CaCl_2$ concentrations for bovine milk substrate in comparable SDS-PAGE profile of whey. Comparison of actinidin and chymosin indicated that the former could be a potential alternative to the latter however, its application as milk coagulant warrants further study.

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