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Jacaratia corumbensis O. Kuntze a New Vegetable Source for Milk-Clotting Enzymes

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ABSTRACT

The partial characterization and purification of milk clotting enzyme obtained from the (root latex) of Jacaratia corumbensis O. kuntze was studied, by fractional precipitation with ammonium sulphate and ion exchange chromatography. The ammonium sulphate precipitate showed five fractions (AS1– 0-20%; AS2 – 20-40%; AS3 – 40-60%; AS4 – 60-80%; AS5 – 80-100%) and among the fractions obtained, the 40-60% fraction (AS3) showed the highest milk clotting activity with a purification factor of 1.2 fold in relation to the crude extract. This fraction when applied on Mono Q column yielded two protein peaks (p1 and p2), but p1 pool showed the best milk-clotting activity. The optimal pH for the crude and partially purified extract was 6.5 and 7.0, respectively. The maximum milk-clotting activity was at 55ºC for the both crude and partially purified extracts. The enzyme was inhibited by iodoacetic acid which suggested that this enzyme was a cysteine protease, with molecular weight of 33 kDa.

Key words: *Jacaratia corumbensis* O. kuntze; Milk clotting enzyme; Characterization; Purification, Vegetable enzyme

INTRODUCTION

Milk-clotting by proteolytic enzymes is very important in dairy technology. The enzymatic coagulation of milk involves a specific hydrolysis of the Phe-Met bond *kappa*-casein covering the protein micelles (Law, 1999).

The traditional product, calf rennet, has always been the reference product because high chymosin concentration that cleaves specifically *kappa*-casein, induced milk-clotting with high specific milk-clotting activity and low general proteolytic activity. It's very important for cheese manufacture because minimize the general non-especific proteolysis and prevents excessive proteolysis during maturation, ensuring the correct ratio between protein and peptides. The excessive

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proteolysis can, moreover, lead to bitter taste (Law, 1999).

In the past, only calf rennet was used for cheese making. The worldwide increase of cheese production and the reduced supply of calf rennet led to search for rennet substitutes (Lopes et al., 1998). Rennet substitutes of plant origin have been increasingly used to manufacture the cheese, especially at the artisanal level. Application of plant coagulants allows target cheese production, and hence contributes to improve the nutritional input of those populations on whom restrictions are improbable by the use of animal rennets (Silva and Malcata, 2005).

Vegetable rennet extracted from *Cynara cardunculus* L. (cardoon flowers) is used for various cheese productions, such as La Serena cheese, a semi-hard Spanish variety manufactured from raw merino ewes' milk. Aqueous extracts of these flowers have been employed for ages in Portugal and bordering regions of Spain for the production of traditional cheeses from raw ewe's milk (Roa et al., 1999) and/or goat's milk (Silva et al., 2003). High levels of proteolytic enzymes in the flowers are responsible for the effective clotting of milk (Lamas et al., 2001).

Plant proteases employed for cheese production in various areas of the world include papain, bromelain, ficin, oryzasin, cucumisin, sodom apple protease and lettuce protease, extracted from *Carica papaya*, *Ananas comosus*, *Ficus glabra*, *Oriza sativa*, *Cucumis melo ssp*, *Calotropis procera* and *Lactuca sativa*, respectively (Uchikaba and Kaneda, 1996; Asakura et al., 1997; Lo Piero et al., 2002).

Wild papaya (*Jacaratia corumbensis* O. kuntze*)* is a shrub occurring in the Brazilian semi-arid region. Its fruits are consumed by wild animals and the tuber is used for animal feed in the drought periods and for making sweet paste by small farmers (Cavalcanti et al., 1999).

In this work, the biochemical properties and partial purification of milk-clotting protease from *Jacaratia corumbensis* O. kuntze was studied.

MATERIALS AND METHODS

Enzyme Extraction

Fresh biological material (*Jacaratia corumbensis* O. kuntze root) was washed several times with distilled water and disinfected with sodium hypochloride (10% v/v). After this, the latex obtained from the root was diluted (1g) in 100 ml 0.9% (w/v) NaCl solution. The solution was then stirred at room temperature (25^{o}C) for 1 h and filtered through a filter paper and maintained at 4°C. This solution was used for the determination of total protein, proteolytic and milk clotting activities.

Protease Assay

Total protease activity was assayed at 25ºC as described by Ginther (1979), using azocasein (1% w/v, Sigma) in 0.1 M Tris-HCl (pH 7.6) containing 10 mM $CaCl_2$ as a substrate. One unit of activity was defined as the amount of enzyme that produces an increase in the absorbance of 1.0 in 1 h at 440 nm. Total protein was determined by the method described by Bradford (1976) using bovine serum albumin as standard.

Milk Clotting Protease Assay

The milk-clotting protease was assayed as described by Arima et al. (1970). A 10 % (w/v) skim milk solution containing 10 mM $CaCl_2$ was used as substrate. One unit of activity was defined as the amount of enzyme that clotted 1 ml of substrate in 40 min at 35ºC.

Partial Purification of Milk Clotting Protease

For the initial purification step, the crude extract containing the enzyme was fractionated using the increasing concentrations of ammonium sulphate as described by Green and Hughens (1955). Fractions named AS1, AS2, AS3, AS4 and AS5 were precipitated with 0-20, 20-40, 40-60, 60-80 and 80-100% ammonium sulphate, respectively at 4ºC. The solution obtained was dissolved, were centrifugation (20, 200 x*g* for 15 min at 4ºC), the precipitates collected were dissolved in 2 ml of 0.1 M phosphate buffer pH 6.5. The enzyme solution obtain was dialyzed against the same buffer for 24 h, at 4ºC. The proteolytic and milk-clotting activities were determined as described previously. The enzyme solution precipitated in 40-60% ammonium sulfate was used as sample for chromatography in Akta system (Pharmacia).

The Mono Q HR 10/10 column (Pharmacia) was equilibrated with 0.1 M phosphate buffer, pH 6.5 as described by the manufacturer. For the elution two buffers were used: Solution A (0.1 M phosphate buffer pH 6.5) and solution B (1 M NaCl) at a flow rate of 1.5 mL/min under linear gradient from 0 to 200 mM buffer B. The protein peaks were detected by the absorbance at 280 nm

and fractions were collected at the outlet stream. The proteolytic and milk-clotting activities of each protein fraction were determined as described previously.

Effects of pH on Protease Activity and Stability

Protease activity was measured at different pH values using azoalbumin as substrate. The azoalbumin solution was prepared in range pH 3.5 – 8.5 in various buffer at 25º C, such as: 100 mM citrate–phosphate buffer (pH 3.5 – 5.8), 100 mM phosphate buffer (pH 5.8 – 7.5), and 100 mM Tris– HCl buffer (pH 7.0 – 8.5). The pH stability was determined by incubation at different pH values (pH 3.5 – 8.5) as described above, with incubation time varying from 30 to 120 minutes. After this time, the protease activity was measured at 25ºC, using azocasein as substrate in100 mM Tris-HCl buffer, pH 7.6, as described previously.

Effect of Temperature on Protease Activity and Stability

The optimum temperature for milk-clotting activity was assayed at various temperatures (35º - 85ºC), using skim milk 10% as substrate.

The enzyme solution was pre-incubated over range of temperature from 35º to 85ºC, with incubation time varied from 30 to 120 minutes for determination of thermal stability. In this experiment, 10% skim milk was used as substrate.

Inhibition Studies

To determine the class and specificity of the protease, enzyme extracts were incubated for 1h at 37ºC with the inhibitors and then added to azocasein as described previously. The inhibitors used were: 0.1mM phenylmethylsulphonylfluoride (PMSF), 0.1 mM pepstatin A, 0.1 mM benzamidine solution in dimethyl sulfoxide (DMSO), 0.1 mM ethylenediamine tetraacetic (EDTA) and 0.1 mM iodoacetic acid.

Electrophoresis

Polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was carried out 5% (w/v) stacking gel and l2.5% (w/v) separating gel. The molecular weight of the milk clotting protease from *Jacaratia corumbensis* O. kuntze was estimated using the protein standards (Sigma): bovine albumin (66.0 kDa), ovalbumin (45.0 kDa), glyceraldehyde 3-phosphate dehydrogenase (36.0 kDa), carbonic anhydrase (29.0 kDa), trypsinogen (24.0 kDa), trypsin inhibitor soybean (20.1 kDa) and lactalbumin (l4.2 kDa).

RESULTS AND DISCUSSION

Protease Purification

Various purification protocol for milk-clotting protease the vegetable have been described, mostly using chromatography. The aim of this work was to achieve one single and effective protocol, using ammonium sulphate precipitation as pre-purification and ion exchange chromatography. Salting out (ammonium sulphate precipitation) of proteins is a widely used technique in enzyme purification, which takes advantages of the desolvation effect caused by high concentrations of salts. This phenomenon is appropriate not so much for the fractionations of proteins, but mainly is their concentration, and is also useful for the removal of some contaminants (Barros et al., 2001). In this case, the ammonium sulphate precipitation of crude extract showed that protease fractionated at 40 to 60% saturation, give a 165.5 U/mg milk-clotting activity, was the best. The yield in terms of protein content was 40%, the recovery in activity of 50% and a purification factor of 1.2 was achieved, in relation to the crude extract. The results were similar to those found for serine protease from lettuce leaves (*Lactuca sativa* L.), which showed that proteolytic activity was recovered in the 55% ammonium sulphate saturation fraction (Lo Piero and Petrone, 1999). A partial purification of the proteolytic enzyme from fig tree latex was carried out between 50 to 85% saturation (Oner and Akar, 1993). The study of proteases from *Cynara cardunculus* showed three proteases with milk clotting activity after fractional ammonium sulphate precipitation between 30 and 80% saturation (Heimgartner et al., 1990). Ammonium sulphate precipitation was chosen in this study because of its simplicity and the economy of the method, but results showed that there was no significant increase in the milk clotting activity. The AS3 fraction (40-60% ammonim sulfate) with milk-clotting activity was applied to a Mono Q HR 10/10 column. Two protein peaks (p1 and p2) were obtained, and only in the first milk-clotting activity was observed. The elution pattern of the chromatography is shown in Fig. 1. After this chromatography, the purified enzyme solution obtained was used as sample for electrophoresis (SDS-PAGE). In Fig. 2

can be observed the SDS-PAGE gel, with one single major band, which revealed a protein with a molecular weight of approximately 33 kDa.

These results can be compared with study of milk clotting protease from flowers of *Cynara carduncul*us with three proteases with milk clotting activity after two steps of the chromatography (DEAE-Sepharose and MonoQ column) (Heimgartner et al., 1990). The purified proteases showed only one band for each protease, with molecular weights of 41, 42 and 45 kDa. Other work showed the presence of three peaks corresponding to Cardosin A and Cardosin B after ion exchange chromatography that purified the acidic dried flowers extract of *C. cardunculus* with gel filtration (Sephacryl S-100 – Pharmacia) and ion exchange chromatography (HiTrap Q HP – Pharmacia) (Silva et al., 2003). The electrophoretogram profile of three peaks showed polypeptides with 30 to 15 kDa for cardosin A and 31 kDa to 14 kDa for cardosin B. The purification of milk clotting protease from lettuce leaves by combination of $(NH_4)SO_4$ fractionation, gel filtration and anionic exchange chromatography gave a single band with an apparent molecular weight of 40 kDa (Lo Piero et al., 2002).

Results indicated that the purification of milk-clotting enzyme from *J. corumbensis* could be achieved in two steps, one pre-purification by ammonium sulfate precipitation and chromatography by using Mono Q HR 10/10 column in Akta system.

Effects of Various Inhibitors

The specific hydrolysis reaction of *kappa*-casein protein in milk can be evaluated by the protease type. The main method for the study of protease types is reaction in the presence of inhibitors. The milk clotting protease from *J. Corumbensis* O. kuntze was inhibited completely by iodoacetic acid but was not affected by other inhibitors, suggesting a cysteine protease (table 1). The milk-clotting protease reported in literature are aspartic proteases, mainly animal and microbial source, but protease from the vegetable can be used too, such as seric proteases such as cucumisin (EC 3.4.21.25), cysteine proteases i.e. papain (EC 3.4.22.2) and ficin (EC 3.4.22.3) (Uchikaba and Kaneda, 1996).

Effect of pH on Proteolytic Activity/Stability

The pH-activity profiles of the enzyme from the crude and purified extract are shown in Fig. 3. The activity of the enzyme was optimal when the assay was carried out at pH 6.5 for the crude extract and pH 7.0 for partially purified extract. At pH 3.5, about 35% and 40% of the maximum activity was obtained for the crude and partially purified, respectively. At pH 8.5, the protease activity decreased 30 and 60%, respectively for the crude and partially purified extract.

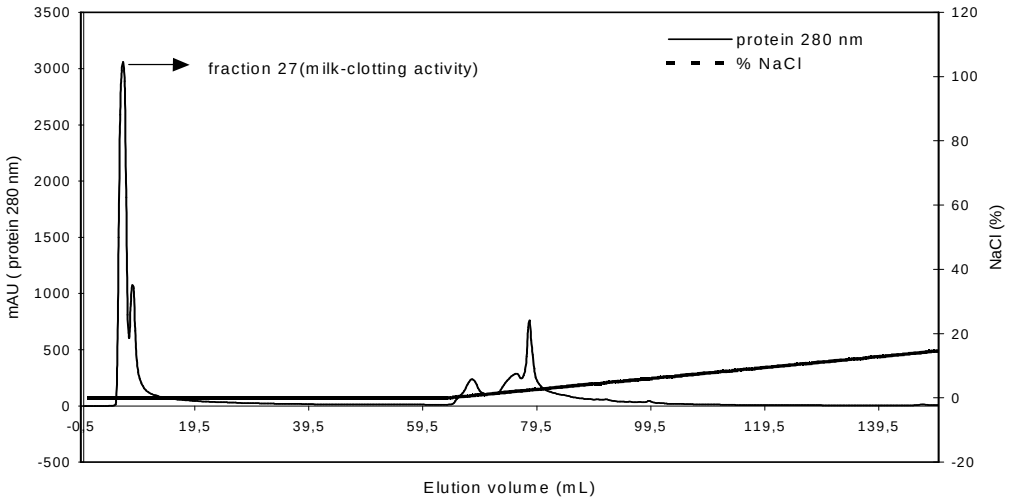

Figure 1 - Elution pattern of the 40-60%, ammonium sulphate fraction from *Jacaratia corumbensis* O. kuntze on Mono Q column.

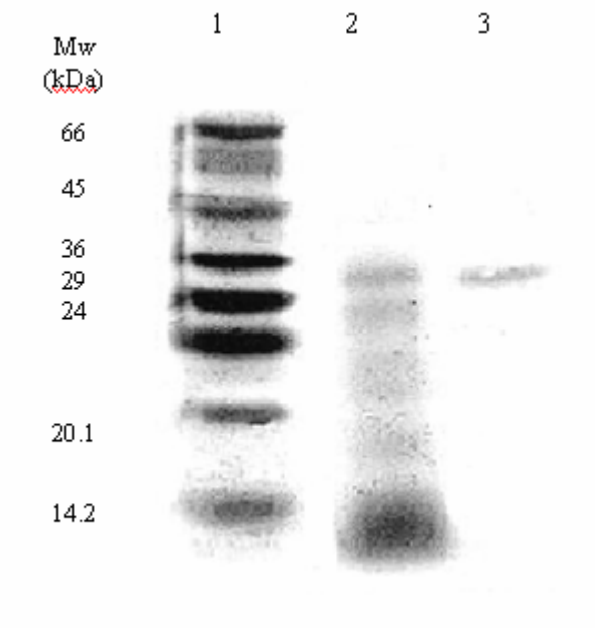

Figure 2 - SDS-PAGE (12.5%) analysis of the purification procedures of the milk-clotting protease from *Jacaratia corumbensis* O. kuntze. Lane 1: molecular mass markers; lane 2: ammonium sulphate precipitated fraction 40-60%, 15 µg; lane 3. fraction 27 (protein peak first) from the step on Mono Q, 15 µg.

Table 1 - Effects of various inhibitors on the milk-clotting protease activity from Jacaratia root latex.

Inhibitors	Inhibition (%)	
	CE[a]	PPE[b]
EDTA	9±1.41	1±0.0
PMSF	20±0.71	11±2.12
Iodoacetic acid	100±0.0	100±0.0
Pepstatin	21±0.0	20±2.12
Benzamidine	11±1.41	6±0.7

[a] crude extract; [b]PPE Partially purified protease

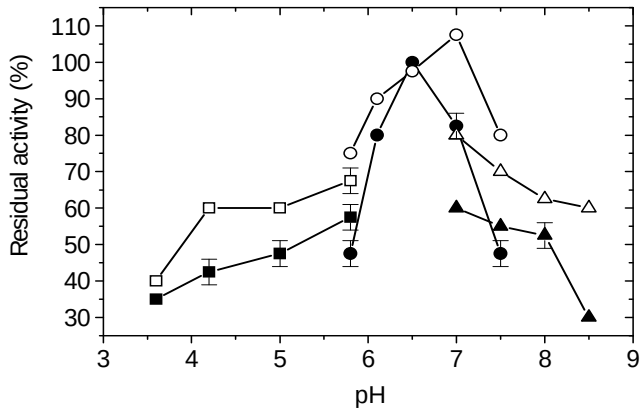

Figure 3 - Effect of pH on activity the milk-clotting protease of the *Jacaratia corumbensis* O. kuntze (Crude Extract)-Buffer solutions 0.1M phosphate citrate (■); phosphate (●) and Tris-HCl (▲), (Partially Purified Extract)-Buffer solutions 0.1M phosphate citrate (□); phosphate (○) and Tris-HCl (△).

These pH profile results could be compared with other work for another milk clotting enzyme from *Lactuca sativa* leaves, where the optimum pH was around 6.5 - 8.0 for several substrates used (Lo Piero et al., 2002). However, the results obtained were different with the milk-clotting enzyme from *Cynara cardunculus*, which showed a maximum activity around pH 5.1. Milk-clotting protease obtained from the latex by *Ficus carica* had maximum activity in the acidic pH range (Fadyloglu, 2001).

The crude and partially purified extracts were stable in the acidic pH range (3.6 – 6.5) and retained 100% of initial proteolytic activity after 120 min (as shown the Fig. 4). In the basic pH range, however, only 60 and 80% (crude and partial purified) of initial proteolytic activity was retained after 120 min in pH 8.5.

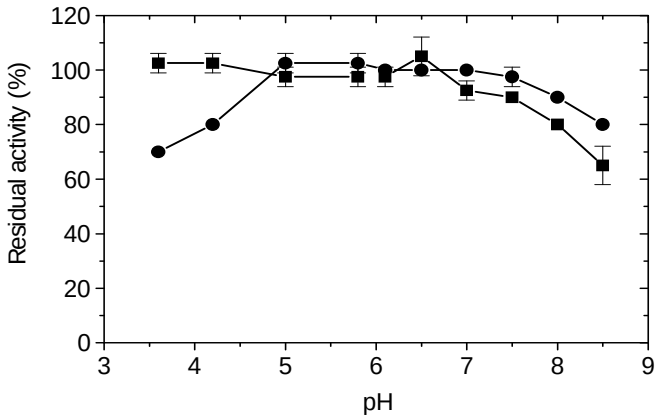

Figure 4 - Effect of pH on stability of crude (•) and partially purified (■) protease from *Jacaratia corumbensis* O. kuntze carried out at 25°C during 120 min. The reaction was carried out in the following buffer solutions 0.1M: phosphate-citrate (3.6 - 5.8), phosphate (6.1 - 7.5) and Tris-HCl (8.0 and 8.5).

Effect of Temperature on Milk-clotting Activity/Stability

The optimal temperature for milk-clotting activity was at 55ºC for both crude and partially purified extract, showing 800% and 270 % of activity (Fig. 5), but their activities were decreased at 75 and 85ºC for both the extracts. The results were similar to those found by Lo Piero et al. (2002) and Asakura et al. (1997) with the proteases from *Lactuca sativa* L. and oryzasin from *Oriza sativa* that exhibited optimal temperature as 50ºC. The crude and partially purified extract of *J. corumbensis* retained approximately 100% milk-clotting activity after 15 and 30 min at 35ºC, respectively (Figs. 6 and 7).

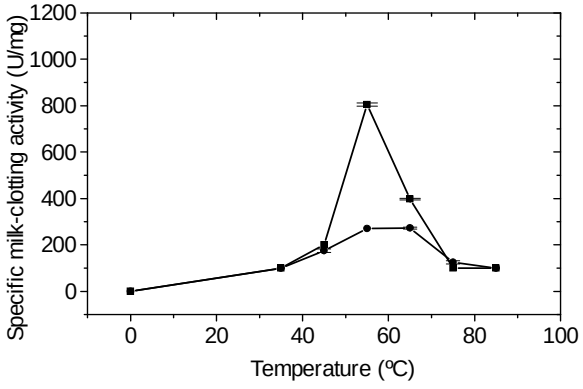

Figure 5 - Effect of temperature on the milk-clotting protease of the *Jacaratia corumbensis* O. kuntze Crude Extract (■); Partially Purified Extract (•).

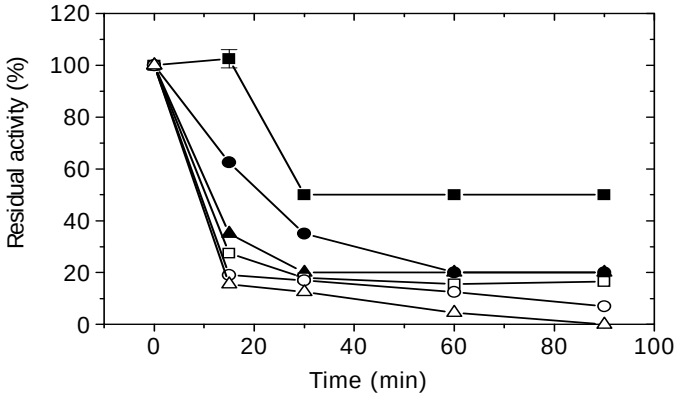

Figure 6 - Thermal stability of proteases from *Jacaratia corumbensis* O. kuntze (Crude Extract) 35º (■); 45º (●); 55º (▲); 65º (□); 75º (△) and 85ºC (○).

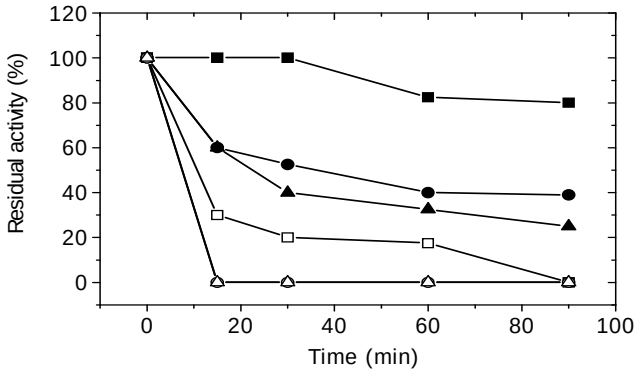

Figure 7 - Thermal stability of proteases from *Jacaratia corumbensis* O. kuntze (Partially Purified Extract) 35º (■); 45º (●); 55º (▲); 65º (□); 75º (△) and 85ºC (○).

However, the activity of crude extract fell to 13% after 15min at 85ºC was inactivated after 90 min at 75ºC. The partially purified extract was inactivated after 15 min at 75 and 85ºC. These results could be compared with other work which did not observe a loss of milk-clotting activity with a crude extract obtained by *Solanum dobium* after 10 min at 40 and 50ºC (Yousief et al., 1996). However, the results reported for lettuce protease leaves showed that incubation at 50ºC did not greatly affect the enzyme, as it retained 80% of original activity (Lo Piero et al., 2002). The partially purified extract retained approximately 80% milk-clotting activity after 90 min at 35ºC, but activity decrease to 20% after 90 min at 55ºC. The partially purified extract was inactivated at 65ºC after 15 min.

The results suggested that the proteases obtained from the latex of *Jacaratia corumbensis* O. kuntze root might be a potential source for enzymes useful in dairy applications.

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RESUMO

A enzima coagulante de leite obtida de látex de raiz de *Jacaratia corumbensis* O. kuntze foi caracterizada parcialmente e purificada, por precipitação fracionária com sulfato de amônio e cromatografia de troca de íon. Foram utilizadas cinco frações de sulfato de amônio (AS1 - 0-20%; AS2 - 20-40%; AS3 - 40-60%; AS4 - 60-80%; AS5 - 80-100%), a fração 40-60% (AS3) mostrou alta atividade coagulante com um fator de purificação de 1,2 vezes em relação ao extrato bruto. Esta fração foi aplicada em coluna Mono Q obtendo dois picos de proteína (p1 e p2), o p1 mostrou melhor atividade coagulante. O pH ótimo para o extrato bruto e parcialmente purificado foi 6,5 e 7,0, respectivamente. A atividade coagulante foi atingida a 55ºC para ambos os extratos, bruto e parcialmente purificado. A enzima foi inibida por ácido iodoacético que sugere que esta enzima é uma cisteína protease, com peso molecular de 33 kDa**.**

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