Scientific Bulletin. Series F. Biotechnologies, Vol. XXV, No. 1, 2021
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

MILK-CLOTTING ENZYMES OBTAINED FROM PLANTS IN CHEESEMAKING - A REVIEW

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Abstract

The paper aimed to present a review of plant proteases used as milk coagulants in cheesemaking. Plant proteases have been used as milk-clotting enzymes since ancient times. These milk-clotting enzymes are starting to become an alternative to the calf rennet. Due to a high price of the calf rennet and a very limited availability, religious restrictions or lacto-vegetarian diet, milk-clotting enzymes obtained from plants are the subject of extensive research. They are present in a various plants and can be obtained from all plant parts: root, stem, leaves, flowers, fruits, seeds. Most research has shown that plant milk-clotting enzymes belong to aspartic proteases but have been reported also enzymes from serine proteases and cysteine proteases with this activity as well. Plant proteases with milk-clotting activity have been researched in terms of coagulation activity and proteolytic activity. Most plant milk- clotting enzymes develop an excessive proteolytic activity leading to lower yields of cheese, defects of texture and bitter flavours. The research will continue in order to meet the increasing global demand for a good and diversified cheese production.

Key words*: cheese, milk-clotting activity, plant proteases.*

INTRODUCTION

Milk is a valuable food, but relatively perishable due to its contamination with microorganisms, since milking. Also, milk production is, in many regions of the globe, seasonal, so there are no constant quantities during the year. Methods of preserving milk in various forms and convenient conditions have been discovered and perfectioned. Thus, at present, the milk components are fractionated: the fat is transformed into butter, which can be preserved by freezing, the dry matter in the milk is transformed into powdered milk with variable fat content, which does not require low temperatures. Milk proteins can be separated by precipitation or separation through membranes and preserved by drying in the form of protein concentrates.

A most common and historical method of preserving milk is processing in cheese, a more complex process that involves concentrating protein along with a variable fraction of fat and minerals, eliminating a significant amount of water and lactose. The cheeses can be stored for a few weeks to several months. The advantages resulting from the possibility to transform the main components of milk into cheeses were arguments for the development of this production: storage stability, relatively easy transport and diversification of the human diet. Worldwide, at present, over 35% of the amount of milk obtained on farms is transformed into cheese (Costin, 2003).

Cheeses are some of the most complex and dynamic foods. They are results of an applied biotechnology, each piece can be considered a bioreactor in which numerous and complicated reactions take place. Finally, is obtained a product with specific sensory and nutritive characteristics. Cheeses are one of the most valuable sources of protein for the global diet and are an excellent source of nutrients such as fats, minerals and vitamins. They can sometimes have a therapeutic role: patients with reduced gastrointestinal absorption and food allergies are currently treated with casein hydrolysate (milk protein). Casein hydrolysate is a biologically active peptide, which plays an important role in various physiological disorders.

Cheeses are dairy products that have played a key role in human nutrition for centuries. Cheese making is essentially a dehydration process, in which milk fat and casein are concentrated 6-10 times (Abebe & Emire,

2020). From ancient times the animal rennet is used in the manufacture of cheese.

Milk coagulation is the main stage for cheese production. Milk-clotting enzymes have been used in cheese making for thousands of years, and they appear to be the oldest known application of enzymes; the earliest indication of cheese making dates back to cave paintings around 5000 BC (Shah et al., 2013). Historically, most enzymatic preparations used for cheese have been extracted from the stomachs of ruminants, but coagulants from microorganisms and plants have also been used at very early dates (Bunty & Nabindra,. 2020). The stomach of the ruminant, especially the calf, is the source of rennet. It contains chymosin (EC 3.4.23.4) as the main enzymatic component and has been widely used in the manufacture of cheeses. Cheese production increased significantly (3.5 times higher than in 1961), but animal rennet production decreased due to the limited availability of herbivore stomachs (Nasr et al., 2017).

The animal rennet is obtained on a commercial scale from the stomachs of young ruminants (calves, lambs, kids). A single calf produces only 5 to 10 g of rennet. The enzyme helps to coagulate casein in milk. The property of the enzyme to coagulate milk is important in terms of cheese quality and yield (Mahajan & Chaudhari, 2014).

Research has long been focused on the discovery of a milk-clotting enzyme that would satisfactorily replace animal rennet. Various factors, such as the high price of animal rennet, various religious restrictions (e.g. Islam and Judaism), diet (lactovegetarians) or the inter-diction on the use of recombinant calf rennet (in France, Germany and the Netherlands) have encouraged the search for alternatives sources of milk-clotting (Roseiro et al., 2003). Research has been directed towards the discovery of milk-clotting enzymes that would satisfactorily replace calf rennet in cheese making, including microbial, recombinant, and herbal enzymes (Mahajan & Chaudhari, 2014).

The most important substitute enzymes that meet the requirements of cheese making include microbial, recombinant enzymes and enzymes that have been isolated from plants.

Animal rennet is a complex of enzymes produced in the stomach of any mammal, including milk-clotting proteases that coagulate milk; belonging to the coagulation process, it produces the separation into a solid part (curd) and a liquid part (whey). The presence of these enzymes is very important in the stomachs of young mammals for digesting the breast milk with which they are breastfed. The main active enzyme in the rennet is chymosin or rennin (EC 3.4.23.4, according with International Union of Biochemistry Enzyme Commission IUB or EC), along with pepsin and lipase.

The natural calf rennet is extracted from the inner mucosa of the fourth chamber of the stomach of young, unweaned calves. The rennet extracted from the old calves, fed with grass or cereals, contains less chymosin or not at all, but more pepsin. This rennet can only be used for some special types of milk and cheese. Each ruminant species produces a special type of rennet to digest the milk of its own species, so there is, for example, a goat's rennet for milk-clotting of goat's milk or a lamb's rennet for milk-clotting of sheep's milk.

There are many alternative sources of milk-clotting enzymes, from plants and microorganisms, that can replace animal rennet. Fermentation produced chymosin (FPC), by applying genetic engineering tools on the microbial organism, is mainly used in the manufacture of cheese in North America and Europe because it is cheaper and has a better quality than animal rennet. Enzymes produced by microorganisms are suitable as milk-clotting substitutes but there has been a lot of interest in coagulation enzymes extracted from plants. These enzymes are present in almost all types of plant tissues and it appears that all proteolytic enzymes have the ability to coagulate milk under appropriate conditions. Almost all enzymes used to coagulate milk belong to aspartic proteases but enzymes from other groups such as cysteine and serine proteases are also used.

Plant extracts have been used since ancient times to coagulate milk to make cheese, especially in Mediterranean countries, West Africa and Southern Europe. Thus, Homer suggested in the Iliad and the Odyssey that the Greeks clotted milk with a fig extract (Ben Amira et al., 2017).

Milk-clotting plant proteases have become a subject of growing interest in cheese industry,

due to their easy availability and simple purification processes. The use of plant proteases in cheese production promotes the greater acceptability by the vegetarians and may improve their nutritional intake (Ben Amira et al, 2017).
The presentation of enzymatic and technological properties of milk-clotting plant proteases, previously studied in literature, could provide a clear vision on key elements for the selection of appropriate plant rennet.
Evaluation of enzymatic activities is the main step in the selection of an appropriate substitute of calf rennet which can be successfully used in cheese making. It is achieved by monitoring of Milk-Clotting Activity (MCA) and Proteolytic Activity (PA).
Milk-Clotting Activity (MCA) is the most important property of enzymes used in cheese making.
It is the ability of the enzyme to specifically hydrolyse of κ-casein. It can be measured by different methods, such as Soxhlet, Berridge and the units used are Soxhlet, Berridge or Rennet Units and International Milk-Clotting Units (IMCU).
Proteolytic activity (PA) expresses the degree of proteolysis of the enzyme.
High proteolytic activity leads to excessive cheese maturation, with advanced hydrolysis of protein chains and formation of nonspecific bitter-tasting peptides.
The MCA/PA ratio is the one that best characterizes a commercially coagulating enzyme: the higher it is, the better the enzyme coagulates the milk, without advanced proteolysis during maturation.
Considering that the researches regarding the use of plant proteases as a substitute for animal rennet, carried out so far in Romania, are in the incipient phase, of pioneering, the present review aims to summarize the latest research findings on plant proteases with milk-clotting activity presenting enzyme chemistry, production and techno-functional properties.

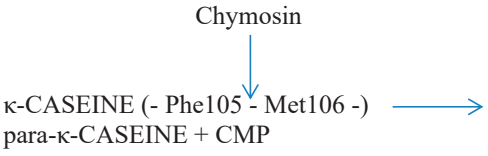
MILK COAGULATION - THE MAIN STAGE OF CHEESE MAKING

The general process of milk coagulation (clotting) into dairy products, such as cheeses and fermented dairy products (yoghurt) is based on the formation of an aggregate protein network, which consists mainly of a certain group of proteins known as caseins. This network includes water, fat and other constituents of milk. Biochemical processes are quite different between cheese and fermented dairy products, as the production of cheeses involves the separation of whey by casein, while in fermented dairy products the entire composition of milk is included in the final product.
Milk coagulation (clotting) in cheese making is of several types, depending on the main agent involved in the biochemical process: enzymatic coagulation, acid coagulation, mixed (enzymatic and acid) coagulation.

Enzymatic coagulation of milk

Mechanism: the biocatalytic action of coagulating enzymes on casein, leads to the formation of "clot" gel.
Enzymatic coagulation of milk represents the modification of casein micelles by limited hydrolysis of casein under the action of milk-clotting enzymes, followed by a network aggregation of micelles induced by the presence of calcium ions (Fox et al., 2004).
The first commercial rennet was prepared, standardized and sold by Chr. Hansen A/S, Denmark in 1874, and was probably the first commercial enzyme of any kind, world-wide used (https://www.chr-hansen.com). Animal rennet is, by definition, an extract of ruminant abomasum, ideally containing mainly chymosin, the enzyme that is specific for the hydrolysis of κ-casein and the destabilization of casein.
However, depending on the age of the calves from which it is extracted, the rennet may contain pepsin, which is an acidic protease with a wider range of action on the casein substrate. Both chymosin and pepsin, and indeed all milk-clotting enzymes used in cheese technology, are classified as aspartic proteinases with Enzyme Commission number (EC) 3.4.23. Because there are now several different types and sources of milk-clotting enzymes on the market, the International Dairy Federation (IDF) officially defines that the name "rennet" is reserved for ruminant stomach enzyme preparations, and other types of milk- clotting

enzymes (especially microbial ones) should be called 'coagulants'. For cheese technology, rennets and coagulants are usefully classified, according to their source, as animal, vegetal, microbial and GMO sources.
Chymosin is the most important and active milk-clotting enzyme, involved in cleaving the Phe105-Met106 peptide bond from κ-CN.
Coagulation of milk induced by chymosin can be described in three phases:
1. During the primary phase, the enzymatic hydrolysis of κ-CN takes place as follows:



The hydrophilic CMP (Casein Macro Peptide) portion is released into the whey. This causes the loss of a negatively charged group and the decrease of steric stabilization. When approximately 70% of k-CN is hydrolysed (Walstra et al., 2006), the colloidal stability of the micelles is low enough to begin the second phase:
2. Spontaneous secondary aggregation phase - gel formation as molecular chains that connect through hydrophobic calcium bonds to form a three-dimensional network, followed by a subsequent solidification.
3. In the third phase, the whey is expelled from casein by syneresis (more cross-linking contraction).
Coagulation is improved by lowering the pH, increasing the calcium concentration and temperature (without aggregation below 20°C). Syneresis is increased by increasing temperature, pH and pressure applied, e.g. stirring (Walstra et al., 2006).

Acid coagulation of milk

Mechanism: selected cultures of lactic acid bacteria ferment lactose from milk and turn it into lactic acid; by lowering the pH to the isoelectric pH of casein, it precipitates and forms a "lactic" gel.
Upon acid coagulation of milk, the micellar casein is modified by the low pH of milk. This causes the colloidal calcium phosphate (CCP) to dissociate from the micelles, the negative charges in the casein micelles being neutralized, with aggregation occurring at the isoelectric point of the micellar casein (pH 4.6). A porous network of weakly bound aggregates is formed. Moreover, the concentration of proteins in the gel network will be increased due to the active participation of denatured whey proteins in the formation of the structure.

In the case of the ***mixed coagulation of milk*** the mechanism undertakes a symbiosis between the two previous procedures.

MILK-CLOTTING PLANT PROTEASES - A GENERAL DESCRIPTION, TYPES AND SOURCES

Proteases are enzymes that perform proteolysis, initiating protein catabolism by hydrolysis of the peptide bonds between the amino acids in the structure of the polypeptide chain that make up the protein. Over time, they have evolved several times so that the same reaction can be performed by different classes of proteases by completely different catalytic mechanisms.
Proteases are found in all organisms, from prokaryotes to eukaryotes and viruses. These enzymes are multifunctional, having many physiological functions in plants and animals. They are involved in various physiological reactions from the simple digestion of proteins in food to extremely well-regulated cascade reactions such as blood clotting or apoptosis. They also act in germination, biological aging, inflammatory processes, etc.
Proteases act either by completely breaking down of peptide into amino acids (unlimited proteolysis) or by breaking down specific peptide bonds (limited proteolysis) depending on the amino acid sequence that makes up the protein. The activity can be a destructive change such as suppressing the function of a protein or digesting the protein to amino acids or it can be an activation of a function, respectively a signal in a signalling pathway.
Proteases are classified, according to the catalytic residue, in the following groups: serine proteases, threonine proteases, cysteine proteases, aspartic proteases, glutamic acid proteases and metalloproteases.
Plant proteases used as milk-clotting enzymes were reported only in aspartic, cysteine and serine proteases.

According to the International Union of Biochemistry Enzyme Commission (IUB or EC), http://www.enzyme-database.org, proteolytic enzymes with milk-clotting activity are part of EC 3 Hydrolases, EC 3.4 acting on peptide bonds (peptidases).
Depending on the groups of the active centre, there are 3 subclasses of endopeptidases with milk-clotting activity (Shah et al., 2014).

Aspartic proteases (EC 3.4.23) (APs)

Aspartic proteases have two aspartic residues in their catalytic centre and are involved in protein degradation during plant development process, protein storage mechanisms, responses to stress and pathogens and plant senescence. They are most active at acid pH and have preferential specificity for cleavage at peptide bonds between hydrophobic amino acid residues responsible for catalytic activity.
Aspartic proteases with milk coagulation activity have been identified in artichokes (*Cynara scolymus*), armory (*Silybum marianum*), rice core, *Centaurea calcitrapa*. Regarding cardoon (*Cynara cardunculus*), flowers are traditionally used in the Mediterranean region in the manufacture of cheese. It produces cardosine and cyprosine, aspartic proteases that have been found to accumulate in mature flowers (petals and pistils) but not in leaves or seeds. Cardosin A is an abundant aspartic protease from *C. cardunculus* pistils (Feijoo-Siota & Villa, 2010).

Cysteine proteases (EC 3.4.22) (CPs)

Cysteine proteases or thiol proteases are some of the largest groups of proteolytic enzymes involved in many processes in both prokaryotes and eukaryotes (e.g., bacteria, parasites, plants, invertebrates and vertebrates).
The catalytic mechanism of these enzymes involves a cysteine group in the active site. Cysteine proteases have great potential for use in the food, biotechnology, and pharmaceutical industries due to their property of being active in a wide range of temperature and pH. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. In plants, they are widespread among different taxonomic groups and prove to be involved in several physiological processes, such as fruit development and ripening, nutrient reserve, degradation of storage proteins in germinated seeds, activation of proenzymes, and degradation of defective proteins. CPs comprise a family of enzymes, which consist of papain and related plant proteases, such as chymopapain, ficin, caricaine, bromelain, actinidine and microsciadin (Rezanejad et al., 2015).

Serine proteases (EC 3.4.21) (SPs)

Serine proteases possess a residue of serine in their active centre and show several biochemical and physiological characteristics. In plants, they are widespread among taxonomic groups, from trees to crops for vegetables and herbs. They are present in almost all components of plants, but most abundantly in fruits. Serine proteases from cucurbits, grains and trees are usually classified together.
Serine plant proteases have been found and extracted from latex, seeds, flowers, stems, leaves and roots.
Shah et al. (2014) described a few serine proteases developed and researched: neriifolin, a type of chymotrypsin serine protease, has been purified from the latex of *Euphorbia neriifolia*; religiosin A, B and C were isolated from latex extracted from *Ficus*; dubiumin was purified from *Solanum dubium* seeds; cucumisin from *Cucumis melo* and lettucine from *Lactuca sativa* were isolated and used as coagulating enzymes.
Research in recent decades has expanded the list of plants or parts of plants that contain enzymes with milk coagulation properties. Milk-clotting plant proteases were characterized and named by their plant sources (Table 1).

Table 1. Milk-clotting plant proteases characterized from different plant sources

Protease	Source	References
Bromelain	Pineapple (*Ananas comosus*)	Harrach et al., 1998
Papain	Papaya (*Carica papaya)*	Mitchel et al., 1970
Ficin	Common fig (*Ficus racemosa)*	Devaraj et al., 2008
Cardosin	Thistle (*Cynara cardunculus)*	Ordiales et.al., 2012
Actinidin	Kiwi (*Actinidia chinensis)*	Katsaros et al., 2010
Cucumisin	Melon (*Cucumis melo)*	Uchikoba & Kaneda, 1996
Lettucine	Lettuce (*Lactuca sativa)*	Lo Piero et al., 2002

ISOLATION AND EVALUATION OF ENZYMATIC ACTIVITY OF MILK-CLOTTING PLANT PROTEASES

Obtaining enzymatic preparations - theoretical considerations regarding the extraction of enzymes

In order to study the structural and / or biochemical properties of an enzyme, the source that best meets the isolation requirements and that meets, as far as possible, the following conditions must be chosen: *maximum catalytic activity* - the enzyme present should not be degraded or inactivated; *maximum possible purity* - it must not contain other large enzymes or molecules; *maximum possible yield* - resulting from the percentage of activity recovered compared to the activity of the original extract, if the purification of the enzyme is also followed.

In the strategy of choosing a biological material for the extraction of an enzyme usable for different purposes or for its study is necessary to consider several factors such as: enzyme *abundance*, because for any study, experiment or application it is necessary to obtain total protein extracts with high concentrations of the enzyme of interest, biological sources rich in the desired enzyme must be chosen; *availability and cost price*: the biological source must be accessible both geographically and economically; *intracellular localization*: of an enzyme is essential to know in order to establish the most convenient method of extraction; *source characterization*: the chosen source must be perfectly characterized; when the chosen source are plants, it is necessary to know them genus, species and variety. It is often necessary to know the area, the climate in which the plant source developed as well as the harvesting period; *comparative studies*. Some enzymes have been studied in some species or in different tissues of some species. In such cases it is very important to study the respective enzymes in different other species or tissues in order to evaluate the evolution of the enzyme, its properties compared to similar ones, primary, tertiary structure, different isoenzymes, etc. The stability of the enzyme and the possible difficulties in handling the source must be considered in all the stages of choosing the source.

Certain intracellular enzymes are used commercially without isolation and purification, but most commercial enzymes are produced extracellularly by microorganisms or plants or must be released from cells in solution and subsequently processed (Figure 1).

Solid/liquid separation is generally required for the initial separation of cell mass, removal of cell debris after cell rupture, and collection of precipitates. This can be done by filtration, centrifugation or aqueous biphasic partition.

In general, filtration or partitioning of aqueous biphasic systems is used to remove unwanted cells or cell debris while centrifugation is the preferred method for collecting the required solid material.

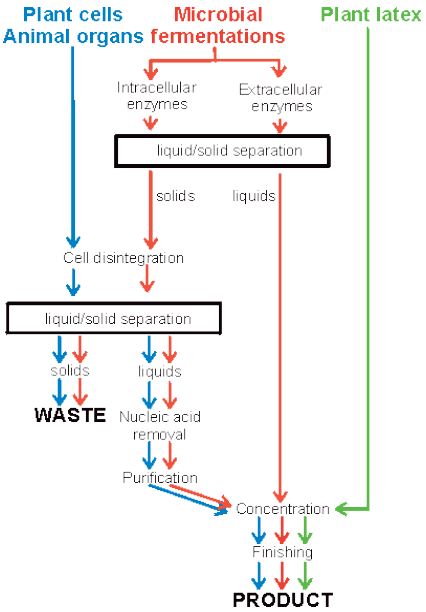


Figure 1. Flow diagram of enzyme preparation (Chaplin & Bucke, 1990)

Evaluation of milk-clotting plant proteases - an important step in validation as a rennet substitute

Experimental research to identify plant proteases with milk-clotting activity can be conducted in a variety of ways but must consider the principles described in the previous paragraph.

In addition, most of the articles written on this topic in the literature, outline the same experimental work procedure:

1) Screening on a wider range of plants with coagulant activity can be achieved, based on similarity, plants from the same family with a plant already proven with milk-clotting activity, on the same plant can be investigated and compared also different parts of the plant (root, stem, leaves, flowers). Oseni & Ekperigin (2013) compared the milk-clotting activity of different parts of Sodom Apple (*Calotropis procera*) (Figure 2).

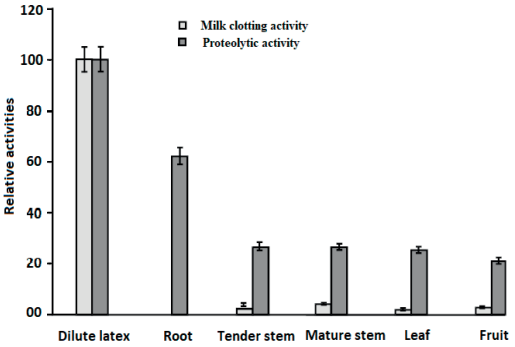


Figure 2. Milk- clotting and proteolytic activity of *Calotropis procera* plant (Oseni & Ekperigin, 2013)

2) Extraction of milk-clotting enzyme from plants, based on flow diagram described (Figure 1).

3) Protein content determination: the protein concentration of each enzyme solution should be determined by different methods in order to report a standardized enzymatic activities per protein unit.

4) Preparation of a standard working substrate (liquid milk from different species, with known fat and protein content, milk reconstituted from powdered milk, corrected with calcium chloride).

5) Defining and applying a working method for determining the MCA e.g. procedure described by IDF (1992) (Da Silva et al., 2013).

6) Proteolytic activity determination by different chemical and biochemical methods.

7) Ratio of milk-clotting to protease activity can accurately indicate which plant proteases should be developed as a milk-clotting enzymes as seen in Table 2 (Dahot et al., 1990).

Table 2. Milk-clotting activity, protease activity and ratio of milk-clotting to protease activity of tested plants (Dahot et al., 1990)

Name of plants	Part of plant tested	MCA[1] (units/ml)	PA[2] (units/ml)	Ratio of MCA/PA
Aloe L. sp.	Stem	190	7	27.14
Euphorbia hista	Whole Plant	360	9	40.00
Cereus triangularis	Stem	160	6	26.67
Euphorbia caducifolia	Stem	600	5	120.00
Euphorbia nivulia	Stem	760	7	108.57
Opuntia phylloclades	Stem with role of leaves	120	5	24.00
Ficus bengalensis	Leaves	380	7	54.29
Ficus carica	Leaves	1200	9	133.33
Ficus elastica	Leaves	490	6	81.67
Calotropis procera	Flowers	170	6	28.33
Calotropis procera	Leaves	390	9	43,33
Carica papaya	Leaves, dried latex	1580	8	197.50

Legend:
1 - The unit of milk-clotting activity was defined as the amount of enzyme which clotted 1.0 ml of milk in one minute at 50°C.
2 - One unit of the protease activity was defined as the amount of enzyme that liberated 1 µg of tyrosine under the standard assay conditions.

Table 3. Ratio of milk-clotting activity/proteolytic activity of some plant proteases and other coagulants (Da Silva et al., 2013)

Name of milk-clotting enzyme	MCA[1] (U/mg)	PA[2] (U/mg)	Ratio of MCA/PA
Chymosin	269	0.08	3363
Rennet from Mucor	438	0.16	2738
Papain	208	0.51	408
Protease of Ginger	314	0.19	1653
Protease of *Euphorbia nivulia*	760	7	109
Protease of Quixaba	917	0.16	5731

Legend:
1 - One milk coagulating unit per millilitre (U ml–1) is defined as 400 t–1; the amount of enzyme that coagulates the milk in one minute has 400 milk coagulating units. The variable *t* is the time required for the first clots of milk to form
2 - One unit of activity is equivalent to a change in optical density of 0.01 nm per minutes at 440 nm.

Within the frame of research for a suitable substitute for calf chymosin, which is strongly recommended enzyme, combining a strong clotting activity with a low general proteolytic activity (Panayotov et al., 2014), should be realized comparative studies of coagulant

activities and MCA/PA ratios of plant proteases with chymosin and microbial rennet as seen in Table 3 (Da Silva et al., 2013).

TECHNOLOGICAL CONSIDERATIONS OF MILK-CLOTTING PLANT PROTEASES USED IN DIFFERENT TYPE OF CHEESES

At this moment, cheeses obtained with milk-clotting plant proteases are normally produced on an artisanal scale, in small dairies.
Roseiro et al. (2003) summarized and described a few technological considerations about this issue, as presented below.

Edam and Cheddar semi-hard cheeses with vegetable proteolytic enzymes

After 8 months of maturation, Cheddar cheese made with *Ficus carica* extract did not show any difference between the control cheese produced with animal rennet; no difference compared to Cheddar cheese obtained with *Withania coagulans* extract, except that the vegetable extract had a slightly light texture. Cheddar cheese obtained with *Streblus asper* extract is known to have bitter and acidic aromas and a delicate texture while cheeses with *Carica papaya* leaf extracts also have flavour defects such as bitterness. The use of cardoon extract has been suggested to produce new types of soft cheeses and for improving the texture of low-fat Cheddar cheese. Edam cheese coagulated with cardoon had a softer texture and the whitish colour of the whey suggested a higher degree of proteolysis and a lower yield (Rao & Matur, 1979).

Camembert and Roquefort mold cheeses with vegetable proteolytic enzymes

Camembert cheese obtained with vegetable coagulant was matured before the one made with animal rennet. There was also reported a slightly astringent taste in cheese made with cardoon at the beginning of ripening, which disappeared towards the end of ripening and which can be explained by the presence of tannins and other substances in flowers. There was a higher proteolytic activity in Camembert sheep's milk cheese and, although the resulting cheese was pleasant to the consumer, it had a softer texture, bitterness and a loss of yield. The main differences in Roquefort cheese were stronger proteolysis and lower production with milk-clotting plant proteases (Everett & Auty, 2008).

Grana and Provolone pasta filata cheeses with milk-clotting plant proteases

Grana and Provolone cheeses made with cardoon extract have a softer texture and a loss of shape. These differences are explained by the activation of enzymes at stretching temperatures (80-85°C) used to make these cheeses. In contrast, chymosin is inactivated by these temperatures. An interesting example is Gran Kinara, a cheese produced in North of Italy with Grana Padano technology, but replacing the animal rennet with vegetable rennet. The use of vegetable rennet extracted from the flowers of *Cynara cardunculus*, the common wild thistle that grows spontaneously also on the mountains, has allowed to offer a rare, sought after alternative to traditional animal rennet, which can contribute decisively to the "zero lactose" of the Gran Kinara and to provide original and pleasing organoleptic characteristics (Sousa, 1998).

CONCLUSIONS

Proteases from plants are used in milk coagulation and cheese-making process especially in Mediterranean countries, Middle East, West Africa and Southern Europe. They currently do not have an industrial use because of high bitterness developed post-coagulation and lower cheese yields. However, some researches and developments were done in the understanding of their action and the control of the various parameters that influence cheese production.
The most important aspects to consider in the study of this kind of plant proteases are type of coagulant, how often the plant source is found in the spontaneous flora, enzymatic activities described by milk-clotting activity (MCA), proteolytic activity (PA) and MCA/PA ratio, as a determinant parameter, concentration of plant proteases in the plant source.
The selection of a suitable plant protease with milk-clotting activity must be based on the best MCA/PA ratio, the use of a low coagulant dose, the optimisation of various coagulation

parameters keeping under control of ripening step.

Plant proteases with milk-clotting activity have been developed to produce cheeses similar to those made with commercial rennet.

The continuous improvements made to counter their proteolytic activity should be based on a good selection of the type of milk for specific cheese variety made with these coagulants, the use of different lactic bacteria cultures which can inhibit the proteolysis.

Good results have been observed for certain types of cheeses from Mediterranean countries as Spain and Portugal, but an industrial scale remains marginal.

Further studies for identification and characterization of the purified plant milk-clotting enzyme would be interesting to start on large scale also in Romania. Future researches have to start on plants from the spontaneous Romanian flora belonging to the same families as those already studied in other countries or which are already known with proteolytic enzymatic activity.

The establishment of efficient formulations of plant milk-clotting enzyme able to replace animal rennet will also be of considerable importance for future uses on an industrial-scale for cheese production.

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