



Assessment of the Proteolytic Activity of a Coagulating Enzyme Extracted from Thistle Flowers

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ABSTRACT

Background: The preparation of cheese curds by milk coagulation using thistle flower extracts is an original technology specific to certain Algerian regions. The aim of this study is to characterize the coagulating power of the enzymatic extract of this plant, in order to make up for the lack of scientific data on this coagulant. This characterization will make it possible to propose technological solutions to replace the commercial coagulating enzyme used by cheese dairies.

Methods: This study is a test of substitution of animal rennet by a vegetable coagulant which will make it possible to produce a cheese curd while respecting the technological standards of coagulating activities and cheese production. It consists of the determination of the coagulating power of a sample of thistle flowers *Cynara cardunculus* in the form of maceration, by the determination of the flocculation time and the coagulation force, used to research the dose of thistle flower extract necessary for optimal coagulation.

Result: In fact, preliminary tests carried out on the plant's flower indicate an interesting coagulant activity. The enzymatic extract obtained from thistle flowers showed good coagulation of a cheese milk obtained from a "low heat" cheese milk powder. However, at the setting time, the gel obtained has the same appearance, with a non-protein nitrogen release rate almost identical to that of commercial rennet. The technological times (setting times), 1820 seconds for the thistle flower enzyme and 1500 seconds for the commercial enzyme, meet the FIL standard set at between 960 seconds and 1920 seconds to obtain a compliant enzymatic cheese curd. The statistical analysis of the results, performed using MINITAB 19 statistical software, yielded significance values greater than 5%, with almost similar coagulant activity for both enzyme extracts, with 0.10 UAC/ml for the thistle flower enzyme extract versus 0.13 UAC/ml for the commercial enzyme. The coagulation kinetics, represented by the NPN/NT percentage, is also almost identical for both enzymes: 5.08% for the thistle flower enzyme versus 5.84% for the commercial enzyme. This enzymatic extract from thistle flowers gave the expected results for the manufacture of a mixed curd cheese. These results enable us to envisage a scientific approach to the industrial application of natural coagulants for the substitution of commercial coagulants in the manufacture of local cheeses and the preservation of their typicality.

Key words: Enzyme extract, Mixed cheese curds, Technological times, Thistle flowers.

INTRODUCTION

Global sales of dairy coagulants represent more than 60% of enzyme sales (CNIEL, 2024). These enzymes play an undeniable role in the pharmaceutical and food industries, as well as in industrial biotechnology. In fact, the dairy industry is the largest user of proteases, which are used in the most important applications, specifically in milk coagulation and cheese making. Cheese processing involves several steps, one of the most fundamental of which is milk coagulation (Germonville, 2003).

This coagulation is traditionally achieved by the action of rennet on milk, an enzyme industrially extracted from the abomasum of unweaned calves (Dagleish and Corredig, 2012). From the industrialization of cheese processing until the 1950s, rennet was the dominant enzyme used in cheesemaking. Global shortages followed due to its reliance on the meat market (CNIEL, 2024). Currently, traditional rennet production only covers 25% of global demand for coagulants (CNIEL, 2024). This global rennet shortage has led to an increase in its price. This situation has led to the search for alternative enzyme preparations of various origins that can coagulate milk in a similar way to rennet. In this context, several research projects have been conducted by

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international firms (DSM Food, DANISCO and CHR HANSEN) to find substitutes for rennet. In this context, microbial proteases, but especially proteases produced by genetically modified microorganisms, have yielded satisfactory results (Benani, 2017; Ramet, 2021; Talantikite-Kellil, 2015).

In recent years, increasing attention has been directed toward natural enzymatic extracts of plant origin as an alternative (Getachew *et al.*, 2024; Mir Khan *et al.*, 2020). These proteases are attracting attention not only because of their proteolytic activity, but also because they are generally active over a wide range of temperatures and pH (Kaur *et al.*, 2024) and can be optimized in the different families of cheeses produced. A large number of studies have been carried out on the effect of plant coagulants on the coagulation of milk from various animal species (bovine, ovine and caprine) and on the proteolysis of cheese curd during ripening. Thus, this present work aims to study the effect of the substitution of a commercial animal enzyme by an enzyme of plant origin extracted from the thistle flower "*Cynara cardunculus*" on milk coagulation and on certain proteolytic activities of the cheese curd obtained, with the aim of allowing an assessment of the interest of natural coagulants as substitutes for commercial enzymes.

MATERIALS AND METHODS

Location and objective of the study

The objective of the study is to test the substitution of animal rennet with a plant coagulant that will allow the production of cheese curd while respecting technological standards for coagulating activities and cheese making. The study consists of determining the coagulating power of a sample of thistle flowers in macerated form, by determining the flocculation time and coagulation strength, used to research the dose of thistle flower extract required for optimal coagulation.

The study was conducted at the Research Laboratory of Sciences and Techniques of Animal Production "LSTPA" of Hassi-Mamèche, Faculty of Natural and Life Sciences, Abdelhamid Ibn Badis University of Mostaganem, Algeria. The experimental period was established from January to March 2025.

Origin of experimental samples

Plant coagulant

The coagulant used in experiment consists of petals from the flower of *Cynara cardunculus*, commonly known as thistle flowers, naturally dried under the sun and harvested in the Sfisifa region, in the province of Naama, Algeria. The petals of flower are known for their milk-clotting properties, induced by a specific protease "cynarase". This variety of flower is exploited in Algeria during the autumn and winter periods till the second half of February.

Milk

The experimental milk used was obtained from the GIPLAIT subsidiary, dairy: «le Littoral», Mostaganem, Algeria in

2025. The milk was collected aseptically in a refrigerated isothermal tank at a temperature of 4°C (Bekihal *et al.*, 2025).

Commercial animal rennet

The commercial enzyme used was a commercial powdered animal rennet is a commercial brand CHY-MAX powder from the CH Hansen, Denmark; purchased from the FLY CHEMICALS group in Oran, Algeria.

The rennet stock solution was prepared at a concentration of 2%. The experimental dilution was established, according to the technical recommendations at 2.5%, defined by the International dairy federation "IDF" (2020), to obtain a flocculation time at 30°C, technological standard, between 8 and 15 minutes.

Preparation of the plant coagulant "*Cynara cardunculus*"

Drying

250 g of thistle flower petals subjected to a second drying in the laboratory in an oven at 37°C for 24 hours, after which they were stored in an airtight jar away from air, moisture and light.

Preparation of the thistle flower extract

Three coagulating enzyme solutions prepared by macerating the thistle flowers followed by filtering the resulting solution through a 40 mm diameter Pyrex™ Buchner glass filter funnel using a vacuum pump to obtain a coagulating extract.

Characterization of reconstituted milk

Determination of titratable acidity and pH measurement

These two parameters are measured according to F.I.L ISO 707 (2018).

Determination of total milk solids

Determination of total milk solids is carried out according to F.I.L ISO 707 (2018). Total solids correspond to the weight of the residue remaining after drying the sample at 105°C in an infrared desiccator (KERN MLS 50-3C). The principle consists of drying 10 ml of milk using infrared radiation and continuously monitoring the weight using an integrated precision balance until a constant weight is reached. Total solids is displayed as a (%) on the desiccator screen.

Determination of total nitrogen (protein) and non-protein nitrogen (NPN)

Total nitrogen and non-protein nitrogen levels were determined on a 5 ml sample of reconstituted milk used for the experiment. The determination of total nitrogen content is carried out using the Kjeldahl method and according to IDF ISO 707 (2020); it consists of mineralizing the reconstituted milk sample and adding sodium hydroxide to the reaction product to release ammonia, which will be titrated with a hydrochloric acid solution in the presence of boric acid. Non-protein nitrogen (NPN) levels were determined after precipitation of milk proteins with a trichloroacetic acid (TCA) solution at a final concentration of 12% followed by filtration.

Characterization of the enzyme extract

Coagulant activity is determined by measuring the flocculation time established by IDF (2020). Flocculation time is the time interval between the moment of renneting and the appearance of the first casein flakes visible to the naked eye. The unit of coagulant activity U.A.C or rennet unit is defined by the quantity of enzyme contained in 1ml which can coagulate 10 ml of milk: 12% w/v of skimmed milk powder dissolved in a 0.01M CaCl₂ solution in 100 seconds at 37°C.

$$\text{U.A.C} = \frac{10 \cdot V}{T \cdot V'}$$

V = Volume of milk.

V' = Volume of enzyme extract.

T = Flocculation time.

Determining setting time

Determining setting time is the time when the first droplets of whey appear (beginning of the exudation of whey). The setting time is generally about twice the time of flocculation: Thus for a flocculation time between 8 and 15 minutes, the setting time is between 16 and 30 minutes (FAO, 2000; Xiuju *et al.*, 2022).

Proteolysis kinetics

The proteolytic activity of a coagulating enzyme results in an increase in the level of non-protein nitrogen (NPN) released into the coagulum mass. Comparing the NPN/NT ratio (where NT represents total nitrogen) between the coagulation of the thistle flower enzyme and the coagulation of the commercial enzyme allows us to assess the difference in proteolysis between the two enzymes.

The non-protein nitrogen level was estimated after precipitation with trichloroacetic acid (TCA 12%) of the experimental milk proteins brought into contact with the coagulating enzyme. After filtration, the nitrogen was determined using the Kjeldahl method. A series of 10 test tubes, each containing 10 ml of experimental milk, was maintained at 37°C for 1 hour in a water bath. At time 0, a 1 ml dose of the enzyme was added to each tube and the timer was started. For each time point of the kinetics, 10 ml of a 12% TCA solution was added and the tube was shaken well (Sousa *et al.*, 2002; Zhao *et al.*, 2019).

It should be noted that we used concentrations of commercial enzyme and enzyme extracted from thistle flowers, ensuring a flocculation time of between 8 and 15 minutes for the entire experiment as described by Bornaz *et al.* (2010). Each tube was filtered and the serum was collected to determine the NPN nitrogen content using the

Kjeldahl method. The kinetics of proteolysis of the two enzymes were studied by measuring the NPN at the setting time. The experiment used to obtain these kinetics was repeated five times and results were expressed as the average curve.

Statistical analysis

The results are the average of the five trials and presented as the mean standard deviation. The statistical analysis using analysis of variance (ANOVA) was performed using MINITAB 19 statistical software (Fguiri *et al.*, 2024).

RESULTS AND DISCUSSION

Physicochemical quality of the experimental milk

The experimental milk prepared at a 12% (w/v) concentration had a moisture content of 88.30%, a protein content of 3.48%, a NPN content of 0.175%, a dornic acidity of 16.2°D and a pH of 6.68. These results are consistent with IDF and FAO standards; Tahlaiti *et al.* (2020).

Quality of the enzyme extract compared to the commercial enzyme

The results of the testing of the clarified thistle flower enzyme extract and the dilution used, as well as those of the commercial rennet, are presented in Table 1. Extraction of the thistle flower enzyme from 100 g of thistle flower petals yielded a volume of 75 ml of clarified enzyme extract.

In this regard, the clarified thistle flower extract gave a mean flocculation time of 125 seconds at 37°C. Dilution to 0.5% (v/v) in sterile distilled water gave a mean flocculation time of 910 seconds (15 minutes) and a mean setting time of 1820 seconds (30 minutes). In comparison, the 1% commercial enzyme solution gave a mean flocculation time of 35.25 seconds. At a calculated dilution of 2.5%, a controlled mean flocculation time of 750 seconds (12.5 minutes) and a mean setting time of 1500 seconds (25 minutes) were obtained.

The coagulant activity unit (C.A.U), which represents the amount of enzyme contained in 1 ml of the enzyme solution to coagulate 10 ml of milk in 100 seconds at 37°C, is 0.8 units/ml for the clarified thistle flower enzyme extract and 2.84 units/ml for the commercial enzyme stock solution (1%).

The flocculation time for the diluted enzymes is between 12.5 and 15 minutes with an average C.A.U between 0.10 and 0.13 units/ml. These results are consistent with successful coagulant activity and kinetics, suitable for any cheese processing specializing in the production of stabilized soft cheeses.

Table 1: Quality of the “thistle flower” enzyme extract.

Check carried out	Enzyme extracted from thistle flower		Commercial enzyme	
	Clarified extract	Dilution 0,5%	Mother solution 1%	Dilution 2,5%
Flocculation time	125.25±5.25	910±2.10	35.25±0.25	750±10
Setting time	250.50±0.5	1820±5.10	70.50±1.5	1500±10
Coagulant activity U.A.C/ml	0.8±0.2	0.10±0.01	2.84±0.01	0.13±0.02

The appearance of the two enzymatic gels formed after enzymatic activity and reaching the technological time "setting time" at a temperature of 37°C appears clearly and shows a good consistency and firmness with the little whey or a low release of soluble NPN. This quality of gel is in high demand in the stabilized soft cheese industry (Dagleish and Corredig, 2012) for which the gel is with mixed coagulation but with dominant enzymatic activity and this gel must be firm and tenacious.

Kinetics of coagulant proteolysis

The kinetics of coagulant proteolysis results in a parallel release of non-protein nitrogen (NPN), soluble in a 12% trichloroacetic acid (TCA) solution (Fox *et al.*, 2005; Zhao *et al.*, 2019). This progressive release of non-protein nitrogen content over time is shown in Table 2, Fig 1 and Fig 2.

Analysis of variance was performed to determine the time at which the difference becomes insignificant between the number of repetitions performed (5 repetitions). The non-protein nitrogen content for both enzymes averages between 0.177 and 0.205. After flocculation, the released non-protein nitrogen content is the same (for the replicates performed) at an average setting time of between 25 and 30 minutes for both enzymes in experimental milk with controlled protein content.

The NPN/TN rate for coagulation made with the thistle flower enzyme extract and coagulation made with the commercial enzyme stabilizes at a respective controlled average of 5.08 to 5.84, which confirms the control of the

dilutions to obtain technological times in accordance with controlled enzymatic coagulation and adapted to a stabilized soft cheese type transformation.

The two figures show two kinetics with identical appearances. The NPN release rates are almost identical for both enzymes. The average obtained for the thistle flower enzyme is 0.177 g/100 ml compared to 0.205 g/100 ml for the commercial enzyme. The NPN rates obtained are consistent with the reference cheese technology and significant due to the control of dilutions giving the same coagulation abilities for both enzymes.

Dagleish and Corredig (2012); Kaur *et al.* (2024) and Xiuju *et al.* (2022) showed that the release of non-protein nitrogen (soluble in a 12% TCA solution) by plant-derived enzymes with a casein structure identical to milk maintained at pH 6.3 was equal to that of chymosin (an animal enzyme) during the first 25 minutes of flocculation time. Furthermore, nitrogen release into the whey at the end of the setting time (estimated at twice the flocculation time) was equal between the two types of enzymes.

The study showed that coagulation with the thistle flower enzyme exhibits the same phases as coagulation with the commercial enzyme. Even though flocculation does not produce the same appearance of primary coagulation flakes with the same milk quality. Studies by Gallacier *et al.* (2018) showed that the differences in milk appearance at flocculation are mainly due to the proteolytic activities of

Table 2: Released non-protein nitrogen content.

Repetition	Thistle flower enzyme		Commercial enzyme	
	NPN (g/100 ml)±SD	% NPN/TN(*)	NPN (g/100 ml)±SD	% NPN/TN (*)
1	0.175±0.005	5.03	0.198±0.002	5.64
2	0.176±0.004	5.06	0.203±0.002	5.78
3	0.177±0.003	5.08	0.208±0.002	5.92
4	0.178±0.002	5.11	0.208±0.002	5.94
5	0.179±0.001	5.14	0.209±0.001	5.98
Average obtained	0.177±0.003	5.08	0.205±0.005	5.84
Total nitrogen TN	3.48±0.02	100.00	3.51±0.05	100.00

*No significant differencee (P>0.05).

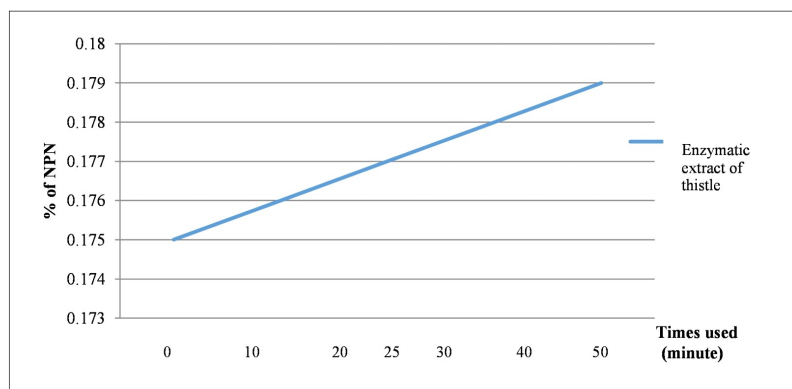


Fig 1: Kinetics of NPN release for coagulation with thistle flower enzyme.

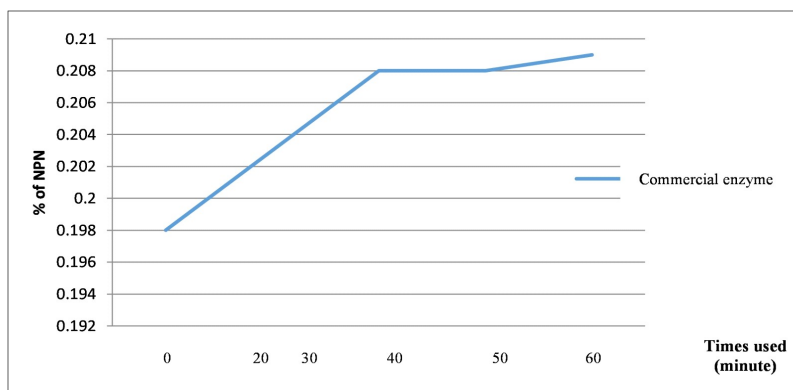


Fig 2: Kinetics of NPN release for commercial enzyme-based coagulation.

the enzymes, which are optimal at pH close to neutral (6.2 to 6.5), whereas commercial enzymes (chymosin-dominated) tolerate moderately acidic pH (5.2).

However, at the setting time, the gel obtained has the same appearance, with a non-protein nitrogen release rate almost identical to that of commercial rennet. The statistical analysis of the results, performed using MINITAB 19 statistical software, yielded significance values greater than 5%, with almost similar coagulant activity for both enzyme extracts, with 0.10 UAC/ml for the thistle flower enzyme extract versus 0.13 UAC/ml for the commercial enzyme. The coagulation kinetics, represented by the NPN/NT percentage, is also almost identical for both enzymes: 5.08% for the thistle flower enzyme versus 5.84% for the commercial enzyme. The technological times (setting times), 1820 seconds for the thistle flower enzyme and 1500 seconds for the commercial enzyme, meet the FIL standard set at between 960 seconds and 1920 seconds to obtain a compliant enzymatic cheese curd.

CONCLUSION

The results obtained in this study constitute an initial assessment of the characterization of the coagulation stages when replacing the commercial enzyme with a plant-based enzyme derived from thistle flowers. The results reveal no disadvantages in replacing the commercial enzyme with the typical plant-based enzyme derived from thistle flowers for a mixed coagulation process with enzyme dominance. On the contrary, we observed advantageous coagulation abilities with controlled technological times (from flocculation to complete coagulum set). 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 for successfully controlling the expected cheese yields. Controlling the enzyme concentration (studied dilution of the enzyme extract) of the thistle flower enzyme extract results in controlled coagulation kinetics with low losses of soluble non-protein nitrogen (NPN) in whey. This study is a contribution in the sense that it provides manufacturers with the scientific approach they need to master cheese processing technology.

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This study deserves to be supplemented by monitoring the behavior of this extracted enzyme during cheese ripening. In the future, it is interesting to continue this study by:

- Determining the yield and cost of extracting the plant enzyme typical of thistle flowers.
- Determining the proteolysis products by electrophoresis during the different coagulation phases.
- Determining the optimal pH and temperature conditions to obtain the best gel characteristics (firmness, syneresis, etc.).
- Applying this enzyme on an industrial scale for its development in cheese formulations.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

I declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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