ASPECTS CONCERNING COAGULATION ENZYMES AND DIFFERENT INDUCING PARAMETERS FOR MILK CURDLING PROCESS

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Abstract: *Cheese making is signing up together all word researcher efforts to find brand new sources of animal protein. One of main sequences in cheese fabrication, the coagulation, could be made even through acidification or biocatalysis. The clotting of milk by enzymes is a key passage in cheese making that, markedly, could affect the characteristics of produced cheese. They differ both on their origin: animal, vegetable, microbial and recombinant from genetically modified microorganism, and their physical state, liquid, powder or paste. The coagulation enzymes used in the cheese industry for milk clotting, being the oldest known application of enzymes in food industry. This paper presents some aspects from a comparative study between different useful types of coagulation enzymes: one of them is from animal origin, that consists pepsine and chymosin and the others two are microbial origin enzymatic prepared, one from Bacillus subtilis and the other one from Aspergillus niger var.awamori. There was also studied various agents of influence and the raw material used was cow milk and sheep milk. Milk coagulation properties could vary greatly among animal species (cattle, buffalo, heep, and goat), among breeds within species, and among individuals. Variations occur in coagulation time, extent of curd firming, and development of syneresis.*

Keywords: *clotting enzymes, cheese, coagulation activity*

1. Introduction

In the last decade, the fraction of total milk destined to cheese production has increased by about 10% in the European Union, and it is now slightly higher than 50% in the former continent and slightly lower in the latter. An increase in the amount of milk used to manufacture cheese has been reported in other European countries, Oceania, and Latin America, and the amount used is much lower in Asia and Africa. Milk coagulation properties vary greatly among ruminant species, among breeds within species, and among individuals. Variations occur in coagulation time, extent of curd firming, and development of syneresis [1,2].

Milk coagulation properties receive much attention in dairy science and industry, mainly because the amount of milk used to manufacture cheese is growing worldwide, and several works have confirmed the importance of milk coagulation properties in terms of cheese processing, yield, and quality [1,3,4]. Milk proteins and their genetic variants strongly affect milk coagulation properties [1,3].

Nowdays, milk clotting properties receive a lot of attention, not only in dairy science but also in other fields of industry, mainly because the amount of milk used in cheese making processes, and also from the point of view of cheese processing, yield, and quality [5].

The coagulation represents one of the capital steps in cheese making that, markedly, could affect the characteristics of produced cheese. There are known two modalities of obtaining: by means of acids,

resulting in soft curd, with low calcium content and high acidity (iso-electrodeposition); by means of coagulation enzymes, so called biocatalysis. Generally, for main types of cheese, it is known mixing types of coagulation. Enzymatic coagulation could uses clotting enzymes animal, vegetable or microbial origin by different physical state, liquid, powder or paste (table 1). Is is very important knowing the nature of coagulation enzymes, that could have a distinct influence through cheese processing.

The animal and microbial enzymes are actually, the most spent.

The main influence parameters for curdling process are presented forward:

- *the temperature*, optimal in the range 40 - 41° C and 25-42 ° C in practice;
- *the quantity of calcium salts*, that may influence the coagulation time and also the quality of curdle; time of coagulation growth with calcium salts decline and also it would have a flossy structure;
- *the level of milk acidity* - the coagulation rate raises up in the same time with ponderate augmenting of acidity;
- *quantity of coagulation enzyme* determines the rate of the process, in certain limits;
- *milk chemical composition*, a high dry matter content, respectively, reffers by enzyme quantity augmentation in order to maintain a certain period of time for coagulation and a normal consistency for the curd;
- *the preliminary heating treatment* for milk could extend the time of coagulation;
- *milk homogenization* could decrease the coagulation time, because of aggregation degree casein augmentation. [6,7]

Table 1

Sources for coagulation enzymes [2, 6, 7, 8, 10]

Origin		Enzymes
Animal	***Ruminant species***	
	Calf	Rennine + pepsine
	Billy goat	88-94% 6-12%
	Lamb	
	Adult cattle	Pepsine + Rennine
	Single gastric species	90-94% 6-10%
	Pig	Pepsine
	Birds	
	Chicken	Pepsine
Vegetable	Fig (juice)	Ficine
	Pineapple (stem)	Bromelline
	Artichoke	
	Carica papaya	Papaine
	Pumpkin, watermelon	
	Calotropis procera	Callotropine
	Cynara cardunculus flowers	
Moulds	*Rhizomucor miehei*	Protease
	Rhizomucor pusillus	Protease
	Cryphonectria parasitica	Protease
	Aspergillus niger v. awamori	„Genetic”rennine
	Thermomucur	
Yeasts	*Kluyveromyces marxianus v. lactis*	„Genetic”rennine
Bacteria	*Escherichia coli*	„Genetic”rennine
	Bacillus subtilis	

In this study were used three different origin types of coagulation enzymes:

- one of animal extraction (consist of pepsine and rennine);
- two microbial type curd:
  - enzyme prepared from *Bacillus subtiliss* biomass (rennine);
  - enzyme prepared from *Aspergillus niger var. awamori* (rennine);

The most used clot derives from the stomach of unweaned calves. It is available as liquid or powder form. In many countries, where sheep and goat breeding is largely diffused, is largely common the use of lamb or kid rennet paste [6]. The active principle consists of chymosin 88-94% and pepsin 6-12% [7]. The specific milk-curdling enzyme present in animal clot is the chymosin (EC 3.4.23.4), that is an acid protease enzyme. Apart from this, a different number of generic proteases, such as pepsin A (EC 3.4.23.1), gastricsin (or pepsin B, or pepsin C) (EC 3.4.23.3) are also present. Milk-clotting enzymes, other than rennet, are called coagulants and are represented by fermentation produced chymosin, which is 100% calf chymosin produced by recombinant DNA technology involving *Aspergillus niger*, *Kluyveromyces lactis* or *Escherichia coli* and by different microbial coagulants, especially the ones from *Rhizomucor miehei*, *Rhizomucor pusillus* and *Cryphonectria parasitica*, *Thermomucor* [8].

The most important difference between calf rennet and lamb or kid rennet prepared is the presence of lipolytic enzymes that are denatured during the activation process of chymosin and pepsin zymogens.

There are few aspects with significant influence through clotting activity, as forward:

- small quantity of enzyme;
- the presence of sunlight or artificial light;
- intense shaking of solution and foam presence;
- overheating, 60°C;
- pH = 6.6-7.4.

Microbial origin rennet, generally, could have intensified proteolytic activity, relative with animal origin rennet, that have bring forward cheese making. They have also revealed higher thermal stability, beside animal or vegetable origin enzyme prepared, but sensorial qualities are constantly under the performances achieved by animal origin enzymes [9, 10].

There are requested some general aspects concerning purity, antibiotic residues absence, inocuity and also a higher level for coagulation activity, for enzymatic prepared. The coagulation capacity is a ratio between enzymatic prepared volume and milk volume in the time of coagulation, in standard conditions [7].

The experimental study consist of comparison between three types of clotting enzymes for cheese making and also the aspects with significant influence to the coagulation process.

2. Experimental

We have studied three rennet type, delivered from a local milk producer:

P_1 – microbial origin coagulation enzymes, from *Bacillus subtilis* biomass, that contains chymosine;

P_2 – animal origin rennet (lamb abomasums), that contain chymosine;

P_3 – clotting enzymes from submerged fermentation of a vegetable substrate with *Aspergillius niger var. awamori*.

The coagulation process has been studied upon two milk samples, their characteristics being related in Table 2.

Table 2
Physico-chemical parameters for milk samples

Physical-chemical characteristics	Cow-milk (L_1)	Sheep-milk (L_2)
Fat content, %	4.02	8.01
Protein, %	3.28	4.85
Acidity, °T	17	22
pH	6.6	6.2

The physical-chemical characteristics for two milk samples were achieved both by Lactoscope method and verified by classical methods (Gerber, Kjeldahl, titration). The coagulation activity was determined by Soxhlet method for all three types of rennet and then the influence of rennet quantity and the coagulation temperature upon the process were studied.

3. Results and Discussions

3.1. The method for coagulation capacity determination

The definition for coagulation activity is the milk volume that can be clotted by an enzyme solution volume, at 35°C, in 40 minutes (2400 seconds). The final moment can be appreciated visually.
We have taken 1 g from each type of rennet and then we're putting in 100 cm^3 calibrated flask.
In 250 cm^3 Erlenmeyer we have introduced 100 cm^3 milk, heated at 35°C, and then was added rapidly and vigorously shaking 1 cm^3 rennet solution 1%.
The time between the moment of rennet adding and the flocks' apparition was measured by chronometer.
The coagulation power for rennet powder was determined by forward formula:

$$P = \frac{V_1 \times 2400}{m \times t} \quad (1)$$

where:
P – unknown coagulation power for rennet, unities per gram;
V_1 – milk quantity tacked in the study, in cm^3;
2400 – theoretically time for coagulation, in seconds;
m – rennet quantity for determination, in grams (0,01g for powder rennet);
t – coagulation time, in seconds.
The results for coagulation power determination by Soxhlet method are synthesized in Table 3.

Table 3
Coagulation activity for clotting enzyme prepared used in the experiment

Clot type	Coagulation activity[cm^3/g],	
	Cow milk [L_1]	Sheep milk [L_2]
P_1	115942	120852
P_2	45282	58624
P_3	126316	138434

From Table 3 it can be observed that the higher coagulation capacity is, in the case of cow-milk, for enzymatic prepared from *Aspergillius niger var. awamori* (P_3) as 2.79 times higher than animal origin rennet (P_2), and 1.09 times higher than enzymatic rennet from *Bacillus subtilis* (P_1). The results for sheep milk are the same: coagulation power for P_3 is 2.36 times higher than for P_2, respective 1.15 times higher than P_1(*Bacillus subtilis*).

The differences have been kept significantly higher for all three types of enzymes, with milk assortments, the advantage being developed for sheep milk.

3.2. The influence of rennet quantity about time coagulation

In the same quantity of milk (50 cm^3), with constant temperature 35°C, we have introduced various quantities of rennet solution 1% and then the time was measured between the moment for adding rennet solution and the time for coagulation starting. In table 4 are presented the results for two samples of milk.

Table 4
The influence of rennet quantity about time coagulation

Nr. crt.	Rennet solution, 1 % [cm^3]	Coagulation time [min.]					
		P_1		P_2		P_3	
		L_1	L_2	L_1	L_2	L_1	L_2
1	1	120	115	215	188	109	97
2	2	70	59	130	120	65	49
3	3	66	46	98	81	37	29
4	4	48	35	80	65	32	26
5	5	37	28	72	50	28	23

The conclusion is that, time for coagulation decrease with rennet quantity augmentation, if it is maintained the same temperature for all three types of rennet.

3.3. The temperature influence on milk coagulation

The coagulation process it takes a higher or lower duration time, for the same conditions of acidity, concentration and percent of rennet solution depend on milk temperature for the coagulation.

We have taken 5 samples of milk of 50 cm^3 that have been heated to: 25°C, 30°C, 35°C, 40°C and 45°C. In each of them it has been added 5 cm^3 rennet solution 1% and then has been kept in water bath, for constant temperature. And then we have measured the duration time between the moment of adding rennet solution and the beginning of coagulation.
Table 5 consists of results for the temperature influence about coagulation time.

Table .5
The temperature influence on coagulation

Nr. crt.	Temperature [°C]	Coagulation time [min.]					
		P_1		P_2		P_3	
		L_1	L_2	L_1	L_2	L_1	L_2
1	25	124	110	270	248	86	72
2	30	118	98	237	210	51	44
3	35	37	32	72	58	28	22
4	40	45	40	87	63	27	20
5	45	57	48	128	64	28	25

The results for two samples of milk: cow-milk and sheep-milk and for three types of rennet from various origin extraction reveals that we

4. Conclusions

Cheese making requires milk coagulation and development of syneresis.

It can be achieved by acidification and/or enzymatic way. The conclusions from this study can be synthesized as:

- coagulation power depends on rennet type, so that microbial origin rennet is more powerful than one of animal origin;
- the most important aspects for coagulation process are temperature of the milk and the rennet quantity;
- as the rennet quantity increase as long the time for coagulation decrease;
- if we didn't respect the optimal temperature with rennet type, it could vary the starting moment for coagulation process;

It has been determined the coagulation capacity for three kinds of coagulation enzymes and also the influence of rennet quantity and coagulation temperature about milk coagulation.

The coagulation power depends with rennet origin (extraction):
Microbial enzymatic preparat (*A.niger*) >Microbial enzymatic prepared (*B.subtilis*)> animal origin chymosin.

5. References

[1]. BITTANTE, G., PENASA, M., CECCHINATO, A., Genetic and modelling of milk coagulation properties, *Journal of Dairy Science*, Vol.95, No.12, 6843-6870 (2012)
[2]. ADETUNGI, V.O., *et al.*, Production of wara, a West African cheese using lemon juice as a coagulant, *LWT* 41, 331-337 (2008)
[3]. BITTANTE, G., Modelling rennet coagulation time and curd firmness of milk, *Journal of Dairy Science*, Vol.94, No.12, 5821-5832 (2011)
[4]. LEITNER, G., MERIN, U., SILANIKOVE, N., Effects of glandular bacterial infection and stage of lactation on milk clotting parameters: Comparison among cows, goats and sheep, *International Dairy Journal*, 21, 279-285 (2011)
[5]. CALVO, M.V., et al., Effect of a hygienized rennet paste and a defined strain starter on proteolysis, texture and sensory properties of semi-hard goat cheese, *Food Chemistry*, 102, 917-924 (2007)
[6]. ADDIS, M., PIREDDA, G., PIRESI, A., The use of lamb rennet paste in traditional sheep milk cheese production, *Small Ruminant Research*, 79, 2-10 (2008)
[7]. COSTIN G.M., Cheese making science and engineering , Editura Academica, Galati (2003)
[8]. MERHEB-DINI, C., Use of a new milk-clotting protease from Thermomucor indicae-seudaticae N31 as coagulant and changes during ripening of Prato cheese, *Food Chemistry*, 130, 859-865 (2012)
[9]. BITTANTE, G., CANTIERO, B., CECCHINATO, A., Prolonged observation and modelling of milk coagulation, curd firming, and syneresis, *International Dairy Journal*, 12 (2012)
[10]. PINO, A., et al., Proteolysis during the ripening of goats' milk cheese made with plant coagulant or calf rennet, *Food Research International*, 42, 324-330 (2009)