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Article

Chemical Characterization of Aspartic Protease from Artichoke Flower (*Cynara cardunculus* L. var *scolymus*) Using MIR Spectroscopy and Analytical Measurements

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Vegetable proteases have been studied as milk coagulants, and artichoke flower (*Cynara cardunculus* L. var *scolymus*) has potential coagulant action as a substitute for microbial chymosin. The objective was to perform chemical characterization of aspartic proteases from artichoke flower (*Cynara cardunculus* L. var *scolymus*) by mid-infrared spectroscopy (MIR) and analytical measurements. Artichoke flower extracts were obtained and crude thistle flower extract and microbial chymosin were used as a reference. Plant extracts and microbial chymosin were analyzed for protein concentration, proteolytic activity (PA), milk clotting activity (MCA), specificity ratio (SR), effects of pH, temperature, NaCl and $CaCl_2$ concentration on MCA and PA and characterized by electrophoretic, spectroscopic, chromatographic and storage stability. The results indicated that the crude extract of artichoke flower showed high MCA (510.08 SU mL^{-1}), low PA (10.50 μg mg^{-1}), SR (48.57) and storage stability for up to 90 days under frozen. The electrophoretic profile of artichoke flower resulted in a protein band with an apparent molecular weight of 32 kDa, associated with cardosin A. Artichoke flower may be a new alternative for commercial plant proteases with active enzymes for milk coagulation.

Keywords: cardosines, enzyme, infrared, milk coagulation, peptidase, rennet

Introduction

Chymosin (EC3.4.23.4) is one of the main aspartic proteases, the oldest and best known in cheesemaking due to its high specificity to cleave the κ-casein bond (Phe_{105}-Met_{106}) and is therefore considered the best milk coagulant.[1-4] However, over the years, the supply of this coagulant from animal sources has decreased and the main reasons involve the complexity in the process of extracting and purifying the enzymes from the stomach of calves and other reasons such as religious (e.g., Judaism and Islam), diet (lactovegetarians), making the supply of the enzyme very laborious and expensive. Because of this, new sources of proteases from microorganisms and/or plants with properties that adequately satisfy the cheese industry are important.[5-7]

In recent years, most of the commercial rennet used in the cheese industry comes from microbial proteases due to the easy cultivation and high productivity of the enzyme. However, plant proteases have attracted attention because of the abundance of plant resources, the convenience of extraction, and the differentiated organoleptic characteristics of cheese appreciated by consumers. In addition, there is a consumer concern regarding genetically modified foods such as recombinant chymosin which is an enzyme preparation obtained from genetically modified microorganisms.[8-10]

Plant proteases as obtained from *Moringa oleifera* seed,[10] *Artocarpus altilis* latex,[11] *Morinda citrifolia* L.,[12] and *Solanum tuberosum* turbot[13] are examples of possible

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alternatives for milk coagulation. According to Gomes *et al.*,[14] some of these plant proteases are highly proteolytic and perform nonspecific hydrolysis of milk caseins, influencing the texture, sensory quality, and the yield of cheese, have a low coagulation activity/milk proteolytic activity ratio, also known as the specificity ratio, and are therefore not suitable for use in the manufacture of some cheeses.

The most commonly used plant coagulants in commercial cheese production are aqueous extracts of thistle flowers (*Cynara cardunculus* L.), used for many years on the Iberian Peninsula to make artisan cheeses from goat and sheep's milk.[15] Thistle flower is a plant that grows spontaneously and is found in abundance in dry, stony and uncultivated soils. Over the years this plant has been characterized in terms of its milk coagulation properties by proteases and the enzymatic composition of the thistle flower can vary according to the ecotypes of the plants and can influence the characteristics of cheeses.[14,16] These extracts are mainly composed of the proteases, cardosin A and cardosin B, which are characterized by high coagulation activity and strong proteolytic activity, leading to the production of cheeses with a creamy, soft texture, a characteristic aroma and a slightly piquant and bitter taste.[1]

Cynara cardunculus L. var *scolymus*, popularly known as artichoke flower, is cultivated mainly for food purposes. It is a plant native to southern Europe, the Mediterranean region, and has been spread worldwide.[17] Appreciated for its bitter-sweet flavor given by the edible inflorescence, it has not been used in other applications. Its use as a coagulant in milk for cheese production can be an economically viable alternative for cheese makers and can add value to the product.[18,19]

Although artichoke is a source with potential application in cheese production, there is little information in the literature about the study and characterization in relation to protein concentration, proteolytic activity (PA), milk clotting activity (MCA), specificity ratio, effects of pH, temperature, concentration of $CaCl_2$ and NaCl on PA and MCA, characterization by electrophoresis and chromatography. In addition, no studies related to spectroscopic characterization by MIR were found. In this sense, the present study aimed to carry out the chemical characterization of aspartic proteases from the artichoke flower (*Cynara cardunculus* L. var *scolymus*) using mid-infrared (MIR) spectroscopy and analytical measurements.

Experimental

Samples

Mature artichoke flowers (*Cynara cardunculus* L. var *scolymus*) were acquired from plants grown in the São Roque region (São Roque, São Paulo, Brazil). As reference were utilized mature flowers of the thistle (*Cynara cardunculus* L. var *sylvestris*) obtained from plants grown in the Alto do Alenteijo region (Campo Maior, Portugal) and microbial rennet (*Aspergillus niger* var *awamori*) (HÁ-LA®, Chr. Hansen, Brazil) acquired in the local trade. The skim milk powder was obtained from Laticínios Bela Vista Ltda. (Goiás, Brazil).

Preparation of coagulant extracts

The artichoke and thistle flower pistils were dried for about 30 days at 25 °C and protected from light. After this period, they were used to prepare aqueous extracts of artichoke and thistle flowers.[14] The pistils (35 g) were macerated in a mortar and added to 0.5 L of distilled water. This mixture was left to rest for 24 h at 25 °C in the dark. The homogenized extracts were filtered in quantitative filter paper (Unifil, C40, 18.5 cm) and used on the same day.[20]

Determination of protein concentration

The protein concentration was determined according to the Coomassie Blue dye binding method,[21] using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) as a standard. Proteins were detected by measuring the absorbance at 595 nm in a spectrophotometer (Shimadzu UV-1800, Duisburg, Germany).

Determination of PA

The PA of the crude plant extracts and microbial chymosin was determined using a modified method of Mohanty *et al.*[22] The substrate was prepared by dissolving 1% (m/v) total casein (Sigma-Aldrich, St. Louis, MO, USA) in 10 mmol L^{-1} sodium phosphate buffer (pH 6.5). The assay was performed by incubating 1 mL of substrate with 100 µL of crude plant extract or microbial chymosin at (37 ± 1 °C) in a thermostatic bath (Tecnal, model Te-184, São Paulo, Brazil) for 30 min. The reaction was stopped by the addition of 3 mL of trichloroacetic acid (TCA, Sigma-Aldrich, St. Louis, MO, USA) at 6.5% (m/v), the solution was centrifuged (centrifuge, MPW-350, Warsaw, Poland) at 5000 × g for 20 min at room temperature (25 ± 1 °C). For the blank assay, substrate was added after inactivation of the enzyme by TCA. The absorbance of the supernatant was measured at 280 nm using a spectrophotometer (Shimadzu UV-1800, Duisburg, Germany). To evaluate proteolytic activity, a standard curve was constructed by measuring the absorbance at 280 nm of serial dilutions of 100 µg mL^{-1} of L-tyrosine solution (Sigma-Aldrich, St. Louis, MO, USA).

One unit of enzyme activity (U) (equation 1) was defined as the amount of enzyme required to produce 1.0 μmol of L-tyrosine equivalent in 1 mL of reaction medium *per* min. The specific activity was expressed in units of enzyme activity *per* μg protein by the ratio of the activity to the concentration of protein in the extract.

$$\text{U/mL} = \frac{\left(\mu\text{g mL}^{-1}\ \text{tyrosine equivalent} \times \text{dilution factor}\right)}{\left(\text{enzyme volume used} \times \text{time}\left(\text{min}\right)\right)} \quad (1)$$

Effect of pH, temperature, NaCl and $CaCl_2$ concentration on PA

The effect of pH on PA was determined by mixing and incubating the crude plant extracts (artichoke and thistle) and microbial chymosin with the bovine casein substrate (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1% (m/v) at 37 ± 1 °C for 30 min. Different pH values were used: sodium acetate 0.05 mol L^{-1} (pH 5.0); sodium phosphate 0.05 mol L^{-1} (pH 5.5, 6.0 and 6.5) and Tris-HCl 0.05 mol L^{-1} (pH 7.0 and 8.0) at 37 ± 1 °C.

The effect of temperature on PA was determined at temperatures 30, 40, 50, 60, 70 and 80 ± 1 °C in a thermostat bath (Tecnal, model Te-184, São Paulo, Brazil) for 30 min at pH 6.5. To determine the effect of NaCl and $CaCl_2$ concentration, NaCl concentrations (0, 100, 250, 500 and 1000 mmol) and $CaCl_2$ (0, 10, 40, 70 and 100 mmol) were used at 37 ± 1 °C and pH 6.5. The proteolytic activity was determined according to sub-section "Determination of PA".

Determination of MCA

The MCA of the crude plant extracts and microbial chymosin were determined using modified method of Luo *et al.*[23] 1 mL of the substrate (12% skim milk in 10 mmol $CaCl_2$, pH 6.5) was incubated at 37 ± 1 °C in a thermostatic bath (Tecnal, model Te-184, São Paulo, Brazil) for 5 min. Then, 100 μL of the plant extracts and microbial chymosin were added. The time from the addition of the enzyme to the first appearance of solid material was measured in seconds and defined as coagulation time. The MCA was expressed in Soxhlet units (SU). The SU represents the volume of milk that can be coagulated by one volume unit of the enzyme extract in 40 min at 37 ± 1 °C and pH of 6.5. The SU were measured using equation 2.

$$\text{MCA}\left(\text{SU mL}^{-1}\right) = \frac{\left(2400 \times \text{V}\right)}{\left(\text{t} \times \text{v}\right)} \quad (2)$$

where 2400 is the 40 min clotting time in seconds; V is the volume of milk (mL); v, the volume of enzyme (mL); and t, the coagulation time in seconds.

Effect of pH, temperature and NaCl and $CaCl_2$ concentration on MCA

The effect of pH on MCA was tested using the crude plant extracts (artichoke and thistle) and microbial chymosin at 37 ± 1 °C in a thermostat bath (Tecnal, model Te-184, São Paulo, Brazil). The pH of the milk was adjusted using 10 mmol L^{-1} of sodium acetate buffer (pH 5.0), sodium phosphate (pH 5.5, 6.0 and 6.5) and Tris-HCl (pH 7.0 and 8.0). The effect of temperature was determined at 30 to 80 ± 1 °C in a thermostat bath (Tecnal, model Te-184, São Paulo, Brazil) at 10 °C intervals. To determine the effect of $CaCl_2$ and NaCl concentration, the concentration of $CaCl_2$ was varied from 10 to 100 mmol and NaCl from 100 to 1000 mmol. The MCA was measured at pH 6.5 and temperature of 37 ± 1 °C. The effect of milk clotting activity was determined according to the sub-section "Determination of MCA".

Determination of specificity ratio (SR)

The milk specificity ratio (SR) of the crude plant extracts and microbial chymosin is the ratio of MCA to PA and was determined according to equation 3.[24]

$$\text{SR} = \frac{\text{MCA}}{\text{PA}} \quad (3)$$

Electrophoretic, spectroscopic and chromatographic characterization

Electrophoretic characterization

Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) was performed on polyacrylamide gel. The stacking gel concentration was 4% and the separation gel concentration was 12% according to Laemmil.[25] Crude plant extracts (artichoke and thistle) and the microbial chymosin were used. Aliquots of 10 μL were transferred to the gels and runs performed at 4 °C for 150 min at 250 V, 30 mA and 15 W (Apelex PS 304 MinipacII, France). The following were used as molecular weight standards: myosin (200.0 kDa), β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.5 kDa, but with apparent molecular weight of 15.5 kDa), and aprotinin (6.5 kDa) (Bio-Rad, Hercules, CA, USA). After the run, the proteins were stained using 0.1% (m/v) Coomassie Blue G-250 (Vetec, Rio de Janeiro, Brazil) and destained with a 30% (v/v) solution of ethanol (Vetec, Rio de Janeiro, Brazil) and 7.5% (v/v) acetic acid (Vetec, Rio de Janeiro, Brazil),

then the gels were scanned. The molecular weights of the gel-migrated proteins were determined according to Iizuka and Faust.[26]

Spectroscopic characterization-MIR

The plant extracts and the freeze-dried microbial chymosin were analyzed in a mid-infrared, FTIR-ATR equipment (Cary 630 FTIR, Agilent Technologies Inc., Santa Clara, CA, USA), equipped with attenuated total reflectance (ATR) with diamond crystal cell. Then, 0.5 g of the samples were placed individually on the accessory compartment where the rays in the infrared range (diamond crystal) hit. The spectra were obtained in absorbance mode with a resolution of 4 cm^{-1} and 64 scanning scans. The software for acquisition of the spectra was the Agilent MicroLab PC software. Before each collection, the background spectrum was read and evaluated in the spectral region with a wavenumber of 4000 to 600 cm^{-1}. For each sample two repetitions were performed, and the room temperature was maintained at around 25 °C.

Chromatographic characterization

The crude extracts of artichoke flower, thistle flower and the microbial chymosin dialyzed at 4 °C were loaded onto a SRT-C SEC 150 Å molecular exclusion column, pore sizes for protein separation MW range 500-150000 (Delaware Technology Park, USA) (4.6 × 300 mm, 5 µm), coupled to a pre-column (ZORBAX SB-C 18, 4.6 mm internal diameter (ID) × 12.5 mm, 5 µm), connected to the Agilent 1260 Infinitty II HP system. A 5 µL aliquot of the sample was injected and proteins were separated by isocratic elution using 20 mmol sodium phosphate buffer (pH 7.0) at a flow rate of 0.2 mL min^{-1} with an analysis time of 30 min. Detection of the proteases was performed at 215 nm on a UV detector. The molecular weights proteins were determined accordingly Iizuka and Faust.[26]

Storage stability

To evaluate storage stability, the proteolytic activity of the coagulant extracts of artichoke and cardoon flowers, and the microbial chymosin were evaluated on the first day they were obtained and after the storage at −18 °C for 30, 60, 90, and 120 days. The proteolytic activity was evaluated according to the topic sub-section "Determination of PA".

Experimental design

The plant extracts were prepared in three repetitions. Assays were performed in triplicate in a completely randomized design. To evaluate the effect of temperature and pH on PA and MCA, two factorial 3 × 6 schemes were performed. The coagulant factor was used for all factorials and presented 3 levels (artichoke extract, thistle extract and microbial chymosin) and the factors temperature (30, 40, 50, 60, 70 and 80 °C) and pH (5.0, 5.5, 6.0, 6.5, 7.0 and 8.0) presented 6 levels.

The effect of NaCl and $CaCl_2$ concentration on PA and MCA was evaluated in two 3 × 5 factorial scheme. The coagulant factor was used for all factorials and presented 3 levels and the factors NaCl concentration (0, 100, 250, 500 and 1000 mmol) and $CaCl_2$ concentration (0, 10, 40, 70 and 100 mmol) presented 5 levels. For storage stability, a 3 × 5 factorial scheme was used, with 3 coagulants × 5 storage times (0, 30, 60, 90 and 120 days). Analysis of variance (ANOVA) was performed to evaluate the effect of treatments on PA, MCA. The independent effects of the coagulants, as well as the interaction between the factors, were also evaluated. A regression was performed at 5% significance level using the Statistical Analysis System (SAS) statistical software program, Student version 9.1.[27]

Results and Discussion

Characterization

Protein content, PA, MCA and SR were determined for crude artichoke extract, crude thistle flower extract and microbial chymosin (Table 1).

The protein content was higher, and the PA was lower for both vegetable coagulants ($p < 0.05$). For a protease to be considered ideal for cheese production, it must have low PA and high MCA, which was found in the present study for artichoke extract.[28]

The MCA of the crude artichoke flower extract was lower than that of the crude thistle extract and the microbial chymosin, and one of the factors that can explain this occurrence is probably the lower concentration of coagulation enzymes present in the crude artichoke flower extract. It should be considered that the artichoke and thistle flowers are of different varieties, which may influence the proteases present.

Some factors can interfere in the characteristics of the plant extract, influencing the concentration of coagulation enzymes and, consequently, in the coagulation properties of the milk, such as: form of maceration, temperature of drying of the pistils, variety of flowers, geographical location, stage of ripeness of the flowers at harvest, or even differences in the enzyme profile.[18]

The specificity ratio is used as an index to evaluate the suitability of an enzyme extract for use as a coagulant in

Table 1. Protein content, specific proteolytic activity, milk clotting activity and specificity ratio for artichoke, thistle and chymosin coagulants (mean ± standard deviation)

Characterization	Artichoke extract	Thistle extract	Microbial chymosin
Protein concentration / (mg mL^{-1})	$2.05^{a} \pm 0.01$	$1.79^{a} \pm 0.09$	$0.68^{b} \pm 0.03$
PA / (μg mg^{-1})	$10.50^{a} \pm 0.22$	$14.15^{a} \pm 0.97$	$41.14^{b} \pm 0.02$
MCA / (SU mL^{-1})	$510.08^{a} \pm 0.38$	$558.92^{b} \pm 0.92$	$1090.66^{c} \pm 0.35$
SR	$48.57^{a} \pm 1.24$	$39.49^{b} \pm 0.93$	$26.51^{c} \pm 0.97$

Means with the same letter in the same row did not differ significantly ($p < 0.05$). PA: proteolytic activity; MCA: clotting activity; SR: specificity ratio; Soxhlet Units (SU): volume of milk (mL) that can be coagulated by one-unit volume of enzyme extract.

cheese making.[28] The crude artichoke flower extract showed a specificity ratio of 48.57, while the thistle extract and microbial chymosin showed 39.49 and 26.51, respectively. El-Salam *et al*.[9] found a value of 103.27 in the studies of purification and characterization of the clotting enzyme from milk from artichoke flowers.

Chymosin is considered the best milk coagulant due to its high specificity, i.e., it has high MCA and low PA, which results in a high specificity ratio, which is the enzyme's ability to hydrolyze κ-casein specifically, and this characteristic is what makes it suitable and most used as a coagulant for cheese production.[29]

A new coagulant to be considered ideal as a replacement for chymosin should have SR equal to or greater than chymosin. Thus, it can form a curd with high yield and low bitterness in the cheese.[5]

Generally, chymosin has a higher SR than most plant coagulants, but in the present study the plant extracts had higher values.[3,13,30] This result suggests that the plant proteases present in the artichoke flower may exhibit specific coagulant activity and without excessive hydrolysis of other proteins. Amira *et al*.[31] evaluated the SR of flowers of *Cynara cardunculus* var. *sylvestris* and found similar result, where plant extract showed higher value of specificity ratio than chymosin. It was also reported by Silva *et al*.,[29] SR in quixaba latex (5731) surpassed chymosin (3363).

The high specificity ratio exhibited by the crude artichoke flower extract, along with its ability to produce milk curd (MCA), make this coagulant useful as a new substitute for microbial chymosin. Llorente *et al*.[8] in their studies confirm this result by producing Gouda cheese with artichoke extract, coagulating the milk in about 30 min, an adequate time for use on an industrial scale.

Effects of pH, temperature and concentration of NaCl and $CaCl_2$ on PA

The interaction between the tested factors (coagulant/pH and temperature) had a significant effect on the proteolytic activity and a quadratic model was fitted ($p < 0.05$). To assess the adequacy of the model, the lack of fit was analyzed, which was not significant indicating that the chosen model is adequate to explain the proteolytic activity as a function of the analyzed treatments. Figure 1 shows the effect of pH and temperature on proteolytic activity, coefficient of determination (R^2) and estimated regression model.

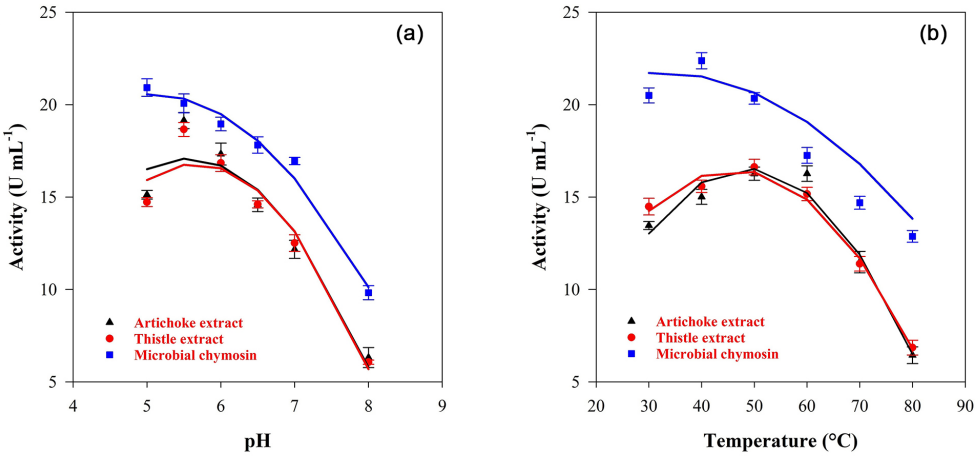
The effect of pH on PA of crude artichoke flower extract can be seen in Figure 1a. Coagulants showed similar effects with pH variation.

To obtain the optimal pH value with the highest proteolytic activity, the model obtained for PA was derived. The crude extract of artichoke flower showed optimal PA (17.08 U mL^{-1}) at pH 5.5, similar to the thistle extract (16.75 U mL^{-1}). The microbial chymosin showed optimal activity at pH 5.0.

Determining the effect of temperature on PA is considered an important measure because it is from this that information can be obtained about the optimal temperature that promotes maximum proteolytic activity.

In Figure 1b, it is possible to verify the loss of activity with the increase in temperature. After model derivation, crude artichoke flower extract (16.55 U mL^{-1}) showed optimal PA at 48.6 °C and crude thistle extract (16.59 U mL^{-1}) at 48.9 °C. At temperatures above this value, the proteases significantly lost their activity. This reduction in activity may be related to enzyme denaturation at temperatures above 60 °C. These results agree with El-Salam *et al*.,[9] who, when performing artichoke extract PA, observed that at temperatures above 65 °C there is a decrease in PA. The microbial chymosin PA reached a maximum value of 21.48 U mL^{-1} at 30.5 °C.

According to de Farias *et al*.,[12] vegetable proteases commonly exhibit high proteolytic activity at higher temperatures, when compared to chymosin. The high PA presented by plant extracts at a temperature of 50 °C is not desirable from a technological point of view, because during the processing of cheese in the coagulation phase



A: $\hat{Y}$= -1.886x^2 + 20.955x - 41.092 R= 0.82
C: $\hat{Y}$= -2.026x^2 + 22.923x - 48.038 R= 0.86
Q: $\hat{Y}$= -1.203x^2 + 12.158x - 10.154 R= 0.96

A: $\hat{Y}$= -0.010x^2 + 0.991x - 7.557 R= 0.88
C: $\hat{Y}$= -0.008x^2 + 0.783x - 1.612 R= 0.96
Q: $\hat{Y}$= -0.003x^2 + 0.224x + 18.102 R= 0.91

Figure 1. Effect of pH (a), temperature (b) on proteolytic activity (U), fitted model and coefficient of determination (R^2). Experimental data (▼ ● ■) and model data (line). A = crude extract of artichoke flower; C = crude extract of thistle flower; Q = microbial chymosin.

it can cause excessive breakage of casein, resulting in a fragile curd with a low technological content.

In addition, low molecular weight peptides are released, which can affect the flavor and texture of cheeses, resulting in an excessively acidic and bitter taste.[12]

Sodium and calcium ions play an important role in the cheese making process, and thus the determination of the effects on PA caused by these solutes are important. The concentration of $CaCl_2$ and NaCl did not influence the PA ($p > 0.05$).

The contents of NaCl and $CaCl_2$ can potentiate the enzymatic activity; however, the concentrations studied are not enough to increase the PA.

Effects of pH, temperature and NaCl and $CaCl_2$ concentration on MCA

Coagulation is a fundamental step in the transformation of milk into cheese, involving the use of coagulants and depending on several parameters (milk composition, coagulant enzyme, pH, temperature, concentration of NaCl and $CaCl_2$).

Based on the experimental values, it was observed that there was an interaction between the coagulants and the treatments studied ($p < 0.05$). A first-order model was fitted for the effect of pH on MCA and second-order models were fitted to assess the effect of temperature, NaCl and $CaCl_2$ concentration on MCA. Figure 2 shows the effect of pH, temperature, NaCl and $CaCl_2$ concentration on the MCA, coefficient of determination and estimated regression model.

The MCA of proteases from artichoke flower crude extract, thistle and microbial chymosin showed a decreasing linear effect (Figure 2a) with high activity at pH 5.0. The increase in pH was accompanied by a gradual loss of milk clotting activity. At pH 7.0 the activity was completely lost, confirming the results obtained in the PA tests, in which plant extracts and chymosin are more active at acidic pH.

Considering the effect of temperature on the coagulation activity of milk (Figure 2b), it was possible to observe a quadratic behavior ($p < 0.05$) for the three coagulants. From the derivation of the model obtained, it was verified that the coagulants exhibited maximum activity at a temperature of 35 °C, a behavior different from the results obtained for the influence of temperature on PA. The increase in temperature resulted in a decrease in coagulation, losing activity.

Temperatures above 70 °C cause the denaturation of whey proteins, especially β-lactoglobulin. From this denaturation, there is the interaction of the –SH group of denatured β-lactoglobulin with κ-casein, by a disulfide bond, forming a complex of κ-casein with the whey protein, which decreased the effective concentration of κ-casein in the substrate, affecting the action of rennet, reducing coagulation.[32] This interaction results in a brittle curd and reduced manufacturing yield due to the loss of fines, which are microscopic pieces of curds lost in the whey.

The increased temperature also causes the calcium and phosphate to transfer from the soluble phase into the colloidal phase of the milk. This shift in the mineral balance contributes to an increase in the coagulation time of the milk, resulting in lower MCA.[33]

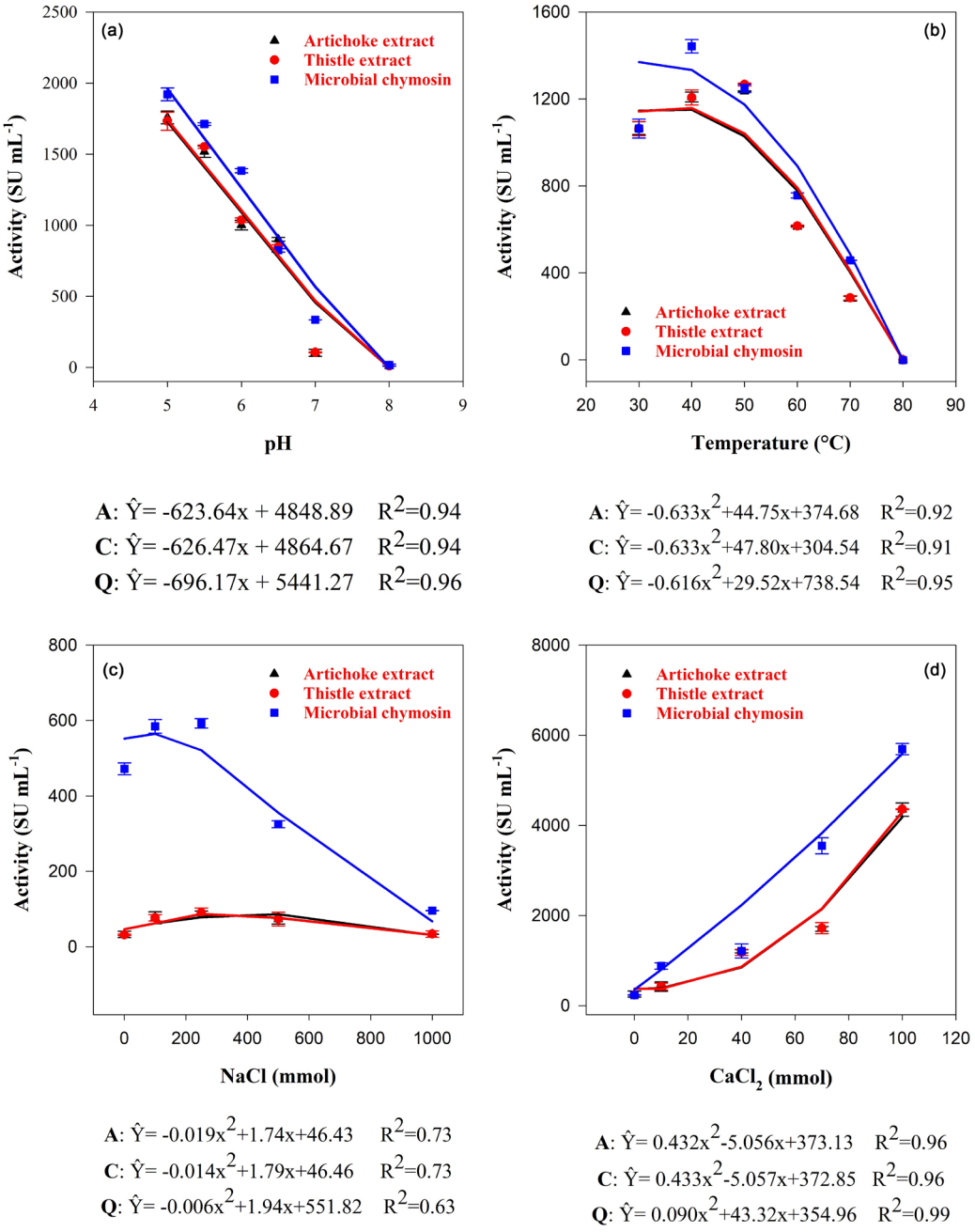


Figure 2. Effects of pH (a), temperature (b) and concentration of NaCl (c) and $CaCl_2$ (d) on MCA, fitted model and coefficient of determination (R^2). Experimental data (▼ ● ■) and model data (line). A = crude extract of artichoke flower; C = crude extract of thistle flower; Q = microbial chymosin.

The effect of sodium chloride concentration on clotting activity showed similar behavior among plant extracts, in chymosin it was found a reduction in MCA with increasing NaCl concentration (Figure 2c). The crude extract of artichoke flower showed MCA of 46.43 SU mL^{-1} when tested without addition of salt, with 500 mmol, it showed maximum activity of 98.62 SU mL^{-1}, and at the concentration of 1000 mmol it showed MCA of 30.83 SU mL^{-1}. These results indicate that at adequate concentrations, NaCl, in association with the studied extract, maximizes the coagulation of milk.

According to Chazarra *et al.*,[34] the addition of NaCl to milk promotes the dissociation of calcium and phosphate from the casein micelles and in solution, affecting the colloidal state of the milk and also the coagulant action. At proper concentration, NaCl decreases the pH of the milk, increases the hydration of casein and leads to a reduction in the zeta potential of the milk, decreasing the coagulation time, resulting in high MCA.[35] The increase in NaCl concentration leads to an increase in the ionic

strength of the milk, resulting in greater protein-protein interaction, decreasing its solubility in aqueous medium, by the salting-out effect, resulting in decreased enzyme action and thereby increasing coagulation time and consequently reducing MCA.

The $CaCl_2$ concentration influenced the milk coagulation activity, with the highest activity occurring at the 100 mmol concentration for all three coagulants tested (Figure 2d). The addition of calcium chloride to milk increases the concentration of calcium in its ionic form (Ca^{2+}) and also of colloidal calcium phosphate, causing a slight decrease in the pH of the milk due to the reaction of some Ca^{2+} ions with the sodium phosphate salts, releasing H^+.[32]

Milk coagulation occurs in two phases, the enzymatic and non-enzymatic phases, also called the secondary phase, in which the aggregation of para-k-casein and macropeptides occurs by association with Ca^{2+} ions, resulting in the formation of para-k-caseinate or curd.[32] As a result, an increase in MCA was observed with an increase in $CaCl_2$.

Electrophoretic, spectroscopic and chromatographic characterization

Electrophoresis

SDS-PAGE analysis was performed to visualize and detect proteins present in the crude extract of artichoke flowers. Electrophoretic patterns of crude artichoke flower extract resulted in a protein band with an apparent molecular weight of 32 kDa (Figure 3). This band probably corresponds to the protease cardosin A.[36]

The crude extract of the thistle flower showed an electrophoretic pattern with 3 protein bands (69, 35 and 32 kDa).[37-39] The band with an apparent molecular weight of 69 kDa indicates that probably the thistle flower has a less developed stage of maturation than the artichoke flower. The proteases present in the flowers of the genus *Cynara cardunculus* L. have active proteases called cardosin A and cardosin B.

Cardosin A is expressed mainly as procardosin A with a molecular weight of 64 kDa in the immature flower, but as the flower matures, procardosin A undergoes a sequence of modifications by proteolytic cleavage, resulting in mature cardosin A. This cardosin has two chains, a heavy and a light chain linked together via hydrophobic interactions and hydrogen bonds. Cardosin B is synthesized as a single-chain precursor and proteolytic modifications take place to generate the mature two-chain form.[37]

According to Alavi and Momen,[37] the crude extract of flowers of the genus *Cynara* contains approximately 75% of cardosin A and 25% of cardosin B. The artichoke flower,

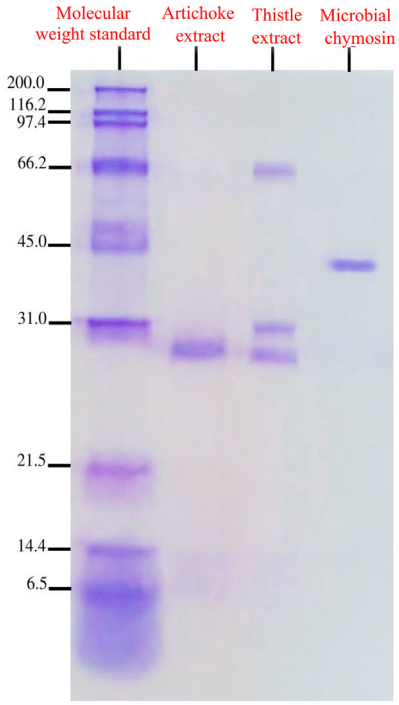


Figure 3. SDS-PAGE electrophoresis of crude artichoke flower extracts, thistle flower and microbial chymosin.

belonging to the genus *Cynara*, presented an intense band (32 kDa) associated with cardosin A. Cardosin B was not visualized.

This result indicates that artichoke flowers produced in Brazil have only the protease characteristic of cardosin A, indicating that the flower production region and cultivation conditions can influence the presence of enzymes. This result differs from that found by Llorente *et al.*,[36] who, when purifying and characterizing the artichoke extract, found two protein fractions with molecular weights of 31 and 34 kDa and Sidrach *et al.*,[40] who found three cardosin enzymes A, B and C when purifying enzymes from artichoke flower.

It is important to emphasize that the crude extract of artichoke flower showed band associated with cardosin with greater intensity, that is, more apparent when compared to the crude extract of thistle flower. The presence of cardosines, in their respective concentrations, generated similar proteolytic and coagulant activities. This result may be associated with the presence of the same types of proteins in plant extracts.

Spectroscopy

The MIR was used in order to obtain information about the structure and molecular vibrations, as well as the chemical composition of the crude extract of the artichoke flower. From the MIR spectra of the plant extracts and chymosin, 6 absorption peaks were observed

(Figure 4) related to the vibrations of functional groups that correspond to the asymmetric stretching of the methylene group (C–H) at 3245 and 2926 cm^{-1}, angular deformation in the plane of the amide II (C–N and N–H) at 1591 cm^{-1}, deformation of the methylene group (C–H) at 1394 cm^{-1}, stretching of the carbonyl group (C–O) at 1256 and 1034 cm^{-1}. The amide related peak describes the behavior of the secondary structure of the proteins.

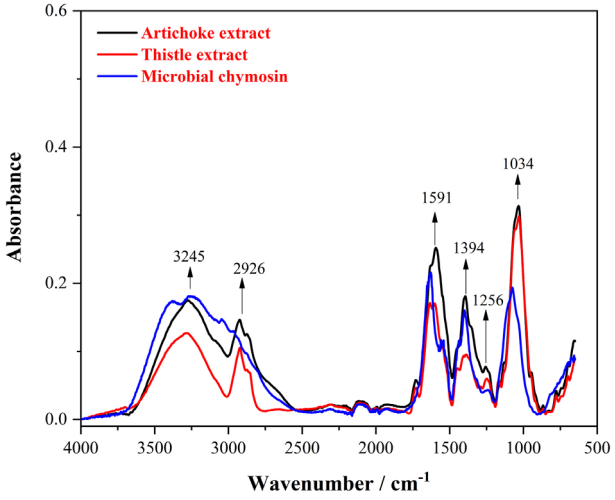


Figure 4. Characterization of the crude artichoke flower extract, thistle flower extract and microbial chymosin by MIR.

The MIR spectra reflect the interaction of electromagnetic radiation with the enzymes present in the plant extracts and microbial chymosin, generating unique vibrational movements resulting from the absorption of energy from the bonds of their functional groups that correspond to a "fingerprint" capable of differentiating the samples analyzed.[19] The studied samples showed similar spectral pattern, but in the crude artichoke extract the functional groups present in the sample absorbed more energy than the other coagulants studied, resulting in more intense peaks, especially the amide II peak at 1591 cm^{-1}, C–H at 1394 cm^{-1} and C–O at 1265 cm^{-1}, indicating a higher concentration of these compounds in the extract and this confirms the high concentration of proteins detected by SDS-PAGE with more intense bands.

Chromatography

The fractionation of the proteins presents in the crude extract of the artichoke flower occurred by molecular weight difference. Figure 5 shows the chromatograms of the crude extracts of artichoke flower, thistle flower and microbial chymosin.

In the chromatogram of the artichoke flower extract it was possible to detect the presence of a peak of higher intensity at the time of 10.50 min, with molecular weight of 36 kDa, and may be associated with the protease cardosin A, responsible for the hydrolysis of casein during milk coagulation. In SDS-PAGE it was also obtained a fraction with molar mass very close to 36 kDa. When compared to the thistle flower extract, it is possible to state that this higher intensity peak may indicate a higher amount of this enzyme in the extract of the artichoke flower. It is also possible to verify the presence of other peaks with lower molecular masses, which although they were not identified, clearly showed the presence of other proteins.

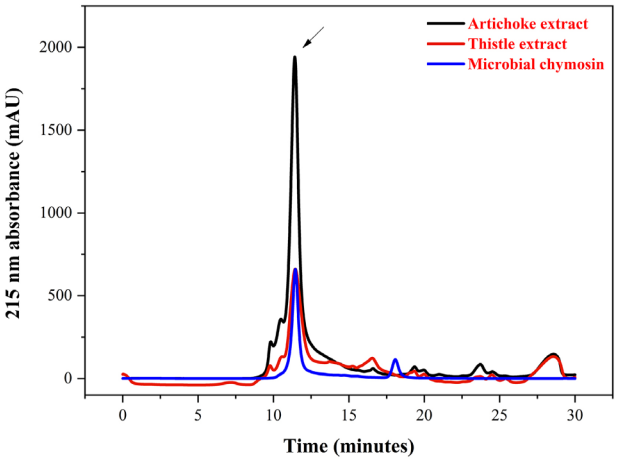


Figure 5. Chromatographic profiles obtained by molecular exclusion of the crude extracts of artichoke, thistle and microbial chymosin.

Comparing the chromatograms, the coagulants studied peaked at almost the same elution times of 10.50, 10.58 and 11.43 min for the crude extract of artichoke, thistle and microbial chymosin, respectively.

The crude plant extracts showed similarities in the chromatographic profiles and differences in peak intensities. These differences can probably be due to the discrepancy in the concentration of proteases present in the extracts, or even, by the presence of other compounds, since the extracts are not pure. From a technological point of view, the use of crude enzyme extracts in the food industry is quite advantageous, because the purification process of enzymes can involve a large number of steps and thus a significant increase in production costs.[8] In this context, it is essential to characterize the crude plant extract and its proteases due to their potential use in food technology.

Assessment of storage stability

When considering a potential new coagulant, especially from plant sources, it is essential to evaluate its storage stability, since many plant protease sources are seasonal and not available all year round, such as artichoke flower, which is harvested between August and November.

Based on the shelf life of the commercial microbial chymosin (180 days), the crude extract of the artichoke flower was stored under freezing for the period of (30 to 120 days) and during this period, every 30 days its proteolytic activity was evaluated.

The PA of the studied coagulants varied significantly ($p < 0.05$) during the storage time, Figure 6. In the first 30 days of storage under freezing there was a loss of approximately 10% of the proteolytic activity of the crude artichoke flower extract, but after this period (30, 60 and 90 days) the proteolytic activity did not differ significantly ($p < 0.05$), with a small loss of activity, remaining practically stable until the time of 90 days of storage. This result suggests that the crude extract of the artichoke flower can be preserved under freezing for a storage period of up to 90 days, with minimal loss of activity. At 120 days there was a 32% loss of activity. The crude extract of the thistle flower showed similar behavior to the artichoke extract.

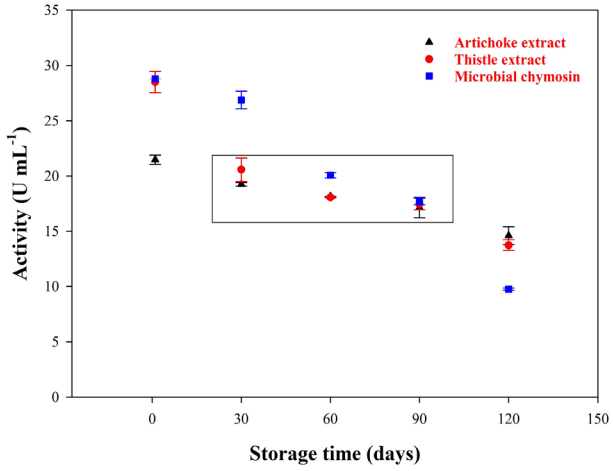


Figure 6. Evaluation of the proteolytic activity of artichoke flower crude extract, thistle flower extract and microbial chymosin during storage under freezing at 0, 30, 60, 90 and 120 days.

A loss of approximately 66% of proteolytic activity occurred in the microbial chymosin after 120 days of storage. The manufacturer of the microbial chymosin indicates a shelf life of 180 days, but the results obtained indicated a reduction in PA after 120 days of storage.

The shelf life of food products is estimated statistically through models and predictive calculations, but the conditions of the food must be the same as in the model, otherwise they will not be applicable, probably some change in the conditions of commercial chymosin may have led to a change in the enzyme stability for storage. In addition, the manufacturer does not make it clear on the label that the PA of the coagulant does not remain stable throughout the storage period, which was found in this article.

Regarding stability, one of the main obstacles to the commercialization of enzymes is the difficulty in keeping them active during long-term storage. The stability of the enzyme during long-term storage facilitates transportation and storage in sales centers. The results obtained indicate that the enzymes present in the crude extract of the artichoke flower remain active during storage under freezing for up to 90 days, suggesting that this is one of the ways to preserve the integrity of proteases for the coagulation of milk, reducing its seasonality and further reinforces that the artichoke flower extract is a viable alternative to replace microbial chymosin for cheese production.

Conclusions

The crude extract of artichoke flower showed caseinolytic and coagulation activity similar to the crude extract of thistle flower. Compared with microbial chymosin, the specificity ratio was higher, proving to be an excellent milk coagulant. From a technological point of view, obtaining crude artichoke extract is a simple and inexpensive procedure, which was used to obtain an active enzymatic preparation.

The electrophoretic profile of the artichoke flower produced in Brazil indicated the presence of only the characteristic band of cardosin A, indicating that the climate, region of flower production and other factors can influence the presence of enzymes. The chromatographic and spectroscopic profiles indicate that the crude artichoke extract has a higher concentration of milk clotting proteases.

The crude artichoke extract remained stable to storage for 90 days, presenting itself as a good milk coagulant agent in the manufacture of cheeses. The use of crude artichoke flower extract will allow the exploration of its technological potential in the production of artisanal and industrial cheeses, resulting in cheeses with unique properties and added value. We emphasize that the production of artichoke flowers has increased in Brazil, making its flowers available for the preparation of an active enzymatic extract, accessible and economically convenient, making it a viable alternative for the market.

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Author Contributions

Josane C. de Jesus was responsible for the formal analysis, data curation, investigation, methodology, project administration and writing original draft; Lucas C. C. Reis and Mateus P. F. Santos for the data curation and investigation; Leandro S. Santos for the conceptualization, formal analysis and supervision; Sibelli P. B. Ferrão for the conceptualization, supervision, project administration and writing review and editing.

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