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Detection of cathepsin D in ewe's milk by Western Blotting method

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Summary

Milk contains about 70 indogene enzymes, while only twenty of them were investigated. One of the less explored is the milk enzyme cathepsin D, proteolytic enzyme located in the lysosomes, which are an integral part of the somatic cells whose number varies depending on the animal's health. Unlike cow's milk, in ewe's milk the presence of cathepsin D was not determined, which could affect the production of dairy products, especially cheese, which are traditionally produced in Croatia and Mediterranean. This paper shows the presence of cathepsin D and its forms in ewe's milk by modified Western Blotting method. The analysis confirmed the presence of procathepsin D, mature and heavy chain of cathepsin D. Pseudocathepsin D and light chain of cathepsin D were not detected.

Key words: milk, cathepsin D, ewe's milk, Western Blotting

Introduction

Milk contains a large number of indigenous enzymes which have different functions such as the impact on dairy products quality and stability of processing procedures (Kelly and Fox, 2006). Among the indigenous enzymes, milk contains two main proteinase systems, plasmin and lysosomal enzymes and other proteolytic enzymes (Fox and Kelly, 2006). Plasmin as alkaline proteinase is the main proteolytic enzyme in raw milk (Andrews, 1983; Kalit et al., 2002) and is associated to casein micelles. One of the main lysosomal enzymes is cathepsin D (EC 3.4.23.5). It belongs to the group of acid, aspartic endopeptidase and is present in lysosomes of all mammalian cells. Cathepsin D (CatD) is originally considered as a "house keeping enzyme" involved in the clearance of unwanted proteins in the human organism (Margaryan et al., 2010). Amino acid sequence (primary structure) of procathepsin D reminds on the amino acid sequences of other aspartic proteases such as renin, pepsin and certain enzymes of fungi (Conner, 1992). According to available research, until now amino acid sequence of procathepsin D has been identified only in some species of mammals such as human, bovine, mouse, rat, porcine and ovine which is shown in Table 1, and dashes in the ovine and porcine sequences indicate that amino acid was not determined (Faust et al., 1985; Diedrich et al., 1990; Fujita et al., 1991; Larsen et al., 1993; Tyynela et al., 2000). Compared to human sequence, there is 70.45 % identity with rat, 70.45 % with bovine, 69.23 % with ovine and 68.18 % with mouse procathepsin D (Benes et al., 2002). CatD is involved in the metabolic degradation of intracellular proteins, activation and degradation of polypeptide hormones and growth factors, activation of enzymatic precursors, processing of enzyme activators and inhibitors, brain antigen processing and the regulation of programmed cell death (Vashishta et. al., 2009). In medical research, CatD is known as a tumour marker and

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interest in this enzyme has risen in recent years due to its involvement in highly prevalent diseases such as hypertension, AIDS, amyloid disease, candidiasis, peptic ulcer disease, breast cancer and malaria (Fialho et al., 2005). Procathepsin D, the proform of lysosomal aspartic peptidase cathepsin D is overexpressed and secreted by various cancer cell lines (Leto et al., 2004).

In milk, CatD fulfil lysosomes placed mostly in the white blood cells. Different conditions like inflammatory udder-pathogenic processes affect the increase of lysosomes, organelles that are an integral part of the macrophages (monocytes) and neutrophils (granulocytes), and thus somatic cells. CatD is related to whey with optimal proteolytic activity at pH 2.8-4.0 and 37 °C (Somers et al., 2003). Most researches were conducted on bovine's milk where four forms of CatD were determined: procathepsin D correspond to molecular masses of 46 and 45 kDa, 43 kDa pseudocathepsin D, 39 kDa mature single and two chained cathepsin D: 31 kDa (heavy chain) and 14 kDa light chain of cathepsin D. On the contrary, in the rats milk, procathepsin D whose molecular weight corresponds to that of 50 kDa has been identified (Benes et al., 2002). The reason of such variation in molecular mass is still not known (Hurley et al., 2000). The major form of cathepsin D in milk is the inactive zymogen, procathepsin D. Both cathepsin D and procathepsin D activity can degrade caseins and cathepsin D can furthermore coagulate milk, albeit slowly (McSweeney et al., 1995; Larsen et al., 1996; Larsen et al., 2006). Increasing levels of proteolytic enzymes from milk will contribute to intensive proteolysis in cheese (Marino et al., 2005; Revilla et al., 2007). Also, different enzymes have different effects on milk and milk products (Table 2), lipolysis and proteolysis are some processes that adversely affect the finished product (taste, smell and texture). So far, the presence of enzyme cathepsin D in ewe's milk by Western Blotting method has not been established. This enzyme could be a problem especially for production of hard cheeses on small farms that are part of livestock production and milk processing in Dalmatia but also in other Mediterranean countries such as Spain, Italy, Greece, etc. (Boyazoglu and Morand-Fehr, 2001) where ewe's milk cheeses are produced traditionally. The aim of the present study was to determinate presence of enzyme cathepsin D in ewe's milk and its forms.

Materials and methods

Milk collection

Samples of ewe's milk were collected at a family farm in central Dalmatia from autochthonous Croatian breed, Dalmatian Pramenka. Milk samples were put into a sterile tubes and stored at -80 °C until its usage. Before analysis, samples of ewe's milk were defrosted and centrifugated for 1 hour at 4000 × g. Medium phase was collected and used for Western Blotting analysis.

Western Blotting analysis

Ewe's milk samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE). To confirm the protocol, positive control was used, human MCF7 Whole Cell Lysate (Santa Cruz Biotechnology). Milk samples and Whole Cell Lysate were diluted in Laemmli sample buffer and heated at 95 °C/3.5 min (Laemmli, 1970). Electrophoresis of the denatured samples were carried out using NuPage 12 % Bis Tris gel ready precast gel (Invitrogen) at 200 V for 1 hour at room temperature in 25 NuPAGE MOPS SDS Running Buffer (20X) (Invitrogen). Proteins were then electrotransferred to PVDF membrane (0.45 μm) (Whatman) at 200 V for an hour and a half in

Table 1. Propeptide sequences of procathepsin D of different mammals (Faust et al., 1985; Diedrich et al., 1990; Fujita et al., 1991; Larsen et al., 1993; Tyynela et al., 2000; Benes et al., 2002)

Human	LVRIPLHKFTSIRRTMSEVGGSVEDLIAKGPVSKYSQAVPAVTE
Rat	LIRIPLRKFTSIRRTMTEVGGSVEDLILKGPITKYSMQSSPRTK
Mouse	IIRIPLRKFTSIRRTMTEVGGSVEDLILKGPITKYSMQSSPKTT
Bovine	VIRIPLHKFTSIRRTMSEAMGPVEHLIAKGPISKYATGEPAVRQ
Ovine	____LHKFTSNRRTMSEAMGPVEHLIAKGPISKYATREPAVRQ
Porcine	LI_IPL_K__I__M____P__LI_K__I_K_____

NuPage transfer buffer (Invitrogen). After blocking for 30 min in 0.5 % PBS Tween 20, pH 7.5 (blocking buffer), the membrane was incubated with custom-prepared human anti-goat cathepsin D monoclonal antibody (Neuromics Antibodies) (1:10000 in 0.05 % PBS Tween 20) for 1 hour at room temperature. The membrane was then washed 3 times with 0.05 % PBS Tween 20 and incubated for an hour and a half with anti-goat IgG-horse radish peroxidase conjugate secondary antibody (Abcam) (1:10000 in 0.05 % PBS Tween 20). After washing the membrane with 0.05 % PBS Tween 20, CatD-antibody complexes were detected using a combination of 4-Chloro-1-naphthol and DAB solution.

Results and discussion

Cathepsin D (Cat D) and its forms were detected in ewe's milk using a modified protocol of Western Blotting method due to the lack of appropriate antibodies. This study confirms that ewe's milk contains the enzyme cathepsin D and its forms: procathepsin D (two forms) 45 and 46 kDa, mature cathepsin D 39 kDa and heavy chain 31 kDa, as shown in example in Figure 1. Benes et al. (2002) detected procathepsin in rat milk while Larsen et al. (1993, 1996) described the bovine cathepsin D and its form in detail.

Although some authors believe that cathepsin D does not have a physiological role in milk, problems arise when cheese-makers wants to produce high quality dairy products manufactured from milk containing an increased number of somatic cells (Andrews, 1983; Fang and Sandholm, 1995; O'Driscoll et al., 1999). Such milk is adverse for further processing primarily for the production of cheese: curd retains more water, cheese is a soft, moist and elastic, resulting in a bitter taste and lower yield (Table 2). Such faults do not represent a problem for large manufacturers (industry), where there is a control of raw material (milk), but can be a problem on small family farms. Traditionally, in Croatia in the area of Dalmatian hinterland, as well as in other low potential rural areas of Mediterranean basin, sheep milk is used for production of high quality cheeses (Barbosa, 1990; Boyazoglu and Morand-Fehr, 2001; Ugarte et al., 2001; Matutinović et al., 2007; Matutinović et al., 2011), and any deviation in quality of ewe's milk can cause significant losses. Further research should certainly go in that direction.

Western Blotting method was used to detect attendance of different forms of enzyme cathepsin D in ewe's milk. The main problem was the absence of adequate antibody which is the reason why for

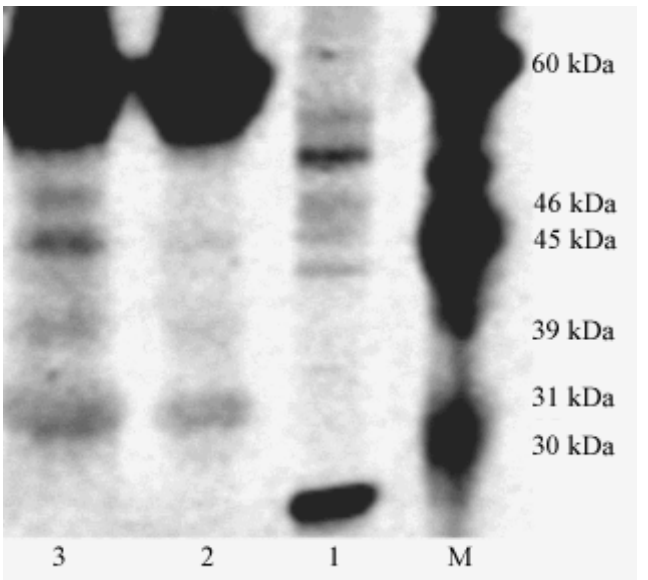

Figure 1. Example of Western Blotting analysis of raw ewe's milk.
(M) Marker, (1) MCF7 Positive control, Human Whole Cell Lysate, (2, 3) different milk samples

Table 2. Known or possible significance of indigenous milk enzymes for the manufacture and/or quality of dairy products (Kelly and Fox, 2006)

Product	Enzyme[a]	Significance
Raw milk	LPO	Antimicrobial effect
	XOR	Antimicrobial effect
Pasteurised milk	Plasmin	Contributes to instability?
	AlP	Index of processing
UHT milk	Plasmin	Contributes to gelation on storage?
	SHOx	Reduces cooked flavour
Cream	Lipase	Can cause rancidity
	LPO	Indicator of heat treatment
Milk powders	Lipase	Can cause rancidity
	Plasmin	Can survive drying and remain active
Yoghurt	LPO	Inhibits post-acidification?
	Plasmin	Affects gel structure and texture?
	AcP	Dephosphorylates proteins and peptides?
Fresh cheese	Plasmin	Affects rennet coagulation of milk
Ripened cheese	Plasmin	Contributes to primary proteolysis
General	Lipase	Contributes to lipolysis
Swiss	Cathepsin D	May contribute to proteolysis due to inactivation of chymosin
Acid	Cathepsin D	May contribute to proteolysis due to low pH
Infant formulae	Lipase	Can cause rancidity
	Plasmin	Can cause bitterness
	XOR	Bactericidal effect
Rennet casein	Plasmin	Survives production and can act in products in which this protein is used

[a]Abbreviations: AlP, alkaline phosphatase; AcP, acid phosphatase; LPO, lactoperoxidase; SHOx, sulphydryl oxidase; XOR, xanthine oxidoreductase

detection of cathepsin D antibodies that are reared in the goat against a human cathepsin D were used. This antibody recognized human cathepsin D in ewe's milk. Secondary antibody, an anti-goat IgG polyclonal antibody was linked to horse-radish peroxidase in order to get better results. Addition of substrate 4-Chloro-1-naphthol or DAB solution gave no reaction so those two substrates were mixed and poured over the membrane.

Although the Western Blotting method is highly sensitive and highly specific, enzyme-linked immunosorbent assay (ELISA) is often used. Many authors in their research customized the method of determination, so, ELISA was adjusted to addition of haemoglobin (Larsen et al., 1993; 1996) or synthetic heptapeptide (O'Driscoll et al., 1999) as substrate (Kelly et al., 2006). The lack of research is certainly one of the reasons that there is still no rapid, reliable and sensitive method for the determination of enzyme cathepsin D and other proteases in milk (O'Driscoll et al., 1999).

Conclusion

Due to the fact that there are 50 unidentified enzymes in milk, determination of the enzyme's presence in milk is crucial for investigation of this part of dairy science. Large numbers of endogenous enzymes in milk have been identified but researches on the presence of the enzyme in other milk types are missing.

These results clearly demonstrate the presence of enzyme cathepsin D and its forms, but further studies are needed to establish whether there is a correlation of enzyme cathepsin D with other parameters of milk and to what extent, and certainly to work on improvement of methods for determination of mentioned enzyme.

Detekcija katepsina D u ovčjem mlijeku Western Blot metodom

Sažetak

Mlijeko sadrži približno 70 indogenih enzima, no samo je njih 20 detaljnije istraženo. Jedan od manje istraženih je katepsin D, proteolitički enzim smješten unutar lizosoma, koji su sastavni dio somatskih stanica čiji broj ovisi o zdravstvenom stanju životinje. Za razliku od kravljeg mlijeka, u ovčjem mlijeku nije utvrđena prisutnost katepsina D koja može utjecati na proizvodnju mliječnih proizvoda prije svega proizvodnju sira koji se tradicijski proizvode u Hrvatskoj i na Mediteranu. U ovom radu prikazana je prisutnost enzima katepsina D i njegovih formi u ovčjem mlijeku pri čemu je korištena modificirana Western blot metoda. Analizom je utvrđena prisutnost prokatepsina D, zrelog i teškog lanca katepsina D. Pseudokatepsin D i laki lanac katepsina D nisu detektirani.

Ključne riječi: mlijeko, katepsin D,
ovčje mlijeko, Western Blot metoda

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