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Milk coagulation – impact on cheese

Mjölkkoagulering – inverkan på ost

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Abstract

Cheesemaking origins from “The fertile crescent”, nowadays southern Turkey, some 8000 years ago. Curd was found in young, still suckling, ruminants’ stomachs after slaughter. All characteristic cheese properties that we think of today, are thought to have evolved through accidents, unlike nowadays, where cheese production rather is planned, designed and centralized to big factories. The purpose of the essay is to, through literature, understand the correlation between; raw milk composition and coagulation properties, which milk coagulating enzymes are available and how they differ, and their joined impact on cheese properties.

The most important parameters of milk composition, in cheese perspective, is protein and fat. Animal rennet, which is most used historically, consists of two proteolytic enzymes, chymosin and pepsin, where chymosin have the most specific activity towards the κ-casein of milk. The available animal rennet cannot provide for the increased demand of cheese, substitutes are therefore necessary. Generally, the substitutes have a higher proteolytic activity, resulting in lower cheese yield and production of bitter flavoured peptides. Plant extracts from *Cynara cardunclulus* have been used historically for cheeses from sheep milk in Spain and Portugal. Microbial substitutes can be either naturally occurring proteases or produced through recombinant DNA-technology. The production of the substitutes needs to be optimized in order to produce consumer approved cheese.

Several aspects are important to control during the design of a specific cheese. It is important to be able to match a specific milk with a correct coagulant under the right conditions, effective breeding programmes are important for all species in order to influence the milk composition. In mail correspondence with an innovation group, working on developing Swedish goat rennet, the need of rennet from Swedish animals is emphasized as there might be an economical boost of farm produced cheeses.

Keywords: Milk, cheese, Milk coagulating enzymes, chymosin, pepsin, rennet, animal origin milk coagulant, microbial milk coagulant, recombinant chymosin, plant-based milk coagulant

Sammanfattning

Ost tros härstamma från ”den bördiga halvmånen” vad som nu är södra Turkiet för 8000 år sedan. Unga, fortfarande diande idisslare hade ostmassa i magen efter slakt. Alla framsteg som har lett till olika ostkaraktärer tros komma ur misstag, tvärt mot dagens produktion som snarare är designad, planerad, standardiserad och centraliserad till stora fabriker. Syftet med uppsatsen är att, genom en litteraturstudie, förstå; råmjölkens sammansättning, samt undersöka vilka olika mjölkkoagulerande enzym som finns och hur de skiljer sig. Samt redogöra för deras gemensamma inverkan på ost.

De viktigaste beståndsdelarna i mjölken, ur ostsynpunkt, är protein och fett. Animalisk löpe vilket är mest använt historiskt, består av chymosin och pepsin, två proteolytiska enzym där chymosin har en mer specifik aktivitet gentemot mjölkens κ-kasein. Dagens utbud av animalisk löpe kommer inte att kunna täcka den ökande efterfrågan på ost och substitut måste därför utforskas. Substituten har generellt en högre proteolytisk aktivitet vilket resulterar i lägre ostutbyte samt bildande av peptider med bitter smak. Växtextrakt av Ulltistel (*Cynara cardunclulus)* har använts historiskt i Spanien och Portugal för fårostar. Mikrobiella substitut kan antingen vara naturligt förekommande proteaser eller producerade genom rekombinant DNA-teknik. Det är viktigt att optimera produktion av substituten för att kunna producera konsumentgodkända ostar.

För att kunna designa en specifik ost är det viktigt att veta hur de olika parametrarna ska anpassas. Det är viktigt att kunna kombinera en viss mjölk med ett visst enzym under rätt förhållande. Ett mer kontrollerat avelsprogram, för alla arter, kan påverka mjölkens sammansättning. I mail-korrespondens med en innovationsgrupp som försöker ta fram svenskt killinglöpe poängteras behovet av svenskproducerad löpe genom den eventuella ekonomiska vinsten för gårdsmejerister.

Nyckelord: Mjölk, ost, mjölkkoagulerande enzym, chymosin, pepsin, löpe, animalisk mjölkkoagulant, mikrobiell mjölkkoagulant, rekombinant chymosin, växtbaserad mjölkkoagulant

Table of contents

Abbreviations

AP aspartic proteases
CP cysteine proteases
MFG Milk fat globules
NSLAB Non-starter lactic acid bacteria
PDO protected designation of origin
SP serine proteases
VOCs volatile organic compounds

1 Introduction

1.1 Cheesemaking

In summary, cheesemaking is a dehydration process (Everett, 2017) turning a liquid into a solid. The basic steps consist of; acidification of the milk with a starter culture. Followed by curdling of the milk, the casein micelle is affected by specific enzymes hydrolysing the peptide causing the micelles to lose their charge and repulsion force of the hydrophilic κ-casein. This results in clotting of the casein micelles due to the lost repulsion. During syneresis, the loss of liquid (whey) as the curd contracts, a gel can lose up to 90% of its volume. Or even more if pressure is applied. The gel is cut and stirred to promote whey loss. And finally ripening where flavour and texture develop.

1.1.1 History

It is believed that cheese origins from "the fertile crescent", nowadays south Turkey, some 8000 years ago (Everett, 2017). It was probably observed that young suckling ruminants had curds in their stomachs after slaughter. Before pottery, the stomach of animals was often used as a container of milk. The stomach still secreted the milk-coagulating enzymes, chymosin and pepsin, which curdled the milk. The transformation from milk to cheese facilitated transport, prolonged the "shelf-life" and diversified the diet for pre-historic humans. They could now utilize the nutrition of milk even if they were lactose intolerant as well as during the non-lactation seasons of the animals.

Almost all varieties of cheeses are thought to have evolved through accidents (Everett, 2017). The variation of cheeses was from the beginning dependent on specific microbiota called, non-starter lactic acid bacteria (NSLAB) or "houseflora", the specific composition of the milk or, accidents during storage, an uncontrolled growth of moulds. The changes became favourable and led to local traditions.

1.1.2 Nowadays

Cheese production is nowadays more standardized and centralized to big factories, though there is an increased interest in locally produced farm products. Nowadays, about 70 % of cheeses produced are made with animal rennet (Everett, 2017). However, the supply of milk coagulating enzymes originating from animals cannot be provided in the amounts needed for the increasing demand of cheese (Amira, Besbes, Attia, & Becker

2017). Substitutes are therefore necessary to explore. High price, ethical and cultural aspects are also reasons why other sources of coagulants are being researched (Shah, Mir, Paray, 2013).

Rennet types

Different enzymes have different milk-clotting activity, although, it is important to realize that conditions such as pH, calcium content and temperature also influence the clotting of milk (Andrén 2011).

Cheeses with plant-based coagulant are mainly found in the Mediterranean, and west Africa. Spain and Portugal have a great variety of cheeses from different animals and areas with *Cynara sp*. (Artichoke genus) as the coagulant. Nigeria and the republic of Benin have cheeses made with *Calotropis procera* (Sodom apple) (Shah et al. 2013).

Recombinant chymosin, produced through fermentation in transgenic microbes is banned in some countries, such as France, Germany and the Netherlands (Shah et al., 2013, consumers generally have a mistrust and a negative attitude towards GMO which also provides a place for plant-based milk coagulants on the market of cheese production.

The aim of this essay is to, through literature, understand the correlation between:

- Raw milk composition and coagulation properties.
- Which milk coagulating enzymes are available and how they differ.
- The impact on cheese properties.

The

2 Methods

book *Cheese: Chemistry, Physics and Microbiology*, Everett, D. W., 2017, have been of great assistance as my main source, due to its thoroughly and profound knowledge about cheese as well as the fact that it is newly re-published hence up to date.

Review articles have been used as a summary of general aspects. The cited articles for the current research, about milk coagulating enzymes and cheese production nowadays have been chosen partly due their time of publication.

Personal contact has been established with a group developing Swedish goat rennet, consisting of Anna-Karin Gidlund, from Gideget, a local Swedish goat dairy outside of Umeå and spokesperson of Swedish farm dairies (Svenska gårdsmejerister). Anders Olsson, CEO of Swedish rennet producer Kemikalia ® as well as the Anders Andrén, scientist at Swedish university of Agriculture (SLU).
Swedish food agency has been contacted for questions about Swedish legislations connected to the dairy market and cheese production.

Keywords: Milk, cheese, Milk coagulating enzymes, chymosin, pepsin, rennet, animal origin milk coagulant, microbial milk coagulant, recombinant chymosin, plant-based milk coagulant

3 Results

3.1 Properties of raw milk

Milk quality is important to the final cheese product. It must be free from antibiotics (Everett, 2017), as it might affect the starter culture and hence the acidification.

Milk is an exceptional medium for the growth of microorganisms. Hence, microbial hygiene and quality are of importance as not all microorganisms are wanted. The pathogens origin from poor milking hygiene, contamination from faeces, soil or human contact. (Everett, 2017). The most important genuses are; *Salmonella spp., Staphylococcus aureus*, *Listeria spp*, *E. coli (STEC)* and spore-forming bacteria such as *Bacillus spp.* and *Clostridium*. The spore-forming bacteria might survive pasteurization otherwise, spoilage organisms tend to be post-pasteurization. Psychotropic strains which survives and grows in cold temperatures are also considered unwanted. All affecting the milk quality negatively.

3.1.1 Properties

All aspects of cheesemaking are affected by the quality and composition of the milk (Everett, 2017). Especially the protein, fat and calcium content as well as the pH. A higher content of protein and fat results in improved coagulation properties; reduced rennet coagulation time, increased curd firmness and cheese yield.

Protein

Increased protein content (2 - 4% of total content) results in improved milk coagulating properties. However, increased protein content to 5 – 8 % of total content may cause rupture of curds during cutting due to too high firmness. The consequence of this is loss of protein and fat, hence lower cheese yield and poor quality (Everett, 2017).

The primary cheese proteins are the caseins. Caseins are a family of four different phosphoproteins; αs1-, αs2-, β- and κ-casein. These proteins differ slightly in phosphor content which effect the binding of Ca (Kumar, Grover, Sharma, & Batish 2010, et al., 2010). The proteins are formed as spherical micelles with hydrophilic κ-casein extending from the structure. (Everett, 2017). The composition of the caseins has an impact on coagulation. A higher content of αs2-, β-caseins and low κ-casein have a lower coagulating rate. During acidification the hydrophilic κ-casein loses their charge and coagulation commence. During enzymatic coagulating the κ-casein are hydrolysed and the micelles aggregate forming a stronger coagulate than during acidification.

The size of the casein micelles has an impact on cheese making as well. Breed, feed and post-translation modifications causes variation in size. Milk with smaller micelles aggregates faster and have firmer coagulum. Smaller micelles have a higher level of κ-casein, which can improve the bridging of proteins and calcium, resulting in a shorter coagulating time and firmer gel (Everett, 2017). Several small casein micelles have an increased surface area compared with fewer large micelles. The increased area leads to greater availability of hydrolytic cleavage, hence, faster aggregation. Smaller micelles also provided better cheese yield (Logan, Leis, Day, Kihlman Øiseth, Puvanenthiran & Augustin, 2015). These results show that it is the size of the micelles rather than the protein concentration that is important.

Proteolysis during ripening is important for texture and flavour. Peptides have a bitter taste, however, proteolysis also produces free amino acids which are important precursors to catabolic reactions producing volatile flavour compounds (Everett, 2017).

Fat content

Fat is important for several reasons, flavour compounds are generally more fat-soluble, triglycerides are important precursors of several flavour compounds. Fat also affects the rheological properties of cheese and the dispersion in the mouth. (Everett, 2017).

The fat in milk are fused together in milk fat globules (MFG). The sizes of the globules vary depending on the individual animal, the feed, stage of lactation, seasonal differences and milking frequency (Logan, et al., 2015). “Cheddar” and “Emmental” cheeses made with bovine milk with smaller MFGs showed improved texture, flavour and sensory characteristics. Larger MFG results in firmer gels, however, if the MFGs are larger than the micropores of the casein network, they might act disruptive to the gel matrix. Desired MFG size can either be obtained naturally or through mechanical separation.

A higher fat content at a constant protein level of 3.3 % reduces the coagulating time and increased the curd firmness (Everett, 2017). However, a too high fat content might dilute the casein and prevents the network formation hence, coagulating. A higher fat content slows the syneresis rate.

Fatty acid (FA) composition has a direct impact of flavour of cheese (Everett, 2017). C_4-C_{10} acids are strongly flavoured. The composition of FAs gives the milk the characteristic flavour of different animals. Buffalo milk have a high amount of C4:0, C16:0, and C18:0 but C6:0 - C14:0, and C14:1 FAs are higher in bovine milk (Everett, 2017). Goat and sheep milk contains branched FAs, and a high content of medium-length FA, which strongly contributes to the specific flavour. Free fatty acids (FFA) are depending on pH, being more volatile at pH below their pKa (5 for short chain fatty acids). Hence, contributing more to flavour in low pH cheeses.

The acids are also precursors to volatile flavour compounds; esters, lactones, secondary alcohols and methyl ketones as well as substrates to ripening bacteria (Everett, 2017). Esters and methyl ketones contributes to the fruity notes of cheeses. Lactones contributes to the "buttery" characteristics of cheese as well as the overall flavour of cheese. Secondary alcohols are involved in overall flavour of blue cheeses.

Lactose

Most lactose is lost during syneresis. The residues are important for flavour development during ripening as it is a precursor for several volatile flavour compounds as well as a substrate for LABs (Everett, 2017). Lactate influences the pH, the growth of secondary flora and the activity of ripening enzymes. Lactate is a substrate for several reactions during ripening, however, not everyone is desired. NSLAB (non-starter lactic acid bacteria) flora, can transform it into Ca-D-lactate crystals which appear as white specs on the surface of mature cheese. *Clostridium tyrobutyricum* can cause "late gas blowing" by producing butyrate, CO_2 and H_2 from lactate. Good hygiene, removal of the spores or addition of $NaNO_3$ or lysozyme can eliminate the problem.

Calcium content

Milk with higher calcium content has a shorter rennet coagulation time. (Everett, 2017). An increase of Ca^{2+} activity can lead to faster aggregation of the casein micelles (Amira, et al., 2017). Free calcium in milk is important for the aggregation of casein micelles (Everett, 2017). The addition of calcium chloride ($CaCl_2$) has showed to improve the coagulation activity of different coagulants in an experimental design to determine differences between recombinant lamb chymosin, recombinant calf chymosin and cow rennet (Kumar, et al., 2010).

pH

Many of the milk coagulating enzymes are more active in the acidic pH range (Kumar, et al., 2010). The acidification of milk from starter cultures affects curd formation in several aspects (Everett, 2017). For example, the activity of the coagulating enzymes, strength of the curd, cheese yield as well as the defence against pathogens and spoilage microorganisms. The enzymatic effect on curd formation from different coagulants depends on pH. However, milk pH can also be affected by coagulant pH (Amira et al., 2017).

Low pH (3 - 4) decreases the curd yield due to increased hydrolytic activity. High pH (6.6 – 6.7) reduces the clotting time and curd firmness (Kumar, et al 2010). At pH 5, the calcium ion activity is increased which lowers the negative charge of the micelles. (Everett, 2017) During the same pH the electrostatic repulsion of the hydrophilic κ-casein are lost.

Seasonal variations

Milk late in lactation shows impaired rennet coagulation and syneretic properties during cheese making. (Everett, 2017). The changes might be due to reduced volume, hence increased concentration of the solids. The ratio of protein and fat content varies during a lactation, affecting the cheese composition, for these reasons standardization is the norm. Seasonal variation has shown differences in MFG size (Logan, et al., 2015).

Beata Nalepa et al. (2018) examined raw milk composition, the microbiota and volatile organic compounds (VOC), over 22 months from September 2012 to July 2014 in north-eastern Poland. The highest content of VOCs was identified in autumn. However, the highest diversity was detected in the spring and summer season. In autumn and winter, only FFA was detected. The bacterial composition in the raw milk varied over seasons. The *Enterobacteriaceae* family had higher numbers in the summer and autumn seasons. The lactococci had its peak in autumn. Correlation between microorganisms and specific VOCs could be observed. Identifying specific VOCs could lead to better understanding of the influence on microbes during production of cheese. The microbes may release lipolytic and proteolytic enzymes as well as VOCs causing defects in aspects such as texture, flavour and appearance in cheese.

When comparing their work with Italian counterpart, differences were detected due to climate and hence different feeding regime. Cows in Poland are fed hay and silage during autumn and winter when Italian cows still can graze outside. (Nalepa, et al., 2018).

Seasonal variation and feeding regime is of great importance in goat cheese. A diet high in forage resulted in higher fat content in milk and cheese. The volatile flavour compounds changed through the seasons, the differences were less pronounced in cheese than in the milk (Everett, 2017).

3.1.2 Differences between species

The differences between the species origins from the need of the species young.

Table 1. Differences in milk properties between different species.

Properties	**Cow**	**Buffalo**	**Sheep**	**Goat**
Water %	87.2	83.2	83.7	88.0
Protein %	3.4	4.7	5.5	3.1
Fat %	4.0	6.7	5.3	3.5
Lactose %	4.8		4.6	4.6
Calcium mg/100 g	117.5 *	196.5*		
Phosphorus mM	19.2	27.2		
Total ash content %			0.9	0.79

*Sources Everett, 2017 and Jensen 1995. Not all values were collected from the same source, resulting in empty squares rather than un-correct number. Cow and buffalo from Everett 2017. Sheep and goat from W.L Hurley ,2010. * Mean value from Everett, 2017 p 1095.*

Buffalo

Buffalo milk stands for 11% of the global milk production (Everett, 2017). Buffalo milk contains higher levels of all types of casein proteins and whey proteins compared with bovine milk (Everett, 2017). The casein micelles and the MFGs are larger than in bovine.

The high content of caseins as well as more inorganic phosphate results in slower acidification.

Milk from small ruminants

Many cheeses are made with a mixture of goat/sheep milk and sometimes cow (Everett, 2017). Most are produced in southern Europe and are protected by PDO (Protected designation of origin) status. The protein content is the most important difference between cow’s and sheep’s milk (Shah, et al. 2013).

The Swedish Landrace goats carries a mutation resulting in milk with low or non α_{S1}-casein content. The lack of α_{S1}-casein gives low cheese yield hence low economic value of the herds (Johansson, Högberg & Andrén 2015). Apart from low cheese yield the low content of α_{S1}-casein content also contributes to less protein retention and less firm curd (Everett, 2017).

Goat and sheep milk contains both branched and linear fatty acids which contributes to the specific flavour of the cheeses (Everett, 2017). The specific taste of goat cheeses origins from medium length FAs. Goat cheeses from animals fed grass had higher acceptability compared with animals fed legumes due to specific number of VOCs.

3.1.3 Milk treatments

Ultra-filtration

Ultra-filtration is a process to standardise or to increase the protein content of milk by removing water (Amira. et al 2017). It reduces the fat retention as the protein content is increased (Everett, 2017). Because of the higher protein content ultra-filtration increases the cheese yield and results in cheeses with a lower moisture. The seasonal variations of milk composition can be equalized by this process.

Standardization

The right ratio between fat and protein should be adjusted to the desired levels. The level of fat is standardized dependently of the cheese type. Standardization results in a consistent production and improve quality of the final product (Everett, 2017).

Pasteurization

A heat treatment to establish microbial hygiene, kill unwanted bacteria, pathogens, spoilage microorganisms as well as inactivate certain lipolytic enzymes (Everett, 2017). Pasteurization facilitates cheesemaking as it evens out the quality of milk. Pasteurized cheese ripens more slowly and tend to develop less intense flavours, probably due to the inactivation of lipolytic enzymes and elimination of the native microbiota.

3.2 Milk coagulating enzymes

There are several different milk coagulating enzymes where animal originated rennet is the most common and most used historically. Artisanal cheeses from the Mediterranean and west Africa have used different plant extracts as coagulant (Andrén, 2011). A higher demand of cheeses results in a shortage of chymosin which is one of the reasons why other alternatives must be explored. The shortage of chymosin also origins from a more effective breeding programme resulting in higher milk yield per cow hence fewer calves to extract chymosin from. A higher demand for beef have led to a higher slaughtering age for the calves, hence lower amount of chymosin (Andrén, 2011). The bovine spongiform encephalopathy (BSE) has reduced both the supply as well as the need for animal rennet (Kumar, et al.,2010).

3.2.1 Animal origin

Chymosin and pepsin

Rennet is a mixture of chymosin and pepsin. Chymosin is a proteolytic enzyme from the young ruminants. It curdles the milk which enhances the nutritional value as it prolongs the time in the intestinal allowing the young animal to extract more nutrients. The amount of chymosin declines only days after birth and becomes replaced by pepsin (Vaclavik & Christian 2008). The ratio between chymosin and pepsin depends on age and feeding regime (Andrén, 2011), (Table 2). Chymosin is a highly specific coagulating enzyme whereas pepsin have a general proteolytic activity which results in a lower cheese yield. Chymosin cleaves the peptide bond between Phe 105 and Met 106 of the κ-casein. It inactivates the κ-casein converting it into insoluble para-κ-casein which forms a curd with calcium. Chymosin is also responsible for textural changes and flavour development during ripening (Kumar, et al., 2010). Both chymosin and pepsin are produced as inactive zymogens and are activated by the low pH in the abomasa (Andrén, 2011) The inactive proenzymes are important for targeting, folding of the protein and control of activation of the zymogens (Kumar et al., 2010).

Chymosin exists in three different genetic forms, A, B and C. B is the most common and C the rarest. The C variant is the newest found and have yet to analyse its amino acid sequence. Only one amino acid residue differentiates the A from B, A have aspartic acid in positions 244 where B have glycine. Chymosin A has a 50 % higher specific milk-clotting activity than chymosin B (Andrén, 2011). Chymosin C have the highest milk clotting activity (Everett, 2017).

Table 2. *Percentage of chymosin and pepsin depending on age and feed.*

Age (months)	Feed	Chymosin %	Pepsin %	Reference
<3	Milk fed	90	10	Andrén 2011
6	Milk and pasture fed	75	25	Andrén 2011
6	Concentrate fed	30	70	Andrén 2011
>24	Concentrate and hay fed	Trace	100	Andrén 2011
Bacterial		100*		Kumar 2010

International milk clothing units (IMCU) correspond to 100% chymosin.

The flavour and texture development of cheeses is determined by the activity of the rennet, which is determined by the production as well as the raw materials (Everett, 2017). The activity is commonly measured in International Milk Clothing Units (IMCU). Most commercial rennet has no lipolytic activity.

Production

Until the 1800, all cheeses were produced on farms using fresh or dried extracts from the calves' abomasa. In the 1850 small dairies were set up which required greater amount of coagulating enzymes. The increasing demand led to industrial production of rennet (Andrén, 2011). Nowadays, animal rennet is produced primarily from frozen abomasa. It is grinded, and the zymogens are extracted with a NaCl brine solution. The zymogens are activated by lowering the pH to 2 for 1 hour and then adjusting to about pH 5.5 before filtering and concentrating the extract. The extract is further filtered to remove bacteria and the concentration of NaCl is then increased to about 20 g per 100 g. As a final step, the rennet is diluted to a certain strength. Modern production increases the activity and yield of chymosin (Everett, 2017).

Rennet paste

Lamb or goat paste, a special animal rennet, extracted from dried milk-filled stomachs of slaughtered lambs and kids. Produces cheeses with strong flavours due to a high amount of lipolytic enzymes increasing the free fatty acids during ripening (Everett, 2017).

Differences between species

Chymosin from different species show higher milk-clotting activity for its own species' milk. Bovine chymosin shows a higher activity for bovine milk, where ovine and caprine chymosin show a lower activity than with sheep and goat milk. Although recombinant camel chymosin shows a 70 % higher specific milk-clotting activity for bovine milk than bovine chymosin (Andrén, 2011). Camel chymosin has a lower general proteolytic activity than bovine chymosin (Everett, 2017). Camel chymosin is more thermostable than buffalo and goat chymosin which are equally stable (Kumar, et al.,2010).

The molecular weight of the enzyme differs between species (Kumar, et al., 2010). The lamb chymosin has similar size, sensitivity to calcium and pH but lower temperature dependency compared with bovine chymosin. Kid chymosin has a higher thermostability and a pH-optimum at 5.5. Buffalo chymosin is identical to bovine but have its highest activity at 30^{o}C. (Everett, 2017). The pH and temperature optimum of chymosin vary depending on species, substrate, the method of production as well as on each other (Kumar, et al., 2010). (See table 3 for further details).

Table 3. *Differences in pH and temperature optimum at maximum relative milk clotting activity (RMCA). Optimum between different origin of chymosin, animal derived or through fermentation, or enzymes of microbial or plant origin*

Origin	pH opt. (RMCA)	Temp. opt. at RMCA ^{o}C

Bovine	6.4 – 6.5	45
Fermented bovine	6.6 – 6.8	45
Buffalo	5.5	55
Buffalo	5.8 – 6.0	45
Goat	5.5	65
Fermented sheep	6.6 – 6.8	40
Rhizmucor miehei	6.6 – 6.8	60
Cynara cardunclus	6.0	37

Sampled from Kumar et al 2010.

3.2.2 Plant origin

The coagulants from plants are natural proteases, involved in plant development which have been found to have milk clotting activity. The milk clotting activity differs between plants as well as their parts from which the enzymes are extracted (Amira, et al., 2017). Many cheeses produced with enzymes from plants have bitter flavours due to excessive proteolytic activity and a production of bitter peptides. This proteolytic activity also has an impact on cheese yield as there is a higher protein loss. For these reasons the industrial use is limited. Plant extracts as milk coagulants are not suitable for long ripened cheeses (Everett, 2017). Plant proteases are divided into various groups based on the catalytic mechanism (Amira, et al., 2017).

<u>Aspartic proteases</u> (AP), are the most common type of milk coagulating enzymes and exists in a great number of plants (Shah et al., 2013). They are most effective in acidic pH. The enzyme-substrate interactions are important, APs easily hydrolyses the Phe-Met-bond in pentapeptides but not in di-, tri-, or tetrapeptides. Showing that the length and residues located around the bond are important (Amira, et al., 2017). *Cynara cardunculus* (Cardoon) is the most commonly used plant as a coagulant in cheesemaking in Spain and Portugal (see table 4 for examples). *C. carduncculus* produces several different aspartic proteases such as cyprosins and cardosin A, B, and C which share hydrolytic characteristics with chymosin and pepsin. The enzymes have been found in flowers but not in leaves or seeds (Shah, et al., 2013).

<u>Serine proteases</u> (SP) with milk coagulating properties have been found and extracted from a wide variety of taxonomic groups as well as from different physiological features (Shah et al. 2013). It is most abundant in fruits but have been extracted from latex, flowers, leaves, roots, stems and seeds. SP exists in trees, legumes, crops and herbs. Lettucine has been extracted from *Lactuca satvia*, Religiosins have been extracted from *Ficus religiosa.*

<u>Cysteine proteases</u> (CP) exists in a wide variety of plants and in all parts but most abundant in fruits (Shah, et al., 2013). Actinidain, a cysteine protease from kiwi fruits, degrades undesired cream milk proteins, resulting in good taste of “Mozzarella” cheeses (Amira, et al., 2017).

Gels with chymosin are more sensitive to temperature changes than gels with plant coagulants (Amira, et al., 2017). A low temperature of milk leads to a higher efficiency of the gelation process with plant coagulants as well as a lower general proteolytic activity.

Production of the plant coagulants

Plant extracts are seldom used in commercial production but rather for local and artisanal cheeses. As the production is not yet standardized the strength and quality of the coagulants are not ideal (Everett, 2017). However, attempts at standardization of plant extracts are underway. A way of optimizing the enzyme during extraction is controlling the pH, as it affects the strength of the final coagulant.

Plant proteases used for milk clotting are either produced through aqueous maceration from their natural source or *in vitro* (Shah, et al., 2013). Different methods of maceration consist of grinding dried flowers and mixing with salt to make a paste or soaking the parts of the plant in water and filtrating the extract. The extracts are often purified. Flowers of *C. cardunculus* are purified through perception with ammonium sulphate. *In vitro* production, cell suspension or callus from the plants are homogenised, centrifuged and freeze-dried before extraction of the crude protein. The in vitro techniques have advantages as a higher yield of enzymes can be obtained as well as overcoming the difficulties of extraction from natural sources.

Table 4. *Table over Cheeses made with plant extracts as coagulants*

Cheese	Milk	Coagulant	Protease	Origin	Reference
Serra da Estrela	Sheep	*C. cardunclus*	AP	Portugal	Amira et al. 2017
Torta del Casar	Sheep	*Cynara spp.*	AP	Spain	Shah et al. 2013
Los Ibores	Goat	*Cynara spp.*	AP	Spain	Shah et al. 2013
Flor de Guia	Goat/cow	*Cynara spp.*	AP	Spain	Shah et al. 2013
Wara	Cow	*Calotropis procera*	CP	Nigeria	Shah et al. 2013
Peshwari	Cow	*Zingiber officinale*	CP	India	Amira et al. 2017

AP – Aspartic proteases, CP – cysteine proteases.

3.2.3 Microbial origin

Coagulants of microbial origin can either be naturally occurring proteolytic enzymes or recombinant chymosin produced through fermentation.

Rhizomucor miehei, a mould with an aspartic protease with milk coagulating properties used by several different producers such as; Chr. Hansen's Lab, Novo Industries Ltd.

etc. (Kumar, et al., 2010). It is the dominating coagulant on the microbial market (Andrén, 2011).

Recombinant chymosin

Recombinant chymosin have several advantages such as predictable coagulation behaviour, low proteolytic activity, hence higher yield since it its 100% pure enzyme. Due to the extensive knowledge about the genome, *E. coli* have been used as host organism of recombinant chymosin (Kumar, et al., 2010). The perks of its ability to grow rapidly and on inexpensive media makes it a suitable protein factory. As well as it large number of cloning vectors and mutant host strains.

Yeast has several advantages as a factory of proteins. Low economic cost during cultivation, extensive knowledge about fermentation and the physiology as well as the genetics of the organism (Kumar et al 2010). *Pichia pastoris* is a commonly used host for protein expression due to the previous mentioned reasons as well as; ease of manipulation, high expression level and its ability to perform eukaryotic post-translation modification. It has been used for the expression of both goat and bovine chymosin.

Production of recombinant chymosin

To express a eukaryotic gene in a prokaryotic host, the mRNA is reversed transcribed to cDNA from which the introns are spliced out. The new gene is then cloned into the host and expressed (Kumar. et al., 2010).

The calf chymosin gene was cloned into *E. coli* k-12, the product was identical to the original, the preparation did not make it unsafe (Kumar, et al., 2010). The production of recombinant pro-chymosin are expressed in inclusion bodies in *E. coli*. Inclusion bodies are cytoplasmic granules often used for storage in bacteria. Production of inclusion bodies could be improved by selection of host, modification of plasmid and optimizing the cultivation conditions. To enhance the yield of chymosin the processing of inclusion bodies should optimize denaturation and renaturation to refold and solubilize the protein. Kumar et al. (2010) further discusses the major problem with inclusion bodies, the poor recovery of the recombinant chymosin in active form in downstream processing which is a major cost during manufacturing. Comparison between animal rennet and recombinant chymosin showed no significant differences in properties such as cheese yield, smell, taste or texture These findings show that the enzymes behave in the same way.

3.3 Impact on cheese

The combination of a specific coagulant, its specific optimum at different pH and temperature, a correct dose of the coagulant, as well as a suitable type of milk are important to produce a specific cheese with favourable characterises. Cheese with plant extracts as coagulants produces slightly bitter flavour due to excessive proteolytic activity. (Everett, 2017). However, this proteolysis leads to a creamy texture, almost liquid. For example, Serra de Estrela from Portugal, produced with *C. cardunculus*, served in wooden boxes, meant to be eaten with a spoon.

Comparison between milk gels with plant coagulants and chymosin showed that the plant coagulants produced less firm gels (Amira, et al., 2017). The gel´s firmness also depends on other factors such as pH, calcium content and temperature (Andrén, 2011), as well as the coagulant dose, previous treatments of the milk (heat treatment or ultra-filtration) (Amira, et al., 2017). The control of these parameters is important to know in the design of a specific cheese. The combination of low pH and low amount of plant enzyme may lower the proteolytic activity which affects both flavour and texture. The low pH reduces the electrostatic repulsion of the casein micelles which promotes the aggregation.

3.3.1 Texture

The feeding regime has an impact on cheesemaking. Low quality and insufficient amount of the feed, results in low milk and cheese yield as well as cheeses with an undesirable high moisture content. (Everett, 2017). A feed high in supplements resulted in cheeses with better textural and sensory properties compared to milk from cows with insufficient supplements. However, feed with too high level of supplements resulted in cheeses with a "hard and gummy texture". The moisture content depends strongly on syneresis (Everett, 2017). Cheeses with high moisture are not cut at all, only placed in moulds. The size of MFGs and casein micelles have an impact on the gel firmness. Large fat globules and small micelles resulted in the firmest curd.

Defects in texture from plant coagulants compared with chymosin are due to residual coagulant and a high proteolysis (Amira, et al., 2017). Curds with kiwi extract had similar rheological properties to those produced with chymosin, elasticity, cohesion and chewiness were similar.

3.3.2 Flavour and taste

As previously stated, plant extracts have a higher proteolytic activity than chymosin, which leads to lower cheese yield and production of bitter flavours (Everett, 2017). However, using purified enzymes the bitterness might be reduced (Amira, et al., 2017). Salting of the cheeses for a longer period (40 h compared to 30h) also reduced the bitterness of plant coagulants. "Mozzarella" cheeses with kiwi extract had no bitter taste, test groups did not notice any difference between commercial mozzarella and cheeses made with kiwi extract (Amira, et al., 2017).

Cheeses produced with bovine milk and coagulant extracts from *Cynara cardunculus* gives an undesirable bitterness. Whereas cheeses produced with *C. cardunculus* but with ovine milk did not. (Amira, et al., 2017). *C. cardunculus.* had higher proteolytic activity towards ovine caseins compared with bovine (Kumar., et al. 2010). However, if the bovine milk were modified to resemble ovine milk in protein content, for example through ultra-filtration, the negative aspects could be reduced (Shah, et al., 2013). These findings show that is important to know which type of milk can be matched with what enzyme to produce a specific cheese.

Many flavour compounds are produced during ripening. Lipolysis is important for flavour development. Depending on type of type of ripening method and which microbes that are present. Gouda and Cheddar, internally ripened with LAB have a low lipolysis but mould-ripened Camembert cheeses have high (Everett, 2017). Type of milk, milk fat composition and previous milk treatments such as homogenization may also affect the lipolysis.

3.3.3 Colour

Colour is an important requirement from consumers. Cheeses from West Africa produced with *Calotropis procera* coagulant had a green colour. The colour depended on the freshness of the leaves and the stems from which the coagulant was extracted (Amira, et al., 2017).

Comparison studies between cheeses with calf rennet and *C. cardunculus* have shown that the plant extract gave an undesired dark yellow colour compared to animal rennet (Amira, et al., 2017). The use of ultra-filtrated milk resulted in a less yellow colour in cheeses produced with *C. cardunculus* coagulant.

Cows fed silage, produced milk containing more colour and volatile compounds, resulting in a more yellowish colour of the cheese compared with than cows fed hay. (Everett, 2017).

4 Discussion

The quality of cheese depends on several factors; the composition of raw milk, animal health, seasonal factors as well as properties during cheesemaking. Somatic cell count is an important parameter of milk quality. High cell count is often associated with increased activity of endogenous proteolytic enzymes, resulting in lower cheese yield and higher moisture content (Everett, 2017). Too high cell count makes the milk unfit for human consumption and an economical loss for the farmer. The numbers are elevated during mastitis, under late lactation and affects negatively by the lactation number. The feed has an important impact on cheese, affecting colour, taste as well as texture.

Breeding programmes are important for all species, as there are several aspects which can be controlled to produce the perfect cheese. For example; the difference between the genetic variation of A, B or C chymosin, the size of MFG and casein micelles depends partly on genetics. Johansson et al (2015) discusses the need of a controlled breeding programme of the Swedish Landrace as several goats carries a mutation which leads to low casein content in the milk, hence lower cheese yield and low economic value of Swedish goat cheese.

The use of animal rennet may be limited for religious, cultural and ethical reasons (Kumar et al. 2010). Even though plant-based coagulants are limited in their industrial use, Amira et al (2017), concludes that the extended knowledge about substitutes of rennet are important for future cheesemaking. Knowing how other physiochemical parameters effects the gels and cheeses, facilitates the design of how to match a specific milk with a specific coagulant. Purified plant extracts are important, as it improves several aspects of cheese quality. Amira et al (2017) also draws the conclusion that both colour and taste can be improved. The colour effect might be reduced as the number of polyphenols are reduced. The production of bitter peptides might also be reduced as the amount of other proteolytic enzymes are reduced. However, the production needs to be optimized and standardized to produce a high yield of favourable extracts with uniform properties.

There are advantages with microbial enzymes, both naturally occurring and recombinant variations. They are cheap to produce and have no requirements during production. Kumar et al (2010) concludes that the expression and activity of recombinant enzymes could be even further improved by exploiting genetic and engineering tools. Enzymes from transgenic organisms cannot be used by dairies with KRAV® or other certifications, nor in some countries due to legislation. GMOs also tend to have a lower acceptance level from consumers.

There is no European legislation regulating which enzymes can be used in cheese production, yet. There is one underway, in the meantime, cheesemakers report which enzymes are used and a list of approved enzymes is formed. Cheesemakers are allowed to use any enzyme as long as they can prove them to be safe for consumer, that there is a technological need and that it’s not confuses consumers. Despite the absence of legislation in the EU there are however some PDO (protected designation of origin) requirements, traditional cheeses that must be made with a specific coagulant.

In personal communication (mail contact) Anders Andrén, a scientist at Swedish Agricultural University, points at the need for rennet from different species made from Swedish animals. It would improve the production of sheep and goat cheeses at small scale dairies. An improvement of the cheeses would im the economic value of farm produced goods which could improve the socioeconomic status of the countryside. However, there are obstacles with the Swedish legislation. The Swedish food Agency cannot easily accept the production of animal rennet with food classification from raw material (*abomasum*) which are not classified as food. Also, due to the low economic value of cheeses from goat and sheep there are problems finding a producer willing to produce goat or lamb rennet, resulting in a vicious circle without progress. Anders Olsson, CEO of Kemikalia ® a Swedish rennet producer, also criticizes the bureaucracy of Swedish legislation as well as points at the increased need of number of animals needed to produce enough amount of rennet from goat. Ca 10-15 times more compared with bovine rennet.

Anna-Karin Gidlund, owner of a Swedish goat dairy *Gide get* and spokesperson of “Swedish farm dairies” (Sveriges gårdsmejerister) is also involved in the development of Swedish rennet. She mentions the ethical dilemma of using animal rennet from countries with uncontrolled slaughtering routines.

It is important to be critical of the sources and of the information provided of this group of persons working on developing Swedish rennet from goat. There is a need as it might improve not only the production and quality of farm produced cheeses, but also boost the socioeconomical status of the countryside, as there is evidence that PDO cheeses from southern Europe has. But at the same time it is important to remember how crucial the Swedish food legislation is to provide safe food, even though they might be criticized of bureaucracy.

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