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Effect of the substitution of rennet by chicken pepsin in the coagulation of milk for cheese processing .

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ABSTRACT

In this study we tried to substitute commercial rennet by pepsin extracted from chicken proventricles as a coagulant agent of milk. In order to compare the effect of pepsin with that of commercial rennet, we approached the technological times of coagulation represented by flocculation and setting, the proteolytic activity was monitored by the kinetics of coagulation. The results obtained showed the conformity of the flocculation times respecting the IDF standard between 8 and 15 minutes. Coagulation by chicken pepsin occurs at a rate of non-protein nitrogen release identical to that of commercial rennet. Using controlled dilutions of the enzyme extracts, the pepsin gel exhibited the same rheological behavior as the rennet gel with flocculation between 12 and 14.5 minutes and an average U.A.C between 0.12 and 0.15 units/ml. No significant difference was observed, which could prevent the substitution of rennet by chicken pepsin during the enzymatic coagulation phase of cheese milks.

1.Introduction

The major interest in processing milk into cheese was to preserve the main constituents of milk (Eck & Gillis, 2006). The first step in cheese making is coagulation, considered the key to the success of any preparation. It consists of the formation of a gel following physico-chemical changes occurring on the casein micelles. Calf rennet is the most widely used coagulating agent for milk coagulation (Lij *et al.*, 2006).

In recent years, rennet has seen an increased demand due to a steady increase in global cheese production. Conversely, its supply has not always kept up with this demand due to the high price and difficulties in supply, as milk calf production is fluctuating while the cheese industry requires sufficient and regular supply (Ziane *et al.*, 2019).

These reasons have caused a lot of research to be undertaken to find efficient and competitive substitutes that can be used industrially. Among these substitutes, proteases of plant origin have a long history of use in traditional preparations such as those from artichoke, thistle, and fig latex (Hamrani *et al.*, 2002).

Other enzymes have been tested such as proteases of fungal origin synthesized by various species. There are also substitutes of animal origin such as bovine pepsins and pepsins extracted from poultry proventricles (Abi Azar, 2007).

Although our country has a production potential in substitutes capable of meeting the needs of coagulating agents, Algeria remains dependent on foreign suppliers in terms of supply and imports almost all the quantities of enzymes needed for the cheese industry. In 2019, about 25 thousand tons of cheese were sold in the Algerian market. According to the National Statistical Office (O.N.S), the Algerian

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cheese industry has used nearly 1.5 tons of rennet and its substitutes (CNIS, 2020).

The high cost of import and the dependence of Algeria on foreign suppliers for the supply of rennet and / or its substitutes, encouraged us to develop local sources for the production of coagulating agents usable in cheese industry. These substitutes that we plan to study can be obtained from local raw materials and with suitable prices because poultry offal is available and is not valued in our country.

2.Material studied, area descriptions, methods and techniques:

2-1. Location

This study was carried out at the Laboratory of Sciences and Techniques of Animal Production of Hassi-Mamèche, Mostaganem, Algeria.

2-2. Chicken proventricles

The samples of chicken proventricles were brought from the slaughterhouse of Zahana belonging to the western poultry group " GAO " Mascara, Algeria.

2-3. Reconstituted milk

The experimental milk was reconstituted at a rate of 12% (w / v) from a skim milk powder low heat enriched with protein recovered from the subsidiary GIPLAIT, the Sahel Mostaganem, Algeria. The milk was enriched with monocalcium phosphate (0.01M) and sodium azide (0.025%). It was then stored in a refrigerator for 10-15 hours for experimental use.

2-4. Extraction of pepsin

The pepsin is extracted from chicken proventricles collected from the slaughterhouse of Zahana belonging to the western poultry group "GAO" Mascara, Algeria. The extraction protocol established by the research team of the laboratory is summarized as follows: Chopping of proventricles, Maceration in sodium carbonate, activation of pepsinogen to pepsin at pH 2, recovery of the pepsin supernatant, regulation of the pH of the extracted pepsin to 6.4 and conservation of the extract at a temperature of 04°C.

2-5. The commercial rennet

The rennet used is a commercial animal rennet powder brand CHR HANSEN Denmark of strength 1/125000 to 650 mg chymosin /100 g.

2-6. Determination of total nitrogen (protein) and non-protein nitrogen (NPN)

The determination of total nitrogen content was performed by Kjeldahl method and according to IDF ISO 707. It consists of mineralization of the reconstituted milk sample by heating in the presence of a mixture of concentrated sulfuric acid, potassium sulfate and copper sulfate, used as catalysts to convert the organic nitrogen in the sample to ammonium sulfate (Mohammad Kamal, 2016). The reaction product is added with soda ash to release ammonia which will be titrated by hydrochloric acid solution in the presence of boric acid

2-7. Characterization of the enzymatic extract

2-7-1. Determination of the coagulant activity: *The coagulant activity is determined by measuring the flocculation time and the setting time (Dahou et al., 2021)*

2-7-2. Determination of the flocculation time: The flocculation time is the time interval between the time of renneting and the appearance of the first casein flakes visible to the naked eye (Dahou *et al.,* 2021).

2-7-3. Determination of setting time: The setting time is the time when the first droplets of whey appear at the beginning of whey exudation (Dahou *et al.,* 2021).

2-7-4. *The unit of coagulant activity U.A.C:* U.A.C is defined by the amount of enzyme contained in 1 ml that can coagulate 10 ml of milk; and calculated by this formula

$$U.A.C = 10.\ V / T.V'$$

Where :

V: volume of milk

V': Volume of the enzyme extract

T: time of flocculation

2-7-5. Kinetics of proteolysis: The proteolytic activity of a coagulating enzyme is reflected by the increase in the rate of non-protein nitrogen (NPN) released in the mass of coagulum.

Comparison of the NPN/NT ratio (where NT represents total nitrogen) between pepsin coagulation and rennet coagulation allows the difference in proteolysis between the two enzymes to be assessed (Davian *et al.,* 2000).

The determination of non-protein nitrogen is estimated after precipitation with trichloroacetic acid (TCA) at 12% final concentration of the proteins of experimental milk in contact with the coagulating enzyme. After filtration, nitrogen is determined by the Kjeldahl method. A series of 10 test tubes containing 10 ml of experimental milk each is maintained at 35°C for 1 hour in a water bath. At time 0, a dose of 1ml of the enzyme is added to each tube, and the stopwatch is activated. For each time of the kinetics, 10 ml of a 12% TCA solution is added, and the tube is shaken well.

Note that we used rennet and pepsin concentrations that ensured a flocculation time between 8 and 15 minutes for the whole experiment. Each tube was filtered, and the serum was collected to determine the NPN content by the Kjeldahl method.

The kinetics of the proteolysis of the two enzymes (rennet and pepsin) is studied by determining the NPN at setting time. The experiment allowing to obtain these kinetics is repeated five times, and our results will express the average curve.

2-8. Statistical analysis

The results are the average of the five trials, and presented in the form of average - standard deviation. The statistical study by analysis of variance (ANOVA) was carried out by the statistical software MINITAB 19.

3. Results and discussion

3-1. Physicochemical quality of the experimental milk used

The experimental milk used prepared at 12% (w/v) presented a moisture of 87.50% , a protein content of 3.63% , a NPN content of

0.185% , a Dornic acidity of 15.8 °D and a pH of 6.65. These results are in accordance with the standards of F.I.L, 2018 and JORA, 2017 (see Table 01).

The enzymatic coagulation of a milk is the first step in the manufacture of cheese that can be considered the result of a process in which casein is concentrated after removal of whey (Vignola *et al.,* 2002 and Mahaut *et al.*, 2011)

For the cheesemaker, a quality milk plays an important role for a good coagulant ability, ensure adequate kinetics of flocculation to the setting of milk, avoid losses of soluble proteins after exudation of whey and the formation of a firm gel (Hyslop, 2003).

Tab. 1. Physico-chemical quality of experimental milk used.

Analysis	Average obtained and standard deviation	Method used
Moisture of reconstituted milk	87,50 ± 0,025	Drying at 105°C
Protein content %	3,63 ± 0,05	Kjeldahl
NPN %	0,185 ± 0,001	Kjeldahl
NPN/NT %	5,0	
Acidity Dornic	15,8 ± 0,25	Dornic
pH	6,65 ± 0,01	pH-metry

3-2. Quality of extracted chicken pepsin compared to commercial rennet

The results of the control of the clarified chicken pepsin extract and the dilution retained, as well as those of the commercial rennet are grouped in table 02

Tab. 2. Quality of the enzymatic extract "chicken pepsin . .

Control performed	Pepsin		Rennet	
	Clarified extract	3% dilution	1% stock solution	2,5% dilution
Flocculation time (s)	55,10 ± 1,10	870 ± 5,50	28,75 ± 1,45	725 ± 12,5
Setting time (s)	96,50 ± 2,45	1560 ± 12,75	48,50 ± 1,60	1490 ± 35,20
Coagulant activity U.A.C/ml	3,25 ± 0,15	0,15 ± 0,002	3,10 ± 0,10	0,12 ± 0,001

The extraction of chicken pepsin from 100 grams of proventriculi gave a volume of 185 ml of enzymatic extract. It should be noted that during our experimentation we opted for a dilution of 3% (v/v) following the preliminary tests carried out to obtain a flocculation time consistent with a good coagulation between 8 and 15 minutes as described by Tanaka et al.,2001

In this sense, the clarified chicken pepsin extract gave an average flocculation time of 55 seconds at 30°C. The 3% (v/v) dilution in sterile distilled water gave an average flocculation time of 870 seconds (14.5 minutes) and an average setting time of 1560 seconds (26 minutes)

In comparison, the 1% rennet solution gave an average flocculation time of 28.75 seconds. At a calculated dilution of 2.5%, an average controlled flocculation time of 725 seconds (12 minutes) and an average setting time of 1490 seconds (approximately 25 minutes) was obtained.

The unit of coagulant activity (U.A.C) which represents the amount of enzyme contained in 1 ml of enzyme solution to coagulate 10 ml of milk in 100 seconds at 30 ° C, is 3.25 unit /ml for the clarified extract of chicken pepsin, and 3.10 unit /ml for the rennet stock solution (1%)

The flocculation time for the 02 enzymes diluted is between 12 and 14.5 minutes with an average U.A.C between 0.12 and 0.15 units/ml

These results obtained are consistent with a successful coagulation activity and kinetics and adapted to any cheese processing.

3-3. Kinetics of coagulant proteolysis:

The kinetics of coagulant proteolysis gives in parallel a release of non-protein nitrogen NPN, soluble in a solution of trichloroacetic acid TCA at a concentration of 12%.

This evolutionary release of the non-protein nitrogen content as a function of time is given in Table 03

Tab. 3. Non-protein nitrogen content released .

Repeat	Pepsin		Rennet	
	NPN (g/100ml) + Standard deviation	% NPN/NT(*)	NPN (g/100ml) + Standard deviation	% NPN/NT (*)
1	0,215 ± 0,0016	5,77	0,210 ± 0,0014	5,64
2	0,218 ± 0,0018	5,86	0,225 ± 0,002	6,05
3	0,221 ± 0,0019	5,94	0,229 ± 0,0025	6,15
4	0,227 ± 0,0022	6,10	0,226 ± 0,0021	6,07
5	0,225 ± 0,002	6,05	0,222 ± 0,002	5,96
Mean obtained	0,221 ± 0,002	5,94	0,222 ± 0,002	5,96
Total nitrogen NT	3,72 ± 0,035	100,00	3,72 ± 0,035	100,00

** No significant difference (P > 0.05)*

The analysis of variance was done to determine the time at which the difference becomes insignificant between the number of repetitions performed (05 repetitions)

The non-protein nitrogen content is similar for both enzymes and is at an average of 0.22. After flocculation, the non-protein nitrogen content released is the same at an average setting time between 25 and 26 minutes for the enzymes on experimental milk controlled for protein content.

The rate of NPN / NT for pepsin coagulation and rennet coagulation is the same and stabilizes at a controlled average of 5.94 -5.96 which confirms the control of dilutions to obtain technological times consistent with an enzymatic coagulation controlled and adapted to any cheese processing.

The rates of NPN released are almost identical for both enzymes. The average obtained for the enzyme chicken pepsin is 0.221 g / 100 ml against 0.222 g/100 ml for the commercial rennet (Figures No. 01 and No. 02).

After 20 minutes of coagulation, setting time of 25 minutes for commercial rennet and 26 minutes for chicken pepsin, the NPN rate is almost identical and significant because of the control of dilutions

giving the same coagulation abilities for the 02 enzymes.

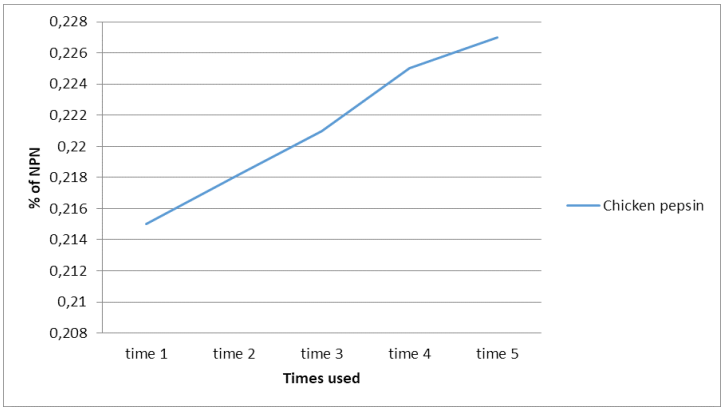

Fig. 1. Kinetics of NPN release for pepsin coagulation.

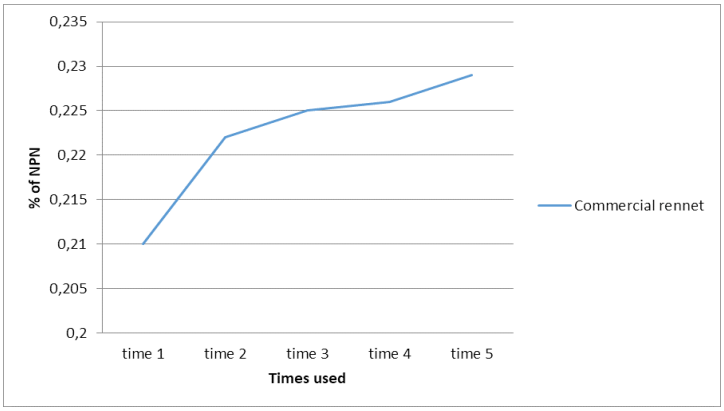

Fig. 2. Kinetics of NPN release for rennet coagulation

Tanaka *et al* (2001), Lucey (2002) and Hamrani (2008) showed that the release of non-protein nitrogen (soluble in 12% TCA solution) by chicken pepsin from a casein solution maintained at pH 5.49, was equal to that of rennet during the first 15 minutes of flocculation time. In addition, the release of nitrogen in the whey at the end of the setting time (estimated at 2 times the flocculation time) was equal between the two types of enzymes.

Our study showed that the coagulation by chicken pepsin presents the same phases as the rennet coagulation, even if the flocculation does not give the same appearance of primary coagulation flakes with the same quality of milk. Studies by Mahaut et al (2011) have shown that the differences in the appearance of milk to flocculation are due mainly to the proteolytic activities of pepsins high for acidic pH (4.6) while rennet (chymosin dominant) tolerate medium acidic pH (5.2)

However, at setting time, the gel obtained has the same appearance with a rate of release of non-protein nitrogen almost identical to that of the commercial rennet.

The statistical analysis of the results, performed by the MINITAB 19 statistical software, gave significance values higher than 5% with an almost similar coagulant activity for the 02 enzymatic extracts with 3.25 UAC/ml for the pepsin extract against 3.10 UAC/ml for the commercial rennet. The kinetics of coagulation represented by the percentage NPN/NT is also almost identical for the 02 enzymes 5.94% for chicken pepsin against 5.96% for rennet. The technological times (setting time), 1560 seconds for pepsin and 1490 seconds for rennet, respect the IDF standard set between 960 seconds and 1800 seconds to obtain a conforming enzymatic cheese curd.

4.Conclusion

The results of our study constitute a first evaluation of the characterization of the coagulation steps during the substitution of rennet by chicken pepsin

The extraction of chicken pepsin allowed to give from 100 g of proventricles 185 ml of enzymatic extract of pepsin, giving an average flocculation time of 55.10 seconds, a setting time of 96.50 seconds and a coagulant activity of 3.25 U.A.C/ml. To obtain a technological flocculation time between 8 and 15 minutes, the extract obtained must be diluted by 3%.

The results do not show any disadvantage to substitute rennet with chicken pepsin during the enzymatic coagulation phase.

On the contrary, we noticed advantageous coagulation abilities with the control of technological times (from flocculation to total coagulum setting)

The whey retention capacity is another advantage with a high hydration rate of the two gels resulting from the two enzymatic activities. This retention capacity is essential to the good control of the expected cheese yields.

The control of the enzymatic concentration (dilution studied of the enzymatic extract) of chicken pepsin gives controlled coagulation kinetics with low losses of non-protein nitrogen NPN soluble in whey

This study deserves to be completed by the follow-up of the behavior of this extracted enzyme at the level of cheese maturation, the determination of the yield and the cost of the extraction of chicken pepsin, the determination of the products of proteolysis by electrophoresis during the different phases of coagulation, the determination of the optimal conditions, of pH, of temperature to obtain the best characteristics of the gel (firmness, syneresis...) and the determination of the type of cheese to be adapted for this enzyme.

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