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Hydrolysis of Ovine and Caprine Caseins by Enzymatic Extract from *Solanum dubium* Seeds

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Abstract: The main aim of the present study was to evaluate the hydrolytic action of *Solanum dubium* serine protease on both ovine and caprine caseins, in order to address an increasing worldwide demand for alternative dairy products with improve organoleptic nutritional and health properties. Two milk types; ovine (sheep milk) and caprine (goat milk) were used in this study to investigate the hydrolysis pattern of their caseins by a purified serine protease from *Solanum dubium* seeds. The proteolytic activity of the enzyme towards caseins prepared from the two milk types via isoelectric precipitation was investigated using sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE. The electrophoretograms showed that they were both sensitive to the action of the enzyme. Ovine caseins were hydrolyzed completely in 6 h, whereas caprine caseins showed slight hydrolysis in 24 h. The enzyme showed high degree of hydrolysis on ovine casein, which it may lead to the production of soft cheese from ovine milk. On the other hand, the enzyme showed low degree of hydrolysis on caprine casein, and hence it could be used for the production of hard cheese from caprine milk.

Key words: Ovine casein, Caprine casein, *Solanum dubium*, Dubiumin, Casein hydrolysis

INTRODUCTION

Cheese is a dairy product that has played a key role in human nutrition for centuries. The main objective has always been and still is to convert milk, which is perishable, into a product with a longer shelf life whilst preserving most of its nutrients. Coagulation of milk is the basic step in the manufacture of cheese. Calf rennet, which contains chymosin (EC 3.4.23.4) as the main enzyme component, has been widely used as a milk-clotting enzyme. Increasing world cheese production and consumption besides the increase of calf rennet's price, along with a reduced supply of calf rennet, has led to a systematic investigation for new rennet substitutes. Much research interest has been directed towards discovering a milk-clotting enzyme which would satisfactory replace calf rennet in cheese manufacture, and numerous enzyme preparations of animal, microbial, and plant origin have been studied. Microbial rennets have proven suitable substitutes for animal rennet, but increasing attention has been directed toward natural rennet extracted from plants such as *Ananas comosus*, *Carica papaya*, *Calotropis porcera*, *Ficus carica*, *Calm viscera*, *Cynara cardunculus*, and *Cynara scolymus*, among others (reviewed in Roseiro *et al*., 2003). Unfortunately, most of the plant rennets were found unsuitable because they produced extremely bitter cheese. An exception to this general rule is represented by the aqueous extract of *Cynara cardunculus* flowers containing two aspartic acids-type proteases, namely cardosin A and B, which has been used for the manufacture of sheep milk cheese in several areas of Portugal and Spain. Some plants of the family Solanaceae such as *Solanum torvum*, and *Solanum dubium* have been tried for the extraction of milk clotting enzymes. The research had done showed positive results using *Solanum torvum* and *Solanum dubium* for the manufacture of white cheese (Habbani, 1992).

Dairy farmers in some parts of the Sudan use the berries of *Solanum dubium* to make white soft cheese using goat and sheep milk (Yousif et al., 1996). We previously reported the isolation and partial characterization of a chymotrypsin-like serine protease (named as dubiumin) from the seeds of *Solanum dubium* Fresen (Mohamed Ahmed *et al*., 2009). Compared to other purification procedures carried out previously, we succeeded in developing a simple and economic purification procedure in our study. Moreover, the high

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stability of dubiumin against autodigestion and under various conditions, in accordance with the availability of raw materials, in addition to its high milk-clotting ability, could therefore pave the way for its uses in the cheese industry as well as other food and biotechnological industries.

Furthermore, analysis of the degradation pattern of bovine casein by serine protease from *Solanum dubium* was studied recently and was even considered as a new source of plant rennet, with distinctive, useful characteristics for the dairy industry (Mohamed Ahmed *et al*., 2010). *Solanum dubium* serine protease hydrolyzes bovine casein fractions in separate forms or in whole casein form very efficiently at different conditions. Even though the independent action of *Solanum dubium* serine protease on bovine caseins has been reported, no data pertaining to the independent action of this serine protease on both caprine and ovine caseins have, to our knowledge, been made available to date. Since proteolysis is the most important biochemical event during cheese ripening, in which the enzymes contributed by the rennet play a relevant role, it is of utmost importance to evaluate the degradation patterns of caseins in model systems that mimic actual cheese making because of their effects on yield, texture and flavor of the final cheese. Therefore, the main aim of this work was to complement that knowledge, via evaluating the action of the *Solanum dubium* serine protease upon the hydrolysis of caprine and ovine caseins, in order to address an increasing worldwide demand for alternative dairy products with improve organoleptic nutritional and health properties. Study of casein breakdown in model systems will likely generate important information to help elucidate the complex processes involved in ripening of cheeses from small ruminants, and eventually contribute toward development of better, non conventional final cheeses.

MATERIALS AND METHODS

Materials:

The *Solanum dubium* serine protease that previously purified was used in this study (Department of Applied Resources Chemistry, Laboratory of Microbial biotechnology, Tottori University, Tottori, Japan). The cow, sheep and goat milk was obtained from University of Khartoum farm. Unless otherwise stated all chemicals used in this study are of reagent grade.

2.2. Methods:

2.2.1. Sodium caseinate preparation:

Whole ovine and caprine caseins were obtained from raw milk, via isoelectric precipitation following acidification to pH 4.25 with 6 M HCl according to the method of Sousa and Malcata (1998) with slight modifications. The mixture of caseins and whey was warmed to 37 °C, and held at that temperature for 30 min. The caseins were recovered by filtration through a clean cloth, and washed several times with deionized water. The caseins were then resuspended in deionized water (to the initial volume), and pH was adjusted to 6.5 with 1 mM NaOH. The suspension was allowed to equilibrate at 4 °C for at least 2 h, freeze-dried and stored until use.

2.2.2. Hydrolysis of caseins:

Whole caseinates were dissolved to a final concentration of 1% (w/v) in 100 mM phosphate buffer (pH 6.5), containing 0.1% (w/v) NaN_3 to prevent protein degradation by adventitious microflora, and allowed to stabilize at 30 °C. The purified enzyme (200mg/ml) was then added at a ratio of 0.5 ml to 10 ml of caseinate (v/v). Aliquots were taken at 5 min, 30 min, 1 h, 3 h, 6 h, 12 h and 24 h. The reaction was quenched by putting the samples in boiling water for 5 min .Controls containing Na-caseinate and Na-azide, at the same concentrations but without addition of enzyme, was also sampled.

2.2.3. SDS-polyacrylamide gel electrophoresis:

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was done using the method of Laemmli (1970), with 15% acrylamide separating gel and 3% acrylamide stacking gel containing 0.1% SDS. Samples were prepared in a tris–glycine loading buffer at pH 8.8 containing 1% SDS and were heated at 100°C for 5 min to inactivate the enzyme. Electrophoresis was done at a current of 20 mA for 2 h in electrophoretic tris–glycine running buffer containing 0.1% SDS. After electrophoresis, the gel sheets were stained for proteins with 0.2% coomassie brilliant blue-R250. Protein stain was destained with 10% acetic acid containing 20% methanol.

RESULTS AND DISCUSSION

Mode of Action of the Purified Enzyme on Ovine Whole Casein:

An attempt was made to study the hydrolysis of ovine casein in order to provide basic information on the possibility of using *Solanum dubium* serine protease as milk coagulant for cheese from small ruminant. The result in Figure 1 showed that ovine casein was hydrolyzed by the purified *Solanum dubium* serine protease. As could be seen the hydrolysis start very early as 1 min and after 1 h of digestion the digested product became more intense as reaction time went on (lanes3-6). A pair of bands of higher electrophoretic mobility denoted as bands (A) and (B), in lanes 2–7 was also produced; band (A) was first visible by 30 min, became much more intense until 3 h, became faint and disappeared completely by 24 h of reaction. On the other hand, band (B) looked very intense by the first hour of hydrolysis, but vanished earlier.

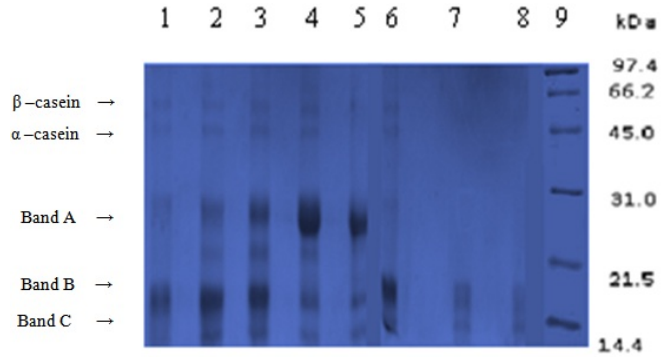

Fig. 1: SDS-PAGE of ovine casein hydrolyzed by *Solanum dubium* serine protease. Lane 9 was SDS low molecular weight standards, lane 1was unhydolyzed whole ovine casein, Lane 2-8, were whole ovine casein hydrolyzed by the enzyme for 5, 30 min, 1, 3, 6, 12, and 24 h, respectively.

Although breakdown of ovine caseinate took place much slower than that of bovine counterpart (Vairo Cavalli *et al*., 2005), a region with bands of greater mobility than α-casein is visible as early as by 30 min (Bands A, B), they became thicker as the hydrolysis time increased and vanished at 24 h. On the other hand (Band-C) also became thicker with time but started to vanish at 12 h hydrolysis and remained till the end of the hydrolysis time. A noticeable decrease of counterpart α-casein from 0 to 24 h of degradation can also be observed. Electrophoresis analysis of water insoluble fractions from La Serena cheese – semi-hard Spanish ewe's milk cheese manufactured with extracts of *C. cardunculus*, showed that α-casein was less susceptible to proteolysis than β-casein (Roa *et al.*, 1999). After ripening Serra da Estrela cheeses produced in Portugal from ovine milk curdled with extracts of flowers of *C. cardunculus*; were characterized by extensive hydrolysis of β- and α-caseins (Macedo & Malcata, 1997). Silva and Malcata (1999) reported that cardosin B degraded both ovine caseins, but not to the same extent; however, when whole ovine caseinate was degraded by cardosin B, α-casein was more susceptible to proteolysis than β-casein, whereas the opposite behavior was observed when the isolated fractions were exposed to hydrolysis. In cheese-like systems, none of the highly susceptible peptide bonds of α-casein were found to be cleaved during the initial 24 h of ripening neither by crude aqueous extracts of *C. cardunculus* nor by purified cardosin A (Silva & Malcata, 2005). Irigoyen *et al.* (2000) found that α-caseins are more acutely hydrolyzed than β-caseins throughout ripening of ovine cheeses that had been curdled with lamb artisan rennet, calf industrial rennet or a mixture of both. The clotting activity of the rennet used is one of the factors that has a major influence upon the degradation extent of caseins; similarly, the origin of the clotting enzyme used (from animal, microbial or plant sources) will constrain the proteolysis degree; plant and microbial clotting enzymes breakdown β-caseins faster than animal ones (Irigoyen *et al*., 2000). The previous results give advantage to the *Solanum dubium* serine protease to be used for the production of soft cheese from ovine milk because it hydrolyzed both α and β caseins of ovine whole casein. Such enzyme preparation could be used for the production of home made cheese particularly in rural areas where people only depending on animal milk as main source of food and they face problem of preserving the fresh milk.

Mode of Action of the Purified Enzyme on Caprine Whole Casein:

Since caprine milk and their products were hugely consumed in rural areas in Sudan, we attempt to

characterize the effect of *Solanum dubium* serine protease on caprine milk caseins. A typical SDS-PAGE electrophoretogram of caprine casein and its fractional degradation by *Solanum dubium* serine protease is depicted in Figure 2. The group of bands with the lowest electrophoretic mobility is accounted for by α-caseins, whereas those of highest mobility were for β-casein. The band of κ-casein was hydrolyzed rapidly at 30 min, whereas complete hydrolysis of ovine α- and β-caseins take place after 3 hours. About four bands (A1, B1, C1, and D1) were appeared ahead of κ-casein on SDS-PAGE electrophoretogram of caprine whole casein hydrolyzed by *Solanum dubium* serine protease. Bands A1 and B1 were remarked as early as 30 min and remain until 12 h and 24 h for bands B1 and A1, respectively. Band A1 was more intense than B1 and remained until the end of the hydrolysis, where as band B1 became less intense when the reaction time elevated. Band C1 appeared as early as 30 min and remained until the end of the hydrolysis. On the other hand, band D1 appeared latter after 3h hydrolysis and remained until the end of the hydrolysis. The degradation of caprine caseins is much differ than that of ovine and bovine caseins, both α and β caseins showed progressive degradation and high molecular weight peptides are produced and remained until the end of the hydrolysis. These results are in contrast with those obtained by (Sousa and Malcata, 1998) which indicates that caprine caseinate underwent more extensive degradation than ovine caseinate under the same conditions (pH 6.5 and pH 5.5). Under all conditions tested, β-casein in ovine and caprine Na-caseinates was hydrolyzed by cardosins to yield a pair of bands of higher electrophoretic mobility, comparable to that of bovine β-casein. This is in agreement with previous reports pertaining to ovine casein hydrolyzed by calf chymosin (Silva and Malcata, 2000). The main advantage for the *Solanum dubium* serine protease is to be used for the production of hard cheese from caprine milk because it hydrolyzed both α and β caseins of caprine whole casein and produced high molecular weight peptides. Such enzyme preparation could be used for the production of home made cheese particularly in rural areas where people only depending on animal milk as main source of food and they face problem of preserving the fresh milk.

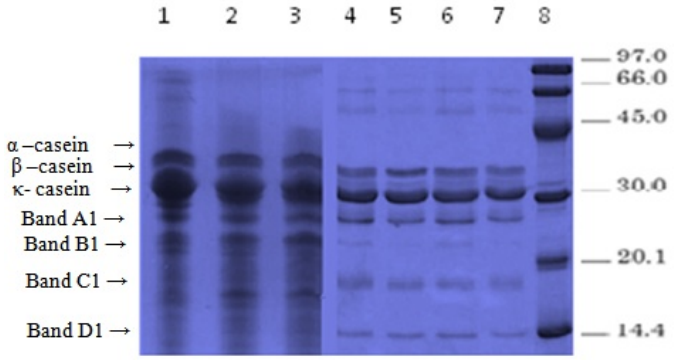

Fig. 2: SDS-PAGE of caprine casein hydrolyzed by *Solanum dubium* serine protease. Lane 8 was SDS low molecular weight standards, lane1 was unhydolyzed whole caprine casein, Lane 2-7, were whole caprine casein hydrolyzed by the enzyme for 30 min, 1, 3, 6, 12, and 24 h, respectively.

Comparison of the Degradation Product of Caprine and Ovine Caseins with Those of Bovine Casein:

Bovine whole casein was hydrolyzed by *Solanum dubium* serine protease into about five bands (A2, B2, C2, D2, and E2) (Fig. 3). The first band (A2) appeared after 5 min hydrolysis and thereafter disappeared after 6 h hydrolysis time. The next four bands (B2, C2 D2 and F2) appeared as early as 5 min hydrolysis time and their intensity increased with increasing incubation time. By the end of the incubation time one of these bands (C2) remain no more hydrolysis up to 24 h, this band had a molecular mass of 16 kDa and assumed to be para-k-casein band. The control sample lane 2 had no enzyme, showed no degradation after 24 h incubation, indicating the absence of indigenous proteinases activity in the casein preparation used. Bovine κ-casein was hydrolyzed rapidly by the enzyme to produce one major band in 5 min hydrolysis (Mohamed Ahmed et al., 2010). At this time, small amount of κ-casein still remained unhydrolyzed. *Solanum dubium* serine protease hydrolyzed bovine whole casein generated peptides, with amount progressively increasing in time. β-casein was completely degraded during 3h incubation, while complete degradation of α-casein occurred after 6 h indicating its slow attack by the enzyme than β-casein (Mohamed Ahmed et al., 2010). It is clear that the three main casein components α-, β-, and κ-caseins, were sensitive to the action of *Solanum dubium* serine protease. Similarly, Egito *et al*., (2007) found that the action of albizia seeds protein extract on α-, β-, and κ-caseins, were stronger than the action of sunflower seeds protein extract and of chymosin and it was also found that

Solanum dubium serine protease was able to hydrolyze β-casein completely by 1h in the separate form, while β-casein in the bovine whole casein completely degraded by 3 h. These results are similar to those observed in the hydrolysis of ovine β-casein by cardosin B (Silva and Malcata 1999).

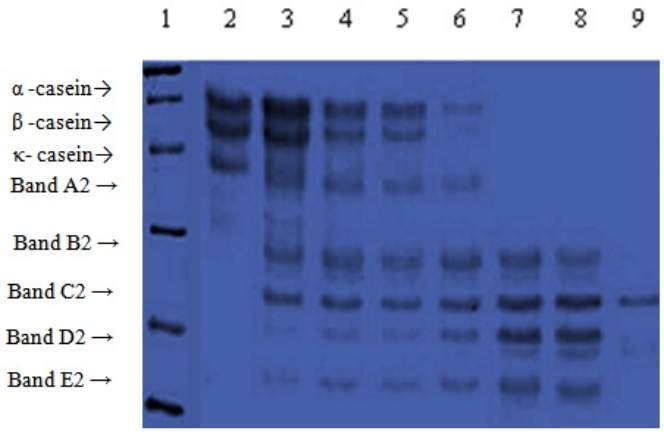

Fig. 3: SDS-PAGE of bovine casein hydrolyzed by *Solanum dubium* serine protease. Lane 1was SDS low molecular weight standards, lane 2 was unhydolyzed whole bovine casein, Lane 3-9, were whole bovine casein hydrolyzed by the enzyme for 5, 30 min, 1, 3, 6, 12, and 24 h, respectively.

Conclusion:

Solanum dubium serine protease hydrolyzes bovine, ovine and caprine caseins and their components each in different way. The enzyme shows several characteristics similar to chymosin, commercial rennet and other plant proteases with some differences in its hydrolysis on casein pattern. These differences may result in different cheese types from various milk types. The main advantage for the *Solanum dubium* serine protease is to be used for the production of hard cheese from caprine milk because it hydrolyzed both α and β caseins of caprine whole casein and produced high molecular weight peptides. Such enzyme preparation could be used for the production of home made cheese particularly in rural areas where people only depending on animal milk as main source of food and they face problem of preserving the fresh milk. According to the data obtained coming researches should be conducted to study the physical, chemical, nutritional and rheological characteristics of cheeses made by *Solanum dubium* serine protease. Economical studies should be done to show the visibility of producing the enzyme as commercial product.

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