Maria Salomé S. Pais

THE CHEESE THOSE ROMANS ALREADY USED TO EAT. FROM TRADITION TO MOLECULAR BIOLOGY AND PLANT BIOTECHNOLOGY

ACADEMIA DAS CIÊNCIAS DE LISBOA

FICHA TÉCNICA

TITULO
THE CHEESE THOSE ROMANS ALREADY USED TO EAT. FROM TRADITION TO MOLECULAR BIOLOGY AND PLANT BIOTECHNOLOGY

AUTOR
MARIA SALOMÉ S. PAIS

EDITOR
ACADEMIA DAS CIÊNCIAS DE LISBOA

EDIÇÃO
ANTÓNIO SANTOS TEIXEIRA
SUSANA PATRÍCIO MARQUES

ISBN
978-972-623-232-2

ORGANIZAÇÃO

Academia das Ciências de Lisboa
R. Academia das Ciências, 19
1249-122 LISBOA
Telefone: 213219730
Correio Eletrónico: geral@acad-ciencias.pt
Internet: www.acad-ciencias.pt

Copyright © Academia das Ciências de Lisboa (ACL), 2015
Proibida a reprodução, no todo ou em parte, por qualquer meio, sem autorização do Editor

THE CHEESE THOSE ROMANS ALREADY USED TO EAT. FROM TRADITION TO MOLECULAR BIOLOGY AND PLANT BIOTECHNOLOGY

Maria Salomé S. Pais

HISTORY OF CHEESE MAKING

Although many studies have been developed on history of cheese making, it is impossible to pinpoint the precise time when the first cheese was made — and what circumstance was responsible for discovering the process of cheese making. The true origin of cheese making may remain uncertain. According to archaeological data, there is evidence that the use to make cheese should come since the earliest days of mankind, soon after the domestication of animals such as cows, sheep and goats. The milk produced by these animals soon has been recognized as a product not only to sustain calves, lambs and kids lives but it would be also important for adults as well. Since the beginning, men from early civilizations realized that liquid milk rapidly spoils, especially in warm conditions, occurring in the regions where civilization emerged. This problem should be responsible for spoilage of this high nutritional value product. In that conditions, man would try to figure out how could control this spoilage in order to benefit of receiving the nutritional benefits of milk.

Cheese would have been produced by accident: — After animal domestication, man used all parts of the slaughtered animal for different purposes (food, clothing, shelter and tools). Animal's dried stomach constituted an excellent flask for transportation of liquids (Harbutt & Denny (1998). Its use was maintained since early times until the early years of 20th century, in some Mediterranean regions.

Horsemen, when performing long journeys in the desert would take their ration of milk in a travelling flask made from the stomach of young animals. The legend tells that an Arab man, travelling over the desert, transported a portion of milk in a pouch made from a sheep's stomach. When the time carne to eat, the traveler discovered his milk curdled and separated into soft lumps of curd and of whey. Thus revising what would have occurred man would have understood that the reason for this to occur would be the effect of the unweaned stomach container. This milk clotting effect has later been considered as resulting from the milk clotting effect of enzymes (also known as rennet) present in the stomach lining of unweaned baby animals.

Cheese making may lies beyond reported history, probably cheese making practices having been developed simultaneously in different parts of the world. Probably, cheese making is as old as 10 000 years BC.

The history of cheese is as old as that of the human species and is linked to the taming of domestic animals 10 000 years BC. The roots of cheese making are not known with certainty. It is believed though, that it originated in Mesopotamia, where it was produced approximately 8000 years ago. It is very likely that its production was completely accidental, occurring during the transport of milk in the stomachs of young animals.

Archaeological studies have revealed that cheese making do exists in Neolith Era, some reports suggesting that the origins of cheese could be found in nomadic Asian people around 6000 years BC.

Milk-curdling vessels dating from 5000 BC have been found on the shore of Lake Neufchatel in Switzerland.

In Mesopotamia, today Iraq, there is evidence by portrayal in the temple of life goddess Ninchursag (3000 BC) not only of milking of cows and milk curdling but also of cheese suggesting that it would already be produced at that time.

In Portugal, archaeological remnants of forms for cheese making have been dated from 2500 BC (Cardoso, 2007). According to this author this type of product is compatible with the «Revolution of secondary products» also called «Neolithic revolution» that corresponds to the diversification of use of certain resources like the use of animals in land preparation or transportation and in the attainment of new products using artisanal techniques, such as cheese production by curdling of milk. It's interesting to notice that the abundance of these forms reveals the importance of this food product. As it varies among the different archaeological stations, this author suggests that cheese making, although being common, would be more intense in regions where cattle breeders were more important while in regions where inhabitants could fish (littoral) or hunt, cheese production would be lower

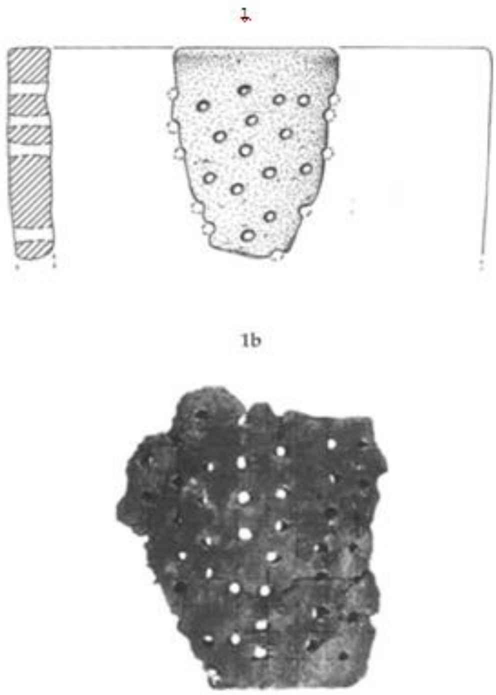

FIGURE la, b
Portion of a ceramic form «forma» used to press curdle milk in Leceia village (Calcolithic period, 2500 BC) in Portugal (by permission of Cardoso, 2007).

In Ancient Egypt, traces of cheese have been found in Egyptian tombs and in murals. Drawings dated from 2000 BC represent steps of cheese and butter making. In ancient Egypt, priests would have been responsible for the development of cheese making techniques from which secrets they would have been the save guardians.

FIGURE 2
Man drawing milk from a goat udder.

Cheese making is mentioned several times in the Old Testament. It can be read that during David's escape across the River Jordan, he was fed with «cheese of cow». It is also written that David was asked to bring «ten cheeses» to the commander of his brothers' unit in the battle against the Philistines. References to cheese are made in many ancient and classic texts. In Greek mythology, it is said that the Gods sent Aristaeus, the son of Apollo, to teach the Greeks how to make cheese, while in Homer's Odyssey we learn on Cyclops Polyphemus (whose baskets were always full of cheese) and his cheese-making techniques. In Ancient Greece abundant references are made to cheese production and consumption, namely in Aristotle's, Pythagoras's and several ancient comedy writers' works. In classical Greece, the strict athlete's diet would contain cheese. It has been reported that around the 4th century BC, the cheeses in Greece would have been flavoured with herbs and spices and used in making of cakes and pies. The Greeks considered cheese a luxury food, accessible only to the wealthy.

Plato considered that the decadence of the Greek empire was due to the luxurious way of living and the great number of banquets. In book II of Republic, Plato instigate the return to a more rigid food regime in which salt, olives, cheese, onions and legumes would be included.

In what concerns cheese making and consumption in Roman Empire, it was an everyday food product. Like in Greece, Romans used to cook with cheese.

Columella (50 AC) describes, with accurate detail, cheese making process, clearly showing the impressive developments of cheese making process in the Roman era. It is interesting to note that, in order to improve the curd-draining process, Romans invented the cheese press, this technique having been later exported to different countries as far as Great Britain. In Ancient Rome a rich cheese market existed, with aromas, spices and a variety of flavours, while later the history is surprisingly full of methodologies for cheese making. It appears that the ripening process has been developed by Romans and they considered that different storage and treatment conditions would result in different flavours and characteristics. Large Roman houses had a separate cheese kitchen, the caseale, and special areas where cheese was matured. In large towns home-made cheese could be taken to a special centre to be smoked.

In the Roman time cheese really carne into its own. Cheese making was done with skill and knowledge and reached a high standard. The importance of cheese in the Roman Empire was so that a rich cheese market still existed, around 13 different types being sold with a variety of aromas, spices and flavours.

Cheese was served on the tables of the nobility. In those days, cheese became a precious commodity, a symbol of high social rank of quality of life at the same time that constituted an exchange currency. While cheeses were, at first a luxury food for nobility, it may have become a staple for masses and legions. At that time, cheese constituted a regular component of the legion's rations. Soldiers would be given with 1 oz cheese ration every day diet. About thirteen varieties of cheeses would have been produced at that time. Around 300 AC, cheese trade and export activities were well established along the Mediterranean coast and along with the roman's invasions, cheese making know how would have been introduced across Europe. The conquering Romans should brought cheese making recipes with them when they occupied France and British Isles. It is considered that France owes one of its famous categories of cheese to invaders. With the decadence of the Roman Empire (around 410 AC), there was no evident progress in cheese producing methodologies (Andrea, 1992).

Hopefully, during the Middle Age, European monks with a true scientific behaviour had function as innovators and developers re-in-venting or improving ripening and ageing techniques for cheese. Producing milder-tasting cheeses, they have been responsible for the development of many of the classic varieties of cheese marketed today and for the refinement of many French cheeses (Brown, 1955). From the Middle Ages one can observe the first steps undertaken for the introduction of cheese-making associations, and probably around the 14th century, cheese production would start the first attempts to be produced as an industry.

FIGURE 3
Rural landscape from Portugal nearby Serra da Estrela.

FIGURE 4
Traditional processing of milk clotting in past times (Serra da Estrela, Portugal).

The traditional way of cheese making and commercializing was probably responsible for the decline of popularity of cheese that was considered unhealthy during the Renaissance period. At this time cheese made part of the rural gastronomy.

FIGURE 5
Italian picture (1494-1507) showing a seller of cheese and sausage in Castle of Issogne (Val de Aosta).

It regained flavour by the nineteenth century, the period when cheeses began to be made in factories instead of in farms. The variety of cheeses available today can be attributed to the various regions in which artisans tried their own hand at cheese making with great success and higher contributions for flavour and characteristics (Carr, 1981). During the Industrial Revolution, workers migrated from the countryside to towns, and dairies were set up for making cheese to supply this new and increasing market. With Pasteur's (1857) discovery of the principle of pasteurizing milk, a new era in cheese making industry was initiated that allowed a more controlled cheese making process at industrial scale, with consistent high quality to be marketable around the world.

CURDLING AGENTS

From animal origin (animal rennet)

From the history of cheese we can well realize that along the history milk clotting was performed using the enzymes complex extracted from stomach of animals that can be either goat, lamb or cow and more recently camel. This natural animal complex, called rennet, is extracted from the inner mucosa of the fourth stomach (abomasums) of young animals. The milk clotting enzyme contained in rennet is called chymosin (a proteolytic and clotting enzyme). The calf extract (rennet), is composed of Chymosin (formerly called rennin) and Pepsin. Chymosin is secreted in the abo¬masums as a pro-chymosin and is inactive. In the abomasums, in presence of H30+ ions (acid environment) the active form of chymosin is obtained.

If the animals are older or if weaning had occur, the abomasums contains less amounts of chymosin and higher amounts of pepsin which makes that this rennet is only appropriate for specific types of cheese. In fact, the proteolytic activity of pepsin is similar to that of chymosin but the clotting activity is very different (Scriban et ah, 1992). This variation in the amount of chymosin makes sense since the natural function of chymosin in animal's stomach is to digest the mother's milk to nourish the baby animals.

Traditionally, rennet is extracted from dried cleaned stomachs that are sliced in small pieces put into salt water, added of vinegar or wine

to lower the pH of the water and kept overnight or along several days. Afterwards the solution is filtered and the remaining rennin is used for milk clotting. It becomes clear that this artisanal way of producing chymosin (formerly rennin) is not compatible with production of high amounts of cheese.

New methods for extraction of rennet have been developed, which enabled the enrichment of the extract in chamois, thus allowing curdling of higher amounts of milk and so, the production of higher amounts of cheese. For comparison the efficiency of the traditional method with the new methods, it can be said that using the first, 1 g of rennet can clot 2-4 1 of milk, while using the 2nd, 1 g rennet can clot 15 000 1 of milk. This difference gives the possibility to change from an artisanal production to the production of higher amounts of cheese. Although most of the cheese is produced in large factories, farmhouse production still exists and is responsible for the maintenance of old traditions and special tastes and aromas (Kosikowski, 1966; Mills, 1988). The farmhouses produced cheeses are becoming popular and many of them constitute gourmet products (Timperley, 1989). Chymosin is an aspartic protease. Aspartic proteases constitute a widely distributed class of enzymes, being found in very diverse organisms such as vertebrates, fungi, bacteria, plants and retroviruses.

The clotting effect of chymosin is due to its ability to cleave specifically the peptide bond of milk k-casein between Phel05-Metl06 (Macedo et ai, 1996). This reaction occurring in the milk, promotes the linkage between the hydrophobic (para casein) and the hydrophilic (acidic glycopeptide) groups of milk casein to be broken. The resulting hydrophobic groups would unite together and form a 3D network that trapping the aqueous phase of the milk, give rise to calcium phosphor-caseinate and in the end of this reaction, precipitation of the curd that separates from the whey occurs, the curd being used in cheese making.

From microbial origin (microbial rennet)

Molds such as *Rhizomucor miehei* and *Mucor miehei* produce proteolytic enzymes able to clot milk. These molds can be grown in fermenters and yield high amounts of microbial rennet. The purified rennet produced by fermented *Mucor miehei* is commonly named vegetable rennet and used to supply kosher and vegetarian's cheese markets. Although being the most widely used mold species for production of microbial clotting enzymes, they need to comply with the criteria of purity and absence of mycotoxins (Scriban et ai, 1992). The cheeses obtained by curdling milk with these molds produced rennet, develop bitterness when maturation period is long, this being a constraint in the use of this microbial rennet (Samson et ai, 2004). Recently a new milk clotting enzyme produced by Bacillus sphaericus has been purified and characterized by Magda et ai, 2007.

From plant origin (plant/vegetable rennet)

Since early days plants have been recognized as having milk clotting properties. Some examples include fig tree (*Ficus carica*), Indian fig (*Opuntia ficus-indica*), nettles (*Urtica* sp.), mallow (*Malva spv Hibiscus* sp.), creeping Charlie (Glechoma bederacea), Lady's Bedstraw (*Galium verum*) and thistles (different species of Compositae: *Cynara cardunculus, C. humilis, Centáurea calcitrapa, Cirsium arietinum, Carlina* spp.), among others. The use of Galium *verum* (also with popular names «Cheese Rennet» and «Cheese Renning?») in curdling milk is known since the sixteenth century.

FIGURE 6
Inflorescences of C. *cardunculus* growing in the wild in south of Portugal.

Its use to curd sheep and goat's milk in Tuscany seems to be due to its capacity to give the cheeses a sweeter and more pleasant taste. This plant would also be used to color this cheese type, this colorant having been probably substituted later by the natural colorant (reddish or yellowish) produced by annatto (*Bixa orellana*). The extracts from stems, leaves or flowers from different species of Compositae and in particular from *Cynara* species have long been used (said since Roman time) for traditional sheep cheese production in Mediterranean countries due to presence of milk clotting enzymes.

FIGURE 7
Flowers of C. cardunculus used to prepare the aqueous extract to clot milk.

The traditional cheeses produced with the extracts mostly from *Cynara cardunculus* constitute DOP products in Portugal and Spain. Although these plant extracts confer to the cheeses special morpho-organoleptic characteristics, the availability of plant material is seasonal, presents a considerable variability in enzyme concentration, largely depending on the harvesting region and on the climatic conditions of the year. Considering these drawbacks of the use of cardoon flowers to attain uniformity of the produced cheeses by clotting milk with the infusion obtained with the flowers, efforts have been done to isolate and identify the enzymes present in flowers and leaves from some Composite species known to be used in milk curdling. It has been demonstrated that the enzymes isolated from the Compositae species, studied so far, have a common characteristic. Those presenting the highest clotting activity are aspartic proteases. They have been designated formerly as Cynarases 1, 2 and 3 (Heimgartner *et al*, 1990) and renamed as Cyprosins, cyprosin B corresponding to cynarase 3, Cardosins (Veríssimo *et al*, 1996), Ceutaurins (Domingues *et al*, 2000). These plant milk clotting enzymes present high homology among them. Like the animal chymosin, ali these enzymes are included in the category of aspartic proteases. They are expressed as a zymogen or pro-enzyme (an inactive enzyme precursor) that by hydrolysis gives rise to the active enzyme. Enzymes isolated from a Cactaceae species (*Opuntia ficus-indica*) designated as Opuntins, belonging to the Cystein proteases category, also present milk clotting activity although lower than those included in the aspartic proteases category

(Teixeira *et al*, 2000). Many other milk clotting enzymes from plant origin have been isolated so far.

In what concerns Cyprosins, they present a molecular weight of 49kDa, are heterodimeric with a small sub-unit of 14-18 kDa and a large sub-unit of 32-34 kDa and contain high mannose-type glycosilations (Heimgartner *et al*, 1990). As other aspartic proteases, cyprosins cleave preferentially peptide bonds between hydrophobic amino acid residues. The milk clotting activity of this enzyme is very similar to that of chymosin but contrarily to chymosin, these plant enzymes cleave α, β , γ and k caseins which is responsible for the production of soft paste texture and a typical aroma (Cordeiro *et al*, 1992). This plant enzyme efficiently clots crude or pasteurized milk from cow, ewe, goat, buffalo and camel milk. This purified enzyme constitutes a very good ingredient for cheese making in medium size industries and is suitable for production of cheese specialties appropriate for gourmets markets. It also may constitute a very good alternative to rennet from fungi origin for consumption by vegetarians who are opposed to GM-derived foods.

FIGURE 8
Cheeses produced using purified cyprosin to clot cow pasteurized milk.

BIOTECHNOLOGY IN THE PRODUCTION OF CURDLING ENZYMES

Before the 20th century, cheese was considered a food specialty, produced in small scale by specialized farmers. With the migration of workers from the countryside to towns, cheese production changed from individual householders to dairies this being the first step for industrial mass cheese production. The industrial revolution, coming across with mechanization, made possible to produce large quantities of many pro-ducts including cheese. Cheese that in the past was rarely eaten recognized an increasing demand. In 1955, 13% of the milk was processed in cheese, this percentage increasing to 31% by 1984. Unfortunately, to supply the cheese industrial production more rennet was needed than could be obtained from calves and other sources did not yield a completely satisfactory coagulant. If in the farmhouses, cheeses can be produced using small volumes of curdling agent, industrial production depends on the availability of high amounts of milk and also of a permanent supply of curdling enzymes. The production of rennin using the animals fourth stomach became not enough for the needs. Otherwise the use of rennet from fungi origin revealed the development of bitterness when maturation period is long. Along with the need to supply the increasing market, the cheese that before was prized for its diversity became to be prized for its uniformity. None of the available rennets (animal, fungi or plant origin) could give rise to fully uniform products. Because of the above constraints, some producers thought on producing a curdling agent able to replace natural chymosin. In the same way, the ability to produce plant clotting enzymes would enable to overcome difficulties with seasonality of the plant material and the lack of uniformity in clotting activity of the natural plant product. The problems outlined above for chymosin and its microbial substitutes could be alleviated by cloning the bovine gene into a suitable production strain and production of the enzyme by fermentation.

RECOMBINANT ANIMAL CHYMOSIN

With the advancement of molecular biology, and the development of biotechnology, it was possible to isolate the genes coding for calf chymosin and engineer bacteria, fungi or yeasts to make them to produce chymosin at a large scale. Bovine chymosin is produced nowadays recombinantly in different organisms (*Escherichia coli*, *Aspergillus niger var awamori*, and *Kluweromices lactis*) as an alternative resource to that one from cows.

Chymosin produced by genetically modified bacteria was the first artificially produced enzyme to be registered. The first example of a commercially available genetically engineered chymosin is Chymax, created by Pfizer in the 1980s by expressing the calf chymosin gene in Escherichia coli cells, the recombinant chymosin production being achieved by fermentation of the E. coli engineered cells (Franke, 1990, Patent n. ° 4935370 USA). This product having been classified as generally re-cognized as safe (GRAS) by the FDA in 1989, was marketed in USA in 1990s. Afterwards it was marketed in several countries including Japan. At a very short term other companies have developed the production of recombinant chymosin by expressing calf chymosin gene in yeasts (Gist--Brocades) approved by FDA in 1992, and by expressing in a filamentous fungus (Danish Company) approved by FDA in 1993 (Fitzmaurice, 1995). Although these last two recombinant chymosins have been available in the market with a small delay they have shared the place in the USA market and worldwide.

In the sequence, several patents have been filled aiming at protecting the large scale production of recombinant chymosin. In one of them (Mule *et al*, 2004; Application Number: 10/599391) the main objective was to provide an efficient process for expressing pro-chymosin gene in E. coli cells, for production of pro-chymosin by fermentation of E. coli cells and its conversion into enzymatically active pure chymosin. A number of genes coding for clotting enzymes (chymosin like) have been isolated from animals other than bovine young animals. In the list can be cited ruminant animals such as deer, buffalo, antelope, giraffe, ovine and caprine species. It is important to refer that also the gene coding for chymosin from *Camelus dromedarius* have been cloned to produce recombinant enzyme to be used as milk clotting agent for cheese manufacturing using cow's milk and milk from any other animal species including camel's milk (Kapeller *et al*, 2004; Application Number: 10/807115). Recombinant chymosin contains only one of the known main chymosin types — either type A or type B. Other chymosin types found in animal rennet do not exist in recombinant chymosin. This may be one reason why in many cases recombinant chymosin is combined with pepsin at different percentages as a way to imitate the complexity of natural rennet and to obtain similar clotting effect as well as the later development of cheese flavor and taste.

Cheese production with genetically engineered chymosin is similar to production with natural calf chymosin. Different types of conventional cheeses have been successfully made by using recombinant rennet on an experimental or pilot scale. No major differences have been detected between cheeses made with recombinant chymosins or natural enzymes, regarding cheese yield, texture, smell, taste and ripening. Biochemical and genetic evidences show that the recombinant chymosins are identical to calf rennet.

FIGURE 9
Industrial cheese production using cow milk and recombinant chymosin.

This biotech product quickly replaced a traditional ingredient (enzyme) with remarkable speed, in one food-making process so important and wide spread over the world as cheese. This shows the power of biotechnology in solving somehow perplexing problems of industries and, in that case, in dairy industry.

RECOMBINANT PLANT CYPROSIN AS A POSSIBLE SUBSTITUTE OF CHYMOSIN

As stated before, the available rennets of plant origin could not give rise to fully uniform products. The isolation of clotting enzymes (cy-prosins) from C. *cardunculus* flowers (Heimgartner *et al*., 1990) and, later on, the isolation of the gene coding for cyprosin B (corresponding to cynarase 3, the enzyme with highest clotting activity (Cordeiro et al., 1994) (Pietzak *et al*, 1992) enabled its cloning in *Saccharomyces cereviseae* (Calixto et al. Patent WO 0075283, 2002). Using the yeast strain W303-1A, Sampaio *et al*. (2008), demonstrated the possibility to produce recombinant cyprosin B by fermentation of the engineered yeast cells. The same authors demonstrated that the recombinant cyprosin B produced is biochemically and genetically similar to the natural enzyme produced by *Cynara cardunculus* flowers.

FIGURE 11
Production of recombinant cyprosin B by fermentation of engineered yeast cells (from F. Sampaio).

The ability to express, for the first time, a plant clotting enzyme (cyprosin B) in yeasts and to produce this enzyme in bioreactor is a promise for the large scale production of a new efficient curdling agent of plant origin and may constitute an efficient alternative to recombinant or natural enzymes from animal or fungal origin. Also, this recombinant plant enzyme, produced in large scale may enable the industrial production of cheeses that until now only could be produced at a small scale. Like for GM chymosins, GM cyprosin is suitable for vegetarians who are not opposed to GM-derived foods.

(Comunicação apresentada à Classe de Ciências
na sessão de 17 de Janeiro de 2002)

REFERENCES

ANDREA, T., *The Strange History of Cheese*, LiveScience Staff Writer, 1992.

BROWN, B., *The Complete Book of Cheese*, Gramercy Publishing, 1955.

CARDOSO, J. L., *As Cerâmicas Decoradas Pré-Campaniformes do Povoado Pré-Histórico de Leceia: Suas Características e Distribuição Estratigráfica*, Estudos Arqueo¬lógicos de Oeiras, Oeiras, 14, pp. 9-276, 2007.

CORDEIRO, M., JACOB, E., PUHAN, Z., PAIS, M. S., and BRODELIUS, P. E., *Milk clotting and proteolytic activities of purified cynarases from «Cynara cardunculus», L.: a comparison to chymosin*, Milchwissenschaft 47: 683-687, 1992.

CARR, S., *The Simon and Schuster Pocket Guide to Cheese*, Simon and Schuster ed., 1981.

DOMINGUES, A., CARDOSO, P. C, XUE, Z.-T., CLEMENTE, A., BRODELIUS, P., and PAIS, M. S., *Purification, cloning and autoproteolytic processing of na asparti̇́c proteinase from «Centáurea calcitrapa»*, Eur. J. Biochem., 267: 6824-6831, 2000.

FRANKE, A. E., *Expression plasmics for Improved expression of heterologous protein in bactéria*, USA, patent n.° 4935370, 1990.

FITZMAURICE, L., *Wisconsin's cheese-making industry benefits from biotechnology*, Wisconsin Biolssues 6(2):10-11, 1995.

HARBUTT, J., and DENNY, R., *The World Encyclopedia of Cheese*, 1998.

HEIMGARTNER, U., PIETRZAK, M., GEERTSEN, R., BRODELIUS, P., SILVA FIGUEIREDO, A. C, and PAIS, M. S., *Purification and partial, characterization of milk clotting proteases from flowers of «Cynara cardunculus» L. (cardoon)*, Phytochemistry 29: 1405--1410, 1990.

KAPPELER, S., FARAH, Z., VAN DEN, B. J. M., RAHBEK-NIELSEN, H., and BUDTZ, P., *Method of producing non-bovine chymosin and use hereof*, patent application n.° 10/ 807115, 2004, publication date: 18-9-2007.

KOSIKOWSKI, F., *Cheese and Fermented Milk Foods*, Cornell University, 1966.

MACEDO, I. Q., FARO, C.]., and PIRES, E. M., *Caseinolitic specificity of Cardosin, na aspartic protease from cardoon Cynara cardunculus: Action on bovina a- and b-casein and compairison with chymosin*, J. Agric. Food Chem. 44: 42-47, 1996.

MILLS, S., *The World Guide to Cheese*, Gallery Books, 1988.

MAGDA, A. El-Bendary, MAYSA, E. M., and THANAA, H. Ali, *Purification and Characterization of Milk Clotting Enzyme Produced by «Bacillus sphaericus»*, J. Appl. Sei. Res., 3(8): 695-699, 2007.

MULE, V. M. R., MYTHILI, P. K., GOPALAKRISHNA, K., RAMANA, Y., and REDDY, D. R. B., *Recombinant calf-chymosin anã a process for producing the same, patent application* n.° 10/599391, 2004.

PAIS, M. S., CALIXTO, F., and PLANTA, R., *Production by yeasts ofaspartic proteínases from plant origin with sheep's, cow's, goafs milk, etc, clotting and proteolytic activity*, USPTO patent applicaton n.° 20060003435.

PIETZAK, M., BRODELIUS, P., XUE, Z.-T., CORDEIRO, M., and PAIS, M. S., «*Cynara carduncuIus» mRNA sequence of «cypro4»*, EMBO Data Library X69672, 1992.

SAMSON, A., SHAOJIANG, C, and JIAN, Z., *Formation ofbitter peptides during ripening of ovine milk cheese made with different coagulants, Lait 84: 567-578, 2004. SCRIBAN, R., Production des Enzymes. In Technique et Documentation, ed. Lavoisier*, Paris, 1982.

TEIXEIRA, G., SANTANA, A. R., PAIS, M. S., and CLEMENTE, A., *Enzymes of Opunha ficus-indica (L) Miller with potential Industrial Applications*, Appl. Biochem. Biotechn. 88: 299-311, 2000.

TIMPERLEY, C, and CECÍLIA, N., A *Gourmefs Guide to Cheese*. HP Books, 1989.

VERÍSSIMO, P., FARO, C, MOIR, A. J. G., LIN, Y., TANG, J., and PIRES, E., *Purification, characterization anã partial aminoacis sequencing of two new aspartic proteinases from fresh flowers of «Cynara carãunculus»*, Eur. J. Biochem., 235: 762-768,1996.