Uma nova enzima coagulante de leite de sementes de noni (Morinda citrifolia L.)

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A new milk-clotting enzyme from noni seeds (*Morinda citrifolia* L)[1]

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Short title: Milk clotting activity in noni seeds

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ABSTRACT – (A new milk-clotting enzyme from noni seeds (*Morinda citrifolia* L)). Proteases are a group of enzymes that catalyze several essential reactions. They are found in all living organisms and in plants has received more attention because of their potential involvement in various industrial processes. Noni plant (*Morinda citrifolia* L.), belongs to the family Rubiaceae, of southwestern Asia origin. The juice made with the fruit is widely used as phytotherapic agent and in combating a range of diseases, while seeds are usually discarded. The objective of this work was to seek active milk clotting proteases in noni seeds. The crude extract (CE) protein was obtained from seeds sprayed and mixed with tris-HCl 50mM buffer (pH 6.0). The results demonstrated the presence of proteases with milk clotting properties in noni seeds, which a high milk clotting activity from 3,891 U/mL to 65°C. This value indicates that 1mL of extract is able to coagulate 3.8 liters of milk in 40 minutes under specific conditions. This property indicates the potential of noni seeds as a natural source of proteolytic enzymes with biotechnological potential for application in the cheese making industry.
Keywords: milk clotting, protease, proteolytic activity, seeds

RESUMO – (Uma nova enzima coagulante de leite de sementes de noni *(Morinda citrifolia* L.)). As proteases são um grupo de enzimas que catalisam várias reações essenciais. Eles são encontrados em todos os organismos vivos e nas plantas, tem recebido mais atenção devido ao seu potencial envolvimento em vários processos industriais. A planta noni (*Morinda citrifolia* L.), pertence à família Rubiaceae, de origem no sudoeste da Ásia. O suco feito com a fruta é amplamente utilizado como agente fitoterápico e no combate a uma série de doenças, enquanto as sementes costumam ser descartadas. O objetivo deste trabalho foi buscar proteases ativas da coagulação do leite em sementes de noni. O extrato bruto proteico (CE) foi obtido de sementes pulverizadas e misturadas com tampão tris-HCl 50mM (pH 6,0). Os resultados demonstraram a presença de proteases com propriedades de coagulação do leite em sementes de noni, que apresentam alta atividade de coagulação do leite de 3.891 U / mL a 65 ° C. Esse valor indica que 1mL de extrato é capaz de coagular 3,8 litros de leite em 40 minutos em condições específicas. Esta propriedade indica o potencial das sementes de noni como fonte natural de enzimas proteolíticas com potencial biotecnológico para aplicação na indústria de produção de queijo.
Palavras chave: atividade coagulante de leite, atividade proteolítica, protease, sementes

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Introduction

Cheese is a dairy product, produced by the coagulation of milk through the action of enzymes contained in animal rennet (chymosin), with the partial extraction of whey (Hickey 2017). One of the most used milk clotting agents is calf rennet. However, the increase in cheese production, associated with the reduction in the supply of rennet of animal origin, as well as the ethical concerns related to the production of these enzymes, has led to an increasing search for alternative sources of enzymes of microbial and plant origin (Jacob *et al*., 2011). There are reports of large production and variety of cheeses produced from vegetable enzymes in Spain and Portugal, which use, for example, *Cynara* sp. as a source of proteases (Roseiro et al., 2003; Shah et al., 2014). Several scientific works have been directed towards the search for milk coagulation enzymes in plants. As a result, proteases with milk coagulant activity have been discovered in Cardoon (*Cynara cardunculus*) (Folgado *et al.*, 2020); sunflower (*Helianthus annuus*) (Nasr *et al*., 2016); Artichoke (*Cynara scolymus*) (Bueno-Gavilá *et al*., 2020) and moringa (*Moringa oleifera*) *(*Sánchez-Muñoz *et al*., 2017). The noni plant (*Morinda citrifolia*) belonging to the Rubiaceae family produces fruits that have been widely used in alternative medicine to combat various types of illnesses (Abou Assi *et al*., 2017), while the seeds are usually discarded. Recently, de Farias *et al.* (2020) reported the presence of cysteine proteases with milk clotting activity in noni (*Morinda citrifolia* L.) fruits. In this work, we report the caseinolytic activity and milk clotting of a noni seed protease, reinforcing the potential of this plant in the cheesemaking process.

Materials and methods

Plant material - Noni seeds were obtained from fruits collected in the counties of Rio de Janeiro and Campos dos Goytacazes. The seeds were obtained from ripe fruits harvested between March and December 2019. The seeds were dried at room temperature and then pulverized using a knife mill (Fortinox). The resulting powder was kept at 25°C, and maximum humidity of 45%, under dark conditions, until use.

Protein extraction - Proteins were extracted from the homogenization of 10 g of seed powder in 10% (w/w) of PVPK-30 added to 50 mL of extraction buffer (50 mM Tris HCl pH 6). The mixture was kept under vigorous stirring for 90 minutes at 4°C. The homogenate was then centrifuged at 12,000 xg for 30 minutes at the same temperature. The sediment was discarded while the supernatant solution was used as crude protein extract (CE).

Protein determination - Protein concentration on protein crude extract (CE) from *M. citrifolia* seeds was performed according to the methodology of Bradford (1976), using the Bradford assay kit and Bovine Serum Albumin (BSA) as standard protein according to the manufacturer's instructions (BioRad).

Milk clotting activity - Detection of milk clotting activity was performed based on the methodology described by Arima *et al*. (1970). Briefly, 900 µl of 10% skimmed milk powder (Molico®) solution in 10mM calcium chloride buffer, pH 6.5, was distributed into microcentrifuge tubes. The reaction was started by adding 100 µl of CE to the tubes. The tubes were kept in a water bath at 65°C and the milk clotting activity time (t) was defined as the time between the beginning of incubation at 65°C and the appearance of milk rennet. One Milk Clotting Activity unit (MCA) was defined as the amount of EB (mL) required to coagulate 100 mL of milk in 40 minutes, under the assay conditions. MCA was calculated using the following equation and expressed in Soxhlet Unit per mL of coagulant (US/mL): MCA (US/mL) =2400/t×S/E,

Where:

t = clotting time (sec);

S = substrate volume (ml);

E = volume of enzyme solution (EB)(mL).

Temperature optimization - The thermal stability of noni seed protease was tested by incubating 100 mL of CE in 900 mL of 10% skimmed milk powder solution in 10 mM calcium chloride buffer, pH 6.5, at different temperatures (30 - 90 °C). The samples were kept in incubation for the time necessary for the first appearance of milk rennet, and then, the MCA was calculated as described above.

Statistical Analysis - Data are presented as the mean (S.D.) of three independent experiments, and the differences from controls were assessed with Student's t-test using GraphPad Prism version 8.0 software and the difference with $P < 0.05$ was considered significant.

Results and discussion

Milk coagulation, using proteolytic enzymes, is an important step in cheesemaking process. The amount and type of protease used in the process are crucial factors because it could affect the final characteristics of the cheeses. Many different parts of the plants have already been studied and the biological activity of protease in them has been detected, demonstrating their milk clotting potential, such as *Bromelia pinguin* fruits (Moreno-Hernández *et al*., 2017), flowers of *Citrus aurantium* (Mazorra-Manzano *et al*., 2013), leaves of *Moringa oleifera* (Shi *et al.*, 2019) and seeds

of *Vallesia glabra* (González-Velázquez et al., 2021). In this study, the protease contained in the crude extract exhibited great potential for breaking down casein, promoting milk coagulation with an MCA of 3,891 SU/mL (figure 1 a). This result is reinforced by the demonstration of the cheese curd produced by the action of the milk coagulation protease contained in the seeds of *M. citrifolia* (figure 1 b). The effect of temperature on milk coagulation by protease activity of noni was evaluated at different temperatures (30 to 90 °C) (figure 1 a). The results showed that the protease contained in noni seed extract is stable at high temperatures. In fact, the milk clotting activity increased at higher temperatures, reaching maximum activity at 65 °C. However, at higher temperatures (>70°C), coagulant activity is reduced, indicating denaturation of the enzyme. Interestingly, enzyme activity rapidly reduces at temperatures <60 °C and > 70 °C (figure 2). However, the enzyme retains approximately 20% residual activity at 90 °C. These data are similar to those observed by Zikiou & Zidoune (2018) who reported an optimal activity of enzymatic extracts of *C. Cardunculus* at 60 °C. In addition, several plant proteases have been reported to show optimal coagulating activity at 60 °C such as *Ficus johannis* (Afsharnezhad *et al.*, 2019), *Zingiber officinale* (Gagaoua *et al*., 2015), *Gracilaria edulis* (Arlene Arbita *et al.*, 2020), indicating that milk clotting proteases found in plants seem to be more suitable for use under different temperature conditions. To the best of our knowledge, this is the first report of the detection of milk coagulant protease in noni (*Morinda citrifolia*) seeds, confirming the possibility of using these seeds as coagulant in cheesemaking process. In the present study, the milk clotting activity of a protease from noni seed was evaluated and partially characterized. Further studies on the purification and biochemical characterization of the protease, together with the evaluation of the quality of the cheese curd produced by its action, will clarify its commercial suitability.

Author Contributions

Julyanne Dantas: Substantial contribution in the concept and design of the study; Contribution to data collection; Contribution to data analysis and interpretation; Contribution to manuscript preparation.

César Siqueira: Substantial contribution in the concept and design of the study; Contribution to data analysis and interpretation; Contribution to manuscript preparation; Contribution to critical revision, adding intellectual content.

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Conflicts of interest

There is no conflict of interest.

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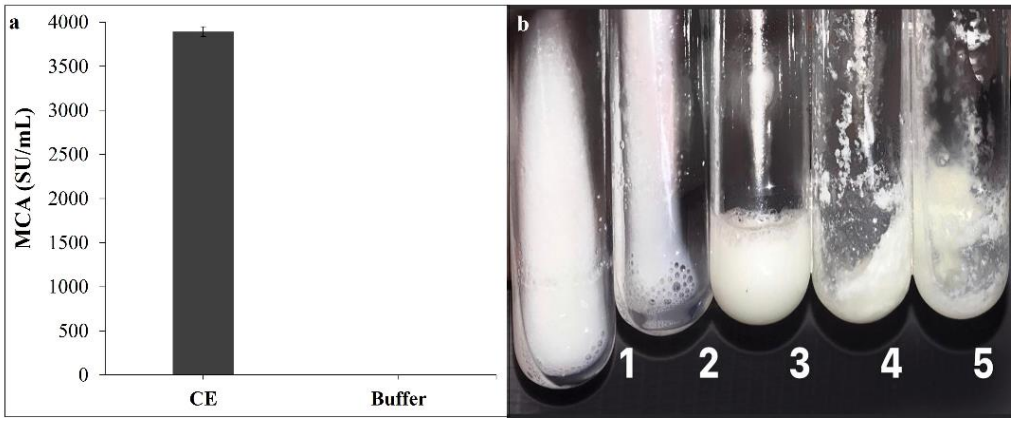

Figure 1. a. Evaluation of milk clotting activity by proteases present in noni seeds (*Morinda citrifolia* L). The reaction took place using 100 µL CE or buffer added to 10% skimmed milk powder in 10 mM calcium chloride buffer, pH 6.5 as substrate. Data are a mean of three independent experiments ratios of standard error to mean were 5% or less. b. Qualitative analysis of the presence of milk clotting proteases in noni seeds: Tube 1 and 2 - 10% skimmed milk powder containing 10 mM $CaCl_2$ incubated with extraction buffer, in the absence of EB; Tube 3-10% skim milk powder containing 10 mM CaCl2 incubated with 10 µL chymosin; Tubes 4 and 5: 10% skim milk powder containing 10 mM CaCl2 incubated with 100 µL CE.

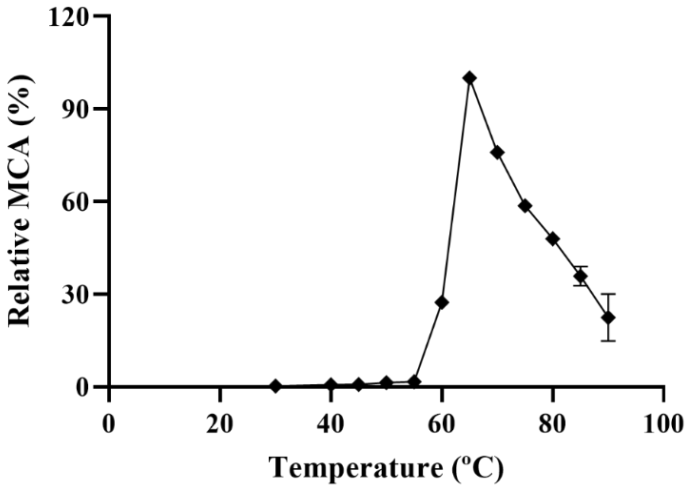

Figure 2. Effect of temperature variation on the milk clotting activity of noni seed proteases (*Morinda citrifolia* L). Each point corresponds to the average of three independent experiments. Milk-clotting activity is reported as a % relative to the maximum observed at the different temperatures. Data are a mean of three independent experiments ratios of standard error to mean were 5% or less.

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