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PURIFICATION AND CHARACTERIZATION OF AN *Onopordum tauricum* PROTEASE WITH MILK CLOTTING ACTIVITY

THESIS TYPE: Research

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Abstract

Plant coagulants have been used for a long time in some Mediterranean countries, such as Spain and Portugal, mainly in artisanal cheese making, especially ovine and caprine cheeses. These coagulants are mainly extracted by thistle plants belonging to *Asteraceae* family, mainly from *Cynara cardunculus*. However, other plants belonging to the same or other families have been studied for the presence of proteases with milk clotting activity. An example is *Onopordum tauricum*, belonging to the same family, which is studied for the first time for the presence of proteases with milk clotting activity. A protease with milk clotting activity was extracted from the flowers of *O. tauricum*, using different extracting agents and methods. This protease was further purified and characterized, using three purification steps (ammonium sulfate precipitation, gel chromatography, and ion-exchange chromatography). SDS-PAGE analysis showed a major band of protein migrating of about 30 000 Da under denaturing conditions.

Purification steps performed in this thesis could successfully remove all the pigments present in the crude extract.

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ACRONYMS AND ABBREVIATIONS

BSA	Bovine Serum Albumin
AP	Aspartic proteases
PA	Proteolytic activity
MCA	Milk-Clotting Activity
AS	Ammonium Sulfate
UF	Ultra-Filtrated
CE	Crude Extract
CMP	Casein Macro-peptide

INTRODUCTION AND SCOPE OF THESIS

Chymosin (animal rennet) is the oldest and most used enzyme in cheese making, dating around 5000 BC (Shah, et al., 2014). This enzyme is found in the stomachs of goat kids, lambs, and calves. The substrate of chymosin is the main protein found on milk, casein. There are 4 groups of caseins, α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein as well as γ-casein which is a C-terminus of β-casein. Caseins in milk are organized in form of micelles, with α and β caseins in the middle surrounded by κ-caseins. The κ-caseins contribute to the stability of other caseins in milk, thus not allowing them to precipitate, since the first three groups of caseins are hydrophobic proteins while κ-casein is both hydrophobic and hydrophilic. The main substrate of chymosin is κ-casein. Chymosin cleavages the peptide bond between Phe105 (Phenylalanine) and Met106 (Methionine) amino acids residues, thus causing the precipitation of other caseins in milk. The product of this reaction is referred to as curd (calcium phosphor-caseinate), which leads to the formation of cheese.

Chymosin can also be used in recombinant form. The recombinant enzyme is produced by microorganisms, where the responsible gene for its production is incorporated. The responsible gene is taken from calves and introduced into a plasmid. The plasmid is then introduced into a microorganism, which will start producing chymosin by transcribing and translating the gene from the plasmid. *Aspergillus niger* and *Kluyveromyces lactis* are mostly used for this purpose.

But these are not the only alternatives. Due to the limited availability, high price of rennet (chymosin), religious factors, diet or ban on recombinant calf rennet in some countries, focus has lately shifted towards vegetable coagulants, which are considered as a potential replacement to animal coagulants. Vegetable coagulants have been used for centuries by people in Portugal and Spain in artisanal cheesemaking, either as crude extract or in purified form (Shah, et al., 2014). They are mostly extracted by thistle plants, like *Cynara cardunculus* and other plants belonging to the same family. Different studies have proven the milk coagulation properties of different plants and several enzymes have been extracted and studied, mainly present in the flowers of these plants. These enzymes are classified into various groups depending on their catalytic mechanism used during hydrolytic process. The main classes of milk-clotting proteases are aspartic, serine and cysteine proteases (Amira, et al., 2017).

Our study is focused on the enzymes which are endowed with milk clotting activity in *Onopordum tauricum*. The scope is to purify and characterize, as well as to study milk coagulation properties of proteases extracted from the flowers of this plant. For this purpose, *O. tauricum* flowers have been collected, and a procedure for the purification of the proteases has been set up.

Chapter 1
BACKGROUND

Cheesemaking is one of the oldest examples of food preservation, dating around 5000 BC. Preservation of milk constituents into cheese, exploits two principles of food preservation, lactic acid fermentation, and reduction of water activity through removal of water or addition of NaCl. There are around 500 varieties of cheese produced throughout the world (McSweeney, et al., 2017).

Cheese is the most diverse group of dairy products and is, arguably, the most academically interesting and challenging. Many dairy products, if properly manufactured and stored, are biologically, biochemically, chemically, and physically very stable, while cheese is biologically and biochemically dynamic, and, consequently, inherently unstable. Cheese production represents a finely orchestrated series of consecutive and concomitant biochemical events which, if managed correctly, lead to the production of cheese with highly desirable aromas and flavors but when not well managed, result in off-flavors and odors. Cheesemaking has two major processes, manufacturing of cheese and ripening. Manufacturing usually requires less time compared to ripening (2 weeks or more), depending on cheese variety. The basic steps of cheese manufacturing are the same for most varieties: acidification, coagulation, dehydration (cutting the coagulum, cooking, stirring, pressing, salting, and other operations that promote gel syneresis), separation of curds and whey, shaping (molding and pressing), and salting. Each of these steps can be modified according to the variety of cheese produced (McSweeney, et al., 2017). Ripening is the second process of cheesemaking that is responsible for the distinct flavor of cheese. The modification of “ripening agents”, determines the features that define many different varieties of cheese, such as texture, taste, and body. Ripening is a complex process that is characterized by a series of physical, chemical, and microbiological changes that include the interaction of indigenous milk enzymes (especially plasmin and lipoprotein lipase), starter bacteria and their enzymes, secondary microbiota and their enzymes, rennet, lipases, added molds or yeasts, and environmental conditions (McSweeney, et al., 2017).

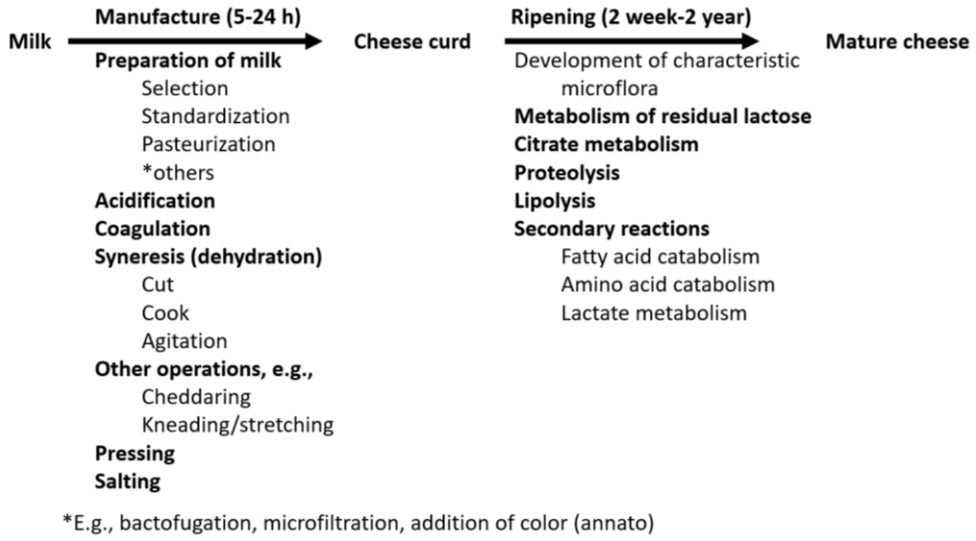

Figure 1-1: Cheesemaking processes and steps (McSweeney, et al., 2017)

An essential step in the manufacturing process of all cheese varieties is coagulation. This step is characterized by coagulation of casein component of the milk protein system to form a gel, which entraps the fat, if present. Coagulation can be achieved by proteolysis by selected proteinases, acidification to pH 4.6, and acidification to pH 5.2 in combination with heating to 90℃. Most varieties of cheese (about 70% of total production) are produced through enzymatic (rennet) coagulation, with a few exceptions in which acid proteases from thistle plants (mainly *Cynara cardunculus*), acid proteinases of animal or fungal origin are used. The primary enzyme in rennet prepared from animal stomachs is chymosin (95% of total milk-clotting activity), with a little pepsin. However, limited supplies of animal rennet, concomitant with a worldwide increase in cheese production, have led to a shortage of calf rennet and consequently rennet substitutes are now widely used in cheesemaking in many countries with satisfactory results (McSweeney, et al., 2017).

Plant-derived coagulants or plant rennet that are extracted from thistle plants (mostly in the family *Asteraceae*) are not so widely used in cheesemaking, but they have gained more interest over the years and are considered as a potential replacement to animal coagulants (animal rennet). Currently, they are only used in artisanal cheesemaking (mainly in goat and sheep cheese). Their industrial use is practically non-existent because of high bitterness and lower cheese yields. However, some developments in the understanding of their action on milk proteins during gelation and the control of various parameters that influence cheese production process suggest a change. In literature, rheological properties of milk gels and sensory characteristics of cheeses produced from plant proteases varied according to the type of coagulant, its enzymatic activities (Milk clotting activity, Proteolytic Activity), and its concentration. Other parameters that can affect the qualities of gels and cheeses, include milk pH, gelation temperature, heat treatment, and

the addition of salt during ripening (Amira, et al., 2017). Each of these parameters and their effect in cheese, as well as challenges and opportunities arising from the application of plant proteases in cheesemaking will be discussed in this thesis.

1.1 Research justification

The problems related to animal rennet availability in many countries, demands of specific customer segments (e.g., lactovegetarians), and the increase in cheese production, has triggered the search for animal rennet substitutes since 1960s. Currently, there are three types of rennet substitutes that are used: microbial-derived coagulants, plant-derived coagulants, and fermentation-produced chymosin (recombinant chymosin). Fermentation-produced chymosin or recombinant chymosin is widely used in cheesemaking, especially in the USA and in the United Kingdom (approximately 80-90%). It was the first food-processing aid made by recombinant DNA technique that was registered by the FDA (McSweeney, et al., 2017). On the other hand, microbial-derived coagulants and plant-derived coagulants are not so widely used. Plant-derived coagulants are characterized by higher proteolytic activity, which induces a slightly bitter taste but also piquant, leading to a buttery, soft texture, and to a liquefication in the center. These characteristics make these coagulants less suitable for long-ripened cheese varieties, which hamper their use in industrial cheesemaking (McSweeney, et al., 2017). However, several improvement strategies have been developed to produce cheeses similar to those made with animal rennet.

The most important source of plant-derived coagulants is undoubtedly *Cynara cardunculus*, but the list of plants that contain enzymes with milk-clotting activity keeps getting longer as other plants with similar characteristics are studied.

The main objective of this study is to characterize proteases from *Onopordum tauricum* (the characteristics of which, as well as the characteristics of *Onopordum* genus, are described below) in their milk-clotting activity and proteolytic activity on casein.

1.2 Onopordum species

Figure 1-2: Onopordum acanthium habitat (Source: Wikipedia)

Onopordum, **cottonthistle**, is a genus of plants in the thistle tribe within the Asteraceae. They are native to southern Europe, northern Africa, the Canary Islands, the Caucasus, and southwest and central Asia. They grow on disturbed land, roadsides, arable land, and pastures.

They are biennials (rarely short-lived perennials) with branched, spinose winged stems, growing 0.5–3 m tall. In the first season they form a basal rosette of gray-green felted leaves and rarely a few flower heads. In the second season they grow rapidly to their final height, flowering extensively, and then die off after seed maturation.

The leaves are dentate or shallowly lobed to compound with several pinnatifid or deeply cut leaflets, and strongly spiny. The terminal flower head is typical for thistles, a semi-spherical to ovoid capitulum with purple (seldom white or pink) disc florets. There are no ray florets. The receptacle is glabrous with dentate margins. The tube of the corolla is slender, sac-shaped and symmetrical. The anthers have awl-shaped outgrowths on the top. The capitula have several overlapping rows of leathery basal simple linear-lanceolate spines. These are smooth to slightly pubescent.

These plants propagate only by seed. The seed heads mature in mid-summer, releasing their seeds. The fruit is a glabrous achene, 4–6 mm long and with 4-50 ribs. The pappus consists of many rows of simple, fine to minutely rough hairs, united in a circular base.

Onopordum species are used as food plants by the larvae of some Lepidoptera species including *Coleophora onopordiella* (feeds exclusively on *O. acanthium*).

Some species of *Onopordum* have been introduced as ornamental plants in the temperate regions of North America and Australia, where they have become naturalised in the wild. In most of these countries, these thistles are considered noxious weeds, especially in Australia where a biological control program has been set up (using the Rosette Crown Weevil, *Trichosirocalus briesei*). In North America, there are also *Trichosirocalus* control programs, but they have proved detrimental to native thistles.

1.2.1 Onopordum tauricum

Onopordum tauricum, the **Taurian thistle** or **bull cottonthistle**, is a species of thistle. It is native to Eurasia and is known in the western United States as an introduced species. It easily becomes a noxious weed, similar to its relative, *Onopordum acanthium*.

This is a biennial herb producing a sticky, glandular, very spiny stem up to 2 meters tall. The spiny, bright light green leaves are up to 25 centimeters long and are divided into triangular lobes. The inflorescence is made up of several large flower heads each up to 7 centimeters wide. They are lined with long, spiny phyllaries and bear pink-purple tubular flowers up to 3 centimeters long.

Figure 1-3: Onopordum tauricum flower (Source: http://pallano.altervista.org/onopordum-tauricum.html)

1.3 Types and sources of plant-derived coagulants

Plant-derived coagulants hold an important position among various coagulants used in cheesemaking. They have become a subject of growing interest, due to their easy availability and simple purification processes. Furthermore, the use of plant proteases in cheese manufacturing promotes the greater acceptability by the vegetarians and may improve their nutritional intake. They have been widely used in some cheese varieties in Mediterranean countries, Southern Europe, and West Africa (Amira, et al., 2017). These coagulants are found in almost all kinds of plant tissues and can be obtained from their natural source or through in vitro culture to ensure a continuous supply of plant proteases. Almost all the enzymes used as milk coagulants belong to aspartic proteases, but enzymes from other groups such as cysteine and serine proteases have also been reported to have milk-clotting activity under proper conditions (Shah, et al., 2014).

The main classes of plant proteases are aspartic, serine and cysteine proteases. The type of enzyme depends on the plant and the part within the same plant (Amira, et al., 2017). Some examples of plant proteases from different plants are presented in Table 1-1.

Aspartic proteases are one of the four groups of proteolytic enzymes, which hydrolyze other proteins into smaller fragments. They are present in all organisms and play important roles (Dunn, 2013). Aspartic proteases get their name from aspartic residues which bind the activated water molecule. In the majority of known aspartic proteases a pair of aspartic residues act together to bind and activate the catalytic water molecule, but in some enzymes, the second Asp is replaced by another amino acid (Rawlings & Salvesen, 2013). They are highly specific endopeptidases. They tend to cleave dipeptide bonds that have hydrophobic residues as well as beta-methylene group. Most of these enzymes are inhibited by pepstatin.

Serine proteases are characterized by the presence of serine residues essential for the catalytic activity. They are widespread in plants and belong to several taxonomic groups. Serine proteases can be found in different parts of the plant, such as fruits, leaves and seeds. Cysteine proteases have also shown to have milk-clotting activity and characterized by many studies, cited in Table 1-1. Their catalytic mechanism involves a cysteine in their active side (Amira, et al., 2017).

Table 1-1*: *Examples of plant proteases: source, classification, and MCA (Amira, et al., 2017)

Plant	Organ	Enzyme			MCA/total MCA/specific MCA	References
		Type	Name	Number and class		
Cynara cardunculus	Flowers	Aspartic protease	Cardosin	8 Cardosin A Q9XFX3/ AJ132884 Cardosin B Q9XFX4/ AJ237674 Cardosin C Cardosin D Cardosin E P85136 Cardosin F P85137 Cardosin G P85138 Cardosin H P85139	Extract: 0.131 ± 0.025 UAC/ml (1 h of maceration) 0.164 ± 0.024 UAC/ml (24 h of maceration) Cardosin A: 1160 UACI/g Cardosin B: 7556 UACI/g	(Silva, et al., 2003) (Sarmento, et al., 2009) (Ordiales, et al., 2012)
Cynara scolymos	Flowers	Aspartic protease	Cynarase	3 Cynarase A Cynarase B Cynarase C	Extract: Between 60 and 70 CAU/mg 30 CAU/mg 100 CAU/mg Between 30 and 40 CAU/mg	(Sidrach, et al., 2005) (Soledad Chazarra, et al., 2007)
Cynara humilis	Flowers	Aspartic protease	Cardosin A like	-	-	(Esteves, et al., 2003)
Silybum marianum	Flowers	Aspartic protease	Enzymatic extract	-	0.083 CAU/ml	(Vairo-Cavalli, et al., 2005)
Oryza sativa	Seeds	Aspartic protease	Oryzasin	1	-	(Asakura, et al., 1997)
Moringa oleifera	Flowers	Aspartic protease	Enzymatic extract	-	1.9 CAU	(Pontual, et al., 2012)
Onopordum acanthium	Flowers	Aspartic protease	Onopordosin	-	0.546 CAU/ml	(Brutti, et al., 2012)
Cirisum vulgare	Flowers	Aspartic protease	Cirisin JN703462	-	-	(Lufrano, et al., 2012)
Cenataurea calcitrapa	Cell suspension	Aspartic protease	Enzymatic extract	-	2.023 U/mg	(Raposo & Domingos, 2008)
Albizia lebbeck	Seeds	Cysteine protease	Enzymatic extract	-	Crude extract: $156\ x\ 10^{-3}$ U/mg Concentrated extract: $591\ x\ 10^{-3}$ U/mg	(A.S.Egito, et al., 2007)
Helianthus annuus	Seeds	Cysteine protease	Enzymatic extract	-	Crude extract: 5.8×10^{-3} U/mg Concentrated extract: 39×10^{-3} U/mg	(A.S.Egito, et al., 2007)

Ficus carica sylvestris	Branches latex	Cysteine protease	Ficin (EC3.4.22.3)	2	1.4 U/mg	(Faccia, et al., 2012) (Raskovic, et al., 2016)
Sideroxylon obtusifolium	Stems latex	Cysteine protease	-	1	917 U/mg	(Silva, et al., 2013)
Actinidia chinensis	Fruits	Cysteine protease	Actinidin	1	Extract: 244 U/mg	(I. Katsaros, et al., 2010) (Puglisi, et al., 2014)
Calotropis gigantea	Latex	Cysteine protease	Calotropain	4 Calotropain FI Calotropain FII Calotropain DI Calotropain DII	Extract: 450 CAU/ml	(Anusha, et al., 2014) (Abraham & Joshi, 1979)
Zingiber officinale	Rhizome	Cysteine protease	Ginger or Zingibain	3 Ginger proteases GP A GP B GP C	442.2 CAU/mg 288.3 CAU/mg	(X.W.Huang, et al., 2011)
Bromelia hieronymi Mez	Fruits	Aspartic and cysteine proteases	Hieronymain	3	Extract: 40 ICAU/ml	(Bruno, et al., 2010)
Solanum dubium Fresen	Seeds	Serine protease	Dubiumin	1	Extract: 880 CAU/ml	(Ahmed, et al., 2009)
Cucumis melo	Fruits	Serine protease	Cucmisin	1	25 CAU (enzyme concentration of 1.5 µM)	(Uchikoba & Kaneda, 1996)
Lactuca sativa	Leaves	Serine protease	Lettucin	1	20 UP (enzyme concentration 1.9 µM) 40 RU (enzyme concentration 1.9-3.8 µM)	(Piero, et al., 2002)
Ficus religiosa		Serine protease	Religiosin	3 Religiosin Religiosin B Religiosin C	387 CAU/ml 803 CAU/ml -	(Kumari, et al., 2010) (Kumari, et al., 2012) (Sharma, et al., 2012)
Streblus asper	Stems latex	Serine protease	Streblin	1	2306 CAU	(Tripathi, et al., 2011)
Balanites aegyptiaca	Pulp of fruits	Aspartic and serine proteases	-	2 Aspartic protease Serine protease	Crude extract: 2.43 CAU/ml Concentrated extract: 6.80 CAU/ml Discolored extract: 6.88 CAU/ml	(Beka, et al., 2014)
Citrus aurantium	Flowers	-	Enzymatic extract	-	AU/ml at 40℃	(Angel, et al., 2013)

1.4 Enzymatic role of proteases in cheesemaking

Enzymatic coagulation is an essential step in cheesemaking. Many characteristics of cheese are affected by this process, specifically from the type of coagulant used, including rheological and sensory characteristics. Thus, the choice of the coagulant to replace animal rennet is very important.

The enzymatic coagulation has two main steps. It begins with an enzymatic cleavage of the phenyalanine$_{105}$-methionine$_{106}$ (Phe105-Met106) peptide bond of κ-casein and is followed by the destabilization of casein micelles, along with a cooperative aggregation, thus resulting in the formation of three-dimensional protein matrix (gel) (Amira, et al., 2017).

The Phe105-Met106 bond is specific for most aspartic proteases. This unique specificity has gained the interest of scientists, who have made some efforts to understand it (Fox, 1993). Studies made by Fox (1993) have shown that the di-, tri-, or tetra-peptides containing Phe-Met bond were not hydrolyzed. However, the penta-peptide (Leu-Ser-Phe-Met-Ala-OMe) was easily hydrolyzed by aspartic proteases. Therefore, the length and the sequence of the peptide around cleavage site (Phe-Met bond) are important for enzyme-substrate reaction. Serine104 is particularly important and its replacement by Glycine or Alanine, makes Phe-Met bond very resistant to hydrolysis by chymosin but not pepsin, whereas the replacement of D-Ser by L-Ser reduces enzyme specificity (Fox, 1993). The residues of amino acids of this penta-peptide help on keeping the substrate in its correct orientation within the active site of the enzyme (Amira, et al., 2017). The products of the enzymatic cleavage of κ-casein are *para*-κ-casein, which remains attached to casein micelles, and the hydrophilic C-terminal fragment, referred to as CMP or glycomacropeptide (GMP), which is released into the milk serum.

The second step is a nonenzymatic reaction that starts rapidly after 90% of κ-casein is hydrolyzed. Some authors suggest that there is a third step which is considered to involve changes in the curd structure once it is formed. Together with the second step, it comprises the rennet gelation process (McSweeney, et al., 2017).

These steps are carried out in the same way even by plant rennet; however, the catalytic reaction can change for some plant proteases. In fact, they can hydrolyze κ-casein in other peptide bonds. As an example, Cardosin A and B can hydrolyze caprine κ-casein in Lys116-Thr117 peptide bond (Table 1-2) (Amira, et al., 2017).

Table 1-2: Action specificity of Cardosin A and Cardosin B from Cynara cardunculus upon isolated bovine, caprine, and ovine caseins (α_{s1}-, β-, and κ) (Amira, et al., 2017)

Endopeptidase	Bovine caseins		
	α_{s1}-casein	**β-casein**	**κ-casein**
Cardosin A	Phe23-Phe24 Phe153-Tyr154 Trp164-Tyr165 Tyr165-Tyr166	Leu127-Thr128 Leu165-Ser166 Leu192-Tyr193	Phe105-Met106
Cardosin B	Phe23-Phe24 Phe150-Arg151 Trp164-Tyr165	Leu165-Ser166 Leu192-Tyr193	Phe105-Met106
	Caprine caseins		
	α_{s1}-casein	β-casein	κ-casein
Cardosin A and Cardosin B	Phe153-Tyr154	Leu127-Thr128 Leu190-Tyr191	Lys116-Thr117
	Ovine caseins		
	α_{s1}-casein	β-casein	κ-casein
Cardosin B	Leu156-Asp157 Trp164-Tyr65	Leu127-Thr128 Leu165-Ser166 Leu90-Tyr91	Phe105-Met106

The hydrolysis of κ-casein and other caseins (α and β caseins) can continue after the first step of coagulation and during ripening either by the coagulant or other enzymes present, including enzymes produced by bacteria and milk enzymes. The degree of this hydrolysis depends on the coagulant, other enzymes present, lactic bacteria as well as environmental conditions. In the case of chymosin, the hydrolysis of κ-casein is limited to the formation of *para*-κ-casein and CMP, but some plant proteases can further hydrolyze it (nonspecifically) in smaller fragments under several conditions. The same thing can be said about α and β caseins. Chymosin can only cleave some peptide bonds of αs1- and β-caseins, as well as αs2 at a significantly lower extent (McSweeney, et al., 2017). One the other hand, plant proteases, with a few exceptions (enzymes from *Cynara cardunculus*), can hydrolyze α and β caseins to a higher extent, leading to the formation of short chain peptides. These peptides can serve as essential substrates for some bacterial microflora, which can degrade them during storage and ripening. The degradation of these peptides by bacteria allows the development of a bitter taste and appearance defects, which represent the major drawbacks of using plant proteases. Therefore, the evaluation of proteolytic activity and its comparison with chymosin's PA is an important process in choosing the right plant coagulant (Amira, et al., 2017).

1.5 Enzymatic activity of plant coagulants

Enzymatic activity is a very important parameter that needs to be evaluated when choosing a coagulant. For this purpose, milk-clotting activity and proteolytic activity of plant coagulants are measured and compared to chymosin. Milk-clotting activity is the ability of a coagulant to coagulate milk by specifically hydrolyzing κ-casein. It can be measured by different methods, such as Berridge 1945, Soxhlet, and IDF Standard 157, using different units such as rennet units (RU or MCU), Soxhlet unit, and the international milk-clotting unit (IMCU), respectively (Amira, et al., 2017). Proteolytic activity is the ability of the enzyme to hydrolyze caseins, both specifically and nonspecifically. It can be determined by measuring the concentration of the casein that is hydrolyzed over time or by measuring the concentration of the product formed over time. PA can be expressed in different units, such as spectrophotometric units, fluorometric units, international units (IU), etc. Different methods can be used to detect the product formed, namely colorimetry, spectrophotometry, and fluorimetry (Nollet & Toldrá, 2015).

MCA and PA can vary depending on the type of coagulant, as well as other factors, namely pH, temperature, milk composition, enzyme concentration, source of enzyme, etc. Many comparisons have been made by researchers between plant proteases and chymosin in terms of MCA and PA (Amira, et al., 2017). These comparisons are significantly important to better understand the mechanism of action of plant proteases on casein and to find the suitable plant coagulant that can successfully replace animal rennet. For a plant coagulant to be suitable for replacing chymosin, it should have high milk-clotting activity and low non-specific proteolytic activity. For this purpose, MCA/PA ratio is evaluated. This ratio has shown to be crucial in evaluating the acceptability of an enzyme as rennet substitute of calf chymosin (Amira, et al., 2017).

Studies made by Ben Amira, A. et al (2017) on cardoon flowers (*Cynara cardunculus* L.) showed that MCA/PA ratio is highly affected by extraction pH. It was noted that flower extracts prepared at different pH, had different values of MCA/PA ratio. This ratio was increased considerably with pH drop and reached a maximum value of 28.71 at pH 3, which exceeds even the value of chymosin (23.59). On the other hand, crude extract prepared at pH 6, had very low values of MCA/PA ratio (4.35), as compared to other extracts and chymosin. The lowest ratio at pH 6 was because, at high pH, the extraction level of other compounds, including non-proteolytic enzymes, is higher. These compounds can promote the development of other reactions which can interfere with the coagulant, thus causing an underestimation of MCA. Furthermore, the high content of phenolic compounds involves their rapid oxidation to form pigments, which may attach to native enzymes, thus leading to their inactivation (Amira, et al., 2017). Another justification was attributed to the fact that, at acidic pH, between 75% and 90% of total extracted enzyme activity correspond to cardosin A, which is shown to be similar to chymosin, in terms of kinetic parameters and specificity. It

can hydrolyze κ-casein in the same peptide bond as chymosin and its activity is promoted at acid pH (Cavalli, et al., 2013).

Most plant proteases are characterized by a low MCA/PA ratio, which makes many of them unsuitable for cheese manufacturing as they produce extremely bitter cheeses (Grozdanovic, et al., 2013). However, some of them have shown higher ratios than chymosin. The best examples are Cardosins from cardoon flowers and Onopordosin from *O. acanthium*. According to Brutti et al. (2012), the latter had higher MCA/PA ratio (9.58) than cardosin (5.34) and was considered by the researchers as the best plant coagulant (Brutti, et al., 2012). Another example is *Sideroxylon obtusifolium* (quixaba latex) which also showed a sufficiently high ratio for commercial production of cheese, exceeding that of chymosin (Amira, et al., 2017).

Plant proteases with a higher MCA/PA ratio can form a good curd, with higher yield and less bitterness during cheese manufacturing, while low ratio may result in poor curd firmness, weaker curd recovery and the production of bitterness, which affects sensory properties of the final product (Figure 1-4) (Amira, et al., 2017).

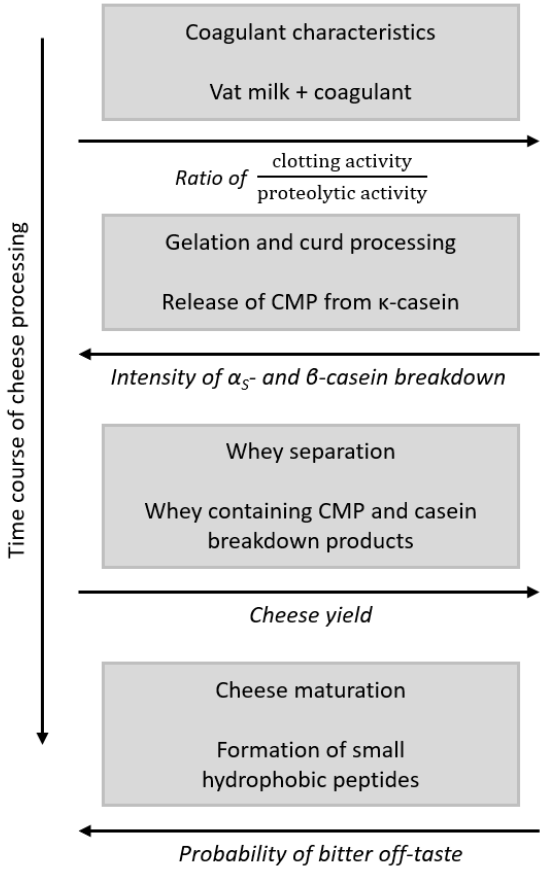

Figure 1-4: Schematic presentation of the main effects of MCA/PA ratio of a coagulant on cheese yield and cheese quality during time course of cheese processing (McSweeney, et al., 2017)

1.6 Effect of plant proteases on rheological properties of gels

The second most important aspect that needs to be analyzed after milk-clotting activity and proteolytic activity, is gel formation. Possibly the most direct way to measure gel formation is to monitor the evolution of rheological properties. These properties are defined by the spatial distribution of casein micelles in the gel network and the strength with which these elements are bound to each-other (McSweeney, et al., 2017).

The most accurate way of monitoring the evolution of rheological properties is dynamic rheology. This method consists in the application of an oscillatory shear stress (τ_0) or strain (γ_0) and measures the response from the developing gel. The measurement yields the elastic or storage modulus (G'), which is a measure of the energy stored per oscillation cycle and reflects how the sample behaves as an elastic solid, and the viscous or loss modulus (G"). which is a measure of the energy dissipated per cycle and indicates how well the sample behaves as a viscous liquid. Their ratio (G"/G') is tan δ, the tangent of the phase angle of the response to the applied stress or strain. When phase angle is higher than 45° or when tan δ is higher than 1, the viscous component dominates, whereas when phase angle is lower than 45° or when tan δ is lower than 1, the sample is more like an elastic solid. On the other hand, phase angle equal to 45° (tan δ=1) represents the transition point in gel formation when gel elasticity develops rapidly, and elastic modulus (G') exceeds viscous modulus (G") (McSweeney, et al., 2017).

From a rheological point of view, cheese is a viscoelastic solid (McSweeney, et al., 2017). Its rheological properties (elasticity and viscosity) are highly dependent on the enzymatic activity of the coagulant on casein. The latter can determine the amount of protein that is incorporated when the gel is initially formed as well as the size of the building blocks of the gel. Therefore, the monitoring of above-mentioned parameters (G', G", tan δ, τ_0, and γ_0) is very important (Esteves, et al., 2001).

Esteves et al. (2001) studied the rheological properties of milk gels coagulated by plant coagulants (*C. cardunculus* L. and *C. humilis* L.) and compared them with chymosin. G' values recorded at the beginning of gelation by plant coagulants were higher than those of chymosin, but after a long incubation time chymosin gels had higher G' values. This difference can be explained by the fact that plant coagulants have a higher proteolytic activity on casein than chymosin, which may alter the degree of proteolysis on κ-casein. It is also possible that during gel formation, hydrolysis of some additional bonds on casein particles by plant coagulants may assist with rearrangements in gel structure, which may lead to an increase in the strength of bonds between adjacent aggregates. These factors can cause a faster initial increase of G' values for plant coagulants compared to chymosin. However, after a longer time, plant coagulants produce G' values lower than chymosin gels (Table 1-3) because they are more proteolytic, which can result in some additional hydrolysis of the network (Esteves, et al., 2001).

Table 1-3: Values of the parameters of Scott Blair model applied to G' as a function of time curves of* C. cardunculus *L.,* C. humilis *L., and chymosin, with milk gelling for ~6 h (Esteves, et al., 2001)

	Coagulant type		
Parameters	*C. cardunculus* L.	*C. humilis* L.	Chymosin
G' (Pa)*	74.6 ± 0.8^{a}	72.7 ± 2.9^{a}	96.0 ± 1.4^{b}
τ (min)**	29.5 ± 3.0^{a}	23.6 ± 2.2^{a}	63.1 ± 4.4^{b}

Means with different superscript within each parameter are different (P<0.0001),
* Value of G' when $t \rightarrow \infty$,
** Time constant of the model (time taken for G' to reach e^{-1} of its final value G'_{∞}).

As for the loss factor (tan δ), a decrease in this parameter is observed when the secondary coagulation phase begins, particularly at the time of transition from milk to gel. After that, tan δ values were slightly increased and tan δ was higher for chymosin, as compared to plant coagulants (Esteves, et al., 2003).

According to Esteves et al. (2003), tan δ is in a reverse proportion with oscillation frequency. It was noted that tan δ was increased (>0.4) with a decrease in frequency (0.002 Hz) for all coagulants (animal and plant coagulants). This indicates an increased susceptibility of the protein-protein bonds to relax for longer, therefore, indicates a greater susceptibility of coagulant-induced gels to undergo syneresis and rearrangements. In addition, the firmness of gels obtained from plant coagulants was compared with chymosin by comparing the yield stress within these gels. The value of this parameter was significantly higher for chymosin, which indicates that chymosin produces firmer and stiffer gels (Esteves, et al., 2003).

To conclude, all these parameters can vary from one coagulant to another and they depend on many factors, such as the origin of the coagulant, its enzymatic activity, and other factors that could intervene in the gelation process and influence the rheological properties of the final product.

1.6.1 Factors affecting rheological properties of gels

Several factors can affect rheological properties of the gel, namely the amount of coagulant, pH and temperature of milk, coagulant pH, and ultra-filtration and heat treatment of milk. Their control is very important as it can improve the gelation process as well as the characteristics of the final product.

Dose and pH of the coagulant. Coagulant dose and pH are of the same importance and they are usually used in combination with each other. They can both affect casein proteolysis, which in return affects the rheological properties of the gels made with plant coagulants. Since plant coagulants are characterized by e higher proteolytic activity on casein, as compared to chymosin, the use of lower doses of coagulant at low pH, can lower the degree of this proteolysis. In return, this can minimize the negative effects of excessive proteolysis on rheological properties of cheese. Studies carried out by Esteves et al. (2003) showed that milk pH had a great impact on the gelation properties of both plant coagulants and chymosin. For all coagulants, by lowering the pH of milk, gelation time became shorter and the rate of increase in G', or rate

of gel firming, increased. This was explained by the fact that a reduction in the charge density of κ-casein causes a reduction in the electrostatic repulsion between casein micelles, which in return promotes particles' aggregation and a faster increase in G' values (Esteves, et al., 2003). When rapid gelation occurred, at pH 6.0 and 6.3, there was a slight increase in tan δ values during gel aging. This can be due to rearrangements in the gel network structure. It was also noted that, at low pH, G' values were decreased during gel aging, especially for plant coagulants, which can be explained by additional proteolysis that occurs in these gels (Esteves, et al., 2003).

A study carried out by Silva et al. (2003) analyzed the effect of pH on gelling properties of plant coagulants, in combination with $CaCl_2$ addition. The addition of $CaCl_2$ at low pHs (6.2 and 6.4) promoted a faster sigmoidal increase of G' in the case of gels produced by plant coagulants (cardosin A and B of *C. cardunculus*). This was explained by an increase in Ca^{2+} activity which could lead to a faster structural rearrangements, and subsequent micelle aggregation (Silva, et al., 2003).

Furthermore, variation in enzymatic behavior towards the curd firming rate also depends on pH. In the same study carried out by Silva et al. (2003), it was noted that the firming rate of chymosin at low pH (6.2 and 6.4), after the addition of $CaCl_2$, exceeded that of plant coagulants. However, at pH 6.6 plant coagulants had a higher firming rate. This suggests that a variation of pH after $CaCl_2$ addition could significantly improve the final firmness of dairy products prepared with plant coagulants (Silva, et al., 2003).

Milk pH. Ben Amira et al. (2017), showed that milk pH can be affected by rennet pH, thus leading to significant variation of all rheological parameters (G', G", and curd firmness) of gels produced by plant coagulants. By lowering rennet pH, milk pH decreased causing a significant rise of the viscoelastic parameters of skim milk gels and faster initial values of G'. The final curd firmness recorded at the end of gelation (after 2 h) ranged, respectively, from 5.78 and 12.10 Pa for crude extract at pH 6 to maximums of 55.73 and 64.16 Pa obtained for CE at pH 3. To explain these results, authors reported that the lowest firmness values of gels made with CE at pH 6 were probably the result of high non-specific proteolytic action, which leads to some hydrolysis in the existing network (Amira, et al., 2017).

Milk temperature. As pH and dose of coagulant, milk temperature also has a high impact on rheological properties. At high temperatures, gels can be more viscous-like, because the rate of rearrangements is greater, the interactions between casein particles are weaker and the apparent plateau in G' values is reached earlier. Moreover, there are indications that the casein networks of various types of milk gels formed at high temperatures are less inter-connected and have dense clusters of aggregated casein particles that may be associated with extensive particle rearrangements. The opposite can happen at low temperatures, as proteolytic activity of the coagulant is lower, which can affect gel firmness and other rheological parameters (G', G", and tan δ). At low temperatures, gels tend to be firmer and the gelation time is higher. However,

this can be beneficial as it can minimize the negative effects of high non-specific proteolytic activity on rheological properties that occurs at high temperatures (Esteves, et al., 2003).

A study carried out by Esteves et al. (2003) on the effects of temperature on rheological properties of gels produced by plant coagulants, showed that plant coagulants are less influenced by temperature changes, as compared to chymosin gels. It was noticed that milk gels induced by plant coagulants had shorter gelation time at 25°C, 35°C, and 40°C, as well as higher initial rate of increase of G' values at all temperatures (Table 1-4). However, the firmest gels were produced by chymosin at 30°C and 32°C. At a gelation temperature of 25°C, the differences in rheological properties and microstructural characteristics between plant coagulants and chymosin were considerable. Plant coagulants had shorter gelation time (t_g) and higher G' values. This represents an advantage of using plant coagulants in cheese production as it gives the possibility to use a wider range of temperatures (Esteves, et al., 2003).

Table 1-4: Effect of gelation temperature on the gelation time, storage modulus (G')[1] and tan δ[2] of milk gels induced by plant coagulants and chymosin (Esteves, et al., 2003)

Rheological parameter	**Temperature (°C)**	**Type of coagulant**		
		Cynara cardunculus L.	*Cynara humilis* L.	Chymosin
Gelation time (s)	25	2350(46)	2413(31)	4547(104)
	30	1342(26)	1489(98)	1583(61)
	32	1136(39)	1112(37)	1123(44)
	35	846(39)	816(26)	1118(42)
	40	688(35)	579(13)	965(37)
Storage modulus, G' (Pa)	25	53.9(1.0)	61.7(2.3)	49.2(0.2)
	30	65.8(1.6)	68.5(2.5)	82.5(2.7)
	32	69.2(2.6)	70.0(2.4)	83.1(1.0)
	35	64.9(3.2)	62.6(1.3)	69.6(1.0)
	40	50.1(0.6)	52.3(1.7)	51.1(1.6)
Tanδ (x $\mathbf{10^{-2}}$)	25	22.7(0.00)	23.2(0.06)	23.2(0.06)
	30	29.0(0.15)	29.6(0.12)	30.6(0.10)
	32	32.3(0.06)	33.4(0.15)	33.5(0.12)
	35	38.8(0.00)	39.4(0.06)	39.9(0.25)
	40	49.7(0.20)	50.2(0.25)	50.4(0.29)

[1] Measurements were taken ~16, ~8.5, ~6, ~4 and ~3 h after coagulation addition to milk at the gelation temperatures 25°C, 30°C, 32°C, 35°C and 40°C, respectively. This corresponds to a steady increase in G' values of milk gels.
[2] Tan δ was obtained at a frequency of 0.1 Hz.
Standard deviation in brackets

Milk ultra-filtration. Milk ultra-filtration is a membrane technique that is used to standardize or to increase protein content in milk and to separate bacteria and spores from milk. This process can minimize the whey drainage and therefore almost all whey proteins can be incorporated into cheese, which can cause an increase in cheese yield. Cheeses made using concentrated ultra-filtrated (UF) milk have different sensorial and functional properties compared to cheeses produced from traditional non-concentrated milk. These differences are due to the incorporation of whey proteins into the matrix. Whey proteins act as inert

fillers in the casein matrix and increase water binding of cheese, therefore making UF cheese to be softer than traditional cheese. The presence of whey proteins has also been suggested to reduce the enzymatic proteolysis of caseins during ripening (Karlsson, et al., 2007).

With increased casein concentration the coagulation properties of milk change. Coagulation time decreases, elasticity increases, level of hydrolyzed κ-casein at the coagulation point is lower and less water and whey proteins are expelled from the gel (Karlsson, et al., 2007). However, UF milk often coagulates prematurely and there is insufficient proteolysis, presumably due to high casein levels. Cheeses produced from UF milk, especially hard and semi-hard varieties, frequently develop a coarse texture and poor flavor. Low et al. (2006) showed that plant coagulants can overcome these problems due to their higher proteolytic activity (Low, et al., 2006).

Heat treatment of milk. Heat treatment of milk at high temperatures (over 55°C), on the other hand, induces the denaturation of whey proteins leading to a complex mixture of whey proteins and casein micelles coated by whey proteins. The presence of whey proteins on the surface of casein micelles can hinder the aggregation of rennet-altered micelles, resulting in a longer coagulation time. Blecker et al (2012) showed that the application of high temperatures induced a decrease in the maximum curd firming rate and an increase in the viscosity of gels. Indeed, when milk was treated at 80°C for 20 min gel-firming rate was significantly decreased (0.0003 Pa), as compared to milk treated at 60°C (0.0175 Pa) and raw skim milk (0.0202 Pa) (Blecker, et al., 2012). For this reason, the application of low temperatures on milk can avoid the decrease in gel firmness.

To conclude, all these factors have a high impact not only on proteolytic activity and rheological properties, but also on texture and appearance of the final product. Thus, controlling them is very important.

1.7 Effect of plant proteases on sensorial properties of cheese

Sensorial properties are essential in evaluating the ability of a plant coagulant to replace calf rennet. Some of these coagulants can produce cheeses with sensorial properties very similar to the ones produced with chymosin, but many of them produce cheeses with very different textures and flavors, due to their high PA and production of bitter peptides. Thus, their evaluation and comparison with chymosin is essential.

1.7.1 Texture, flavor, and taste

Texture, flavor, and taste can vary depending on the type of coagulant (animal coagulant, plant coagulant, microbial coagulant) used in cheesemaking. Cheeses produced with plant coagulants are characterized by a softer texture, bitter taste, and a bit spicy in some cases. However, this depends on the plant coagulant used as each of them has different effects on sensorial properties of cheese.

Many comparisons have been made by researchers between cheeses produced with plant coagulants and chymosin in terms of sensory properties. A good example is *Cynara cardunculus* which has been used for a very long time in Mediterranean countries. Seven out of 12 Portuguese sheep or goat cheeses are produced using crude extract of dried flowers of *C. cardunculus* L. as coagulant. These cheeses are also under Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) status, and are known for their exceptional organoleptic, rheological properties and their authenticity in terms of regional origin or traditional production (Regulation (EU) No. 1151/2012). The most common characteristic of these cheeses (particularly those produced with sheep milk) is the soft texture with an exquisite flavor, slightly bitter and sometimes piquant. Indeed, sensory evaluation of sheep cheeses produced with cardoon extracts revealed enhanced flavor properties with a low perception of bitterness when compared with cheeses produced with microbial or calf rennet (Almeida & Simoes, 2018).

Another example is *Onopordum acanthium*, a closer relative of *Onopordum tauricum* (the object of this research). A semi-hard cheese prepared with the enzymes extracted from this plant (Onopordosin), showed similar characteristics to other commercial cheeses of the same type. This cheese was characterized by an intense and slightly bitter flavor. This characteristic is mainly attributed to the excessive casein breakdown and to the accumulation of high molecular weight bitter peptides (Brutti, et al., 2012).

Examples are many as other plants are being studied for the presence of enzymes with milk clotting activity. The sensorial properties of cheeses produced with plant coagulants can vary. In a semi-hard cheese known as "Peshwari" made from wholly or partially skimmed cow's milk using ginger rhizomes as a coagulant, was noticed that the texture was creamy, but no flavor defects were detected during storage, unlike cheese produced with Onopordosine (Hashim, et al., 2011). Similar results were obtained with "Murcia al Vino" cheese prepared with a plant coagulant obtained from cardoon flowers. This cheese was softer, less grainy, and creamier than the same cheese prepared with calf rennet (TEJADA, et al., 2006). Similar results were also obtained in other cheese varieties prepared with plant coagulants, like cheese prepared with *Bromelia hieronymi* coagulant and sheep milk cheese prepared with cardoon flowers (*C. cardunculus*). The texture of cheese prepared from *Bromelia hieronymi* was not very satisfactory as compared to cheese prepare with chymosin, but it did not develop undesirable flavor or bitterness. These texture defects were mainly due to excessive casein proteolysis (TEJADA, et al., 2007).

In contrast to this information, several plant coagulants can successfully replace calf rennet in terms of texture properties. For example, cheese prepared with Kiwi extract and chymosin were characterized by similar values of the parameters describing elasticity (7.10 and 6.38 mm), cohesion (0.328 and 0.349), and chewiness (135 and 122 mJ), respectively. They also had the highest hardness values (6.19 and 5.52 N) (Mazorra-Manzano, et al., 2013). Kiwi extract was also used to produce commercial "Mozzarella" cheese. In a sensory analysis, panelists did not notice any difference between "Mozzarella" produced with plant

coagulant and the one produced with calf rennet. Results showed the absence of a bitter taste. The good taste of this cheese was associated with the ability of actinidin to degrade undesired cream milk proteins (Puglisi, et al., 2014). In addition, extract from *S. dubium* was used to produce a cheese named "Gibna bayda", which did not differ from cheese produced with calf rennet in terms of sensory characteristics, particularly flavor. It gave better nutritional values to consumers and it had a slightly bitter taste (Kheir, et al., 2011).

Sensory characteristics of cheeses produced with plant coagulants are mainly related to the excessive proteolytic activity of these coagulants on casein. Indeed, bitterness is associated with the presence of bitter peptides originating from the hydrolysis of α_{s1}- and β-casein by residual rennet (Gomez, et al., 1997). These peptides are composed of aromatic amino acids residues with elevated hydrophobicity average, and their high presence in cheese can cause undesirable bitterness (Ney, 1971). However, proteolytic activity of plant coagulant is not the only factor that causes sensory defects in cheese. Researchers have reported that process steps and parameters can highly influence sensory properties, therefore, controlling of these parameters during cheesemaking can help in the reduction and prevention of any sensory defects. Yousif et al. (1996) showed that bitterness can be reduced by using purified enzyme and optimum concentration of coagulant extract (Yousif, et al., 1996).

Ripening is an important process that influences sensory properties of cheese as it is characterized by a primary and a secondary proteolysis. In case of cheese prepared with plant coagulants, it has been noticed that if ripening is avoided and a coagulant with a high MCA/PA ratio is used, the characteristics of the final product could strongly resemble those of the same cheese made with calf rennet (Yousif, et al., 1996). However, the same properties can be achieved, thanks to the mastery of some parameters during preparation and ripening. A good example is the extract of *Cynara scolymus* flowers. This coagulant was considered as a good substitute of animal rennet in the production of Gouda-type cheese, as the physicochemical parameters analyzed during ripening as well as organoleptic properties were very similar. Furthermore, cheese salting for a longer period (40 h) during ripening prevents the development of bitterness (Llorente, et al., 2014).

Generally, taste of cheeses produced with plant coagulants can be influenced by the type of milk and its composition. Studies carried out in cheeses produced with extracts of *C. cardunculus* using goat, sheep, and cow's milk, showed that cheese produced with goat and sheep milk did not develop a high bitter taste (Pélissier & Manchon, 1976). However, the opposite happened with cow's milk cheese, which always tends to develop an undesirable bitter taste (Barbosa, et al., 1981). This was explained by the fact that, ovine caseins are generally less sensitive than bovine caseins to form hydrophobic bitter peptides following proteolytic action. Thus, a good choice of milk can avoid some undesirable taste defects (Pélissier & Manchon, 1976).

1.7.2 Color

Visual quality, mainly color, is of great importance for cheese produced with plant coagulants. Improvement of such qualities is important to meet consumers requirements (Amira, et al., 2017).

Cheeses produced with plant coagulants, especially the ones prepared with crude extracts of plants, can have different colors depending on the plant and the part of the plant from which the coagulant is extracted. An example is a West African soft cheese (*wagashie*) prepared with fresh and dried extracts of *Calotropis procera* leaves and stems, which had a green color due to the presence of chlorophyll in the leaves. The intensity of the color changed from slightly green to green for the cheese prepared with dried crude extract and from lightly green to very green for the one prepared with fresh crude extract. These changes were due to an increase in crude extract concentration from 2 g to 7 g (dry matter) (Chikpah, et al., 2014). Another example is a cheese prepared with lemon fruit, which had a yellowish color (Akinloye & Adewumi, 2014). The same color was also observed in a cheese prepared with *C. cardunculus* extract, which had a darker and more yellow color that the same cheese prepared with calf rennet (Agboola, et al., 2009).

The presence of color in cheese prepared with plant coagulants is due to the presence of pigments in crude extracts which are extracted together with the coagulant during extraction. Cheese color can be improved by changing the concentration of the extract or by using purified extracts. The latter can reduce the amount of polyphenols and colored pigments added to milk, thus improving cheese color (Chikpah, et al., 2014).

1.8 Effect of plant proteases on cheese yield

Cheese yield is a very important parameter that needs to be considered when choosing an appropriate coagulant to substitute calf rennet. Several plant enzymes have been studied for their effect on cheese yield. Most of these enzymes are not suitable for cheese production in an industrial scale because of great loss of protein during cheesemaking. This loss of protein is caused by excessive proteolytic activity of plant proteases on casein (Khan & Musad, 2013). Some examples of cheese yields of some cheeses prepared with plant coagulants are presented in Table 1-5.

Table 1-5: Examples of cheese yields of some cheeses prepared with plant coagulants, as compared with those made with commercial rennet (Chymosin) (Amira, et al., 2017)

Plant	Cheese yield (or curd yield)			References
	Cheese	**Plant rennet**	**Chymosin**	
Actinidia chinensis	Curd	17.8 ± 1.6 g/100 g Fw	20.2 ± 2.7 g/100 g Fw	(Mazorra-Manzano, et al., 2013)
Cucumis melo		15.1 ± 0.9 g/100 g Fw		
Zingiber officinale		15.4 ± 1.9 g/100 g Fw		
Bromelia hieronymi	Miniature cheese	14.52 ± 0.31 g/100 g Fw 8.63 ± 0.43 g/100 g Dw	16.05 ± 0.06 g/100 g Fw 9.26 ± 0.46 g/100 g Dw	(Bruno, et al., 2010)
Withania coagulans	White cheese	268 g/L	234 g/L	(Khan & Musad, 2013)
Calotropis procera	Soft cheese	237.4 g/L	-	(Chikpah, et al., 2014)

Chapter 2
MATERIALS AND METHODS

2.1 Reagents and reference compounds

0.1 M and 25 mM sodium acetate pH 5.5, 0.1 M sodium citrate pH 3, 25 mM TRIS-HCl pH 7.6, Bradford (Coomassie Brilliant Blue G-250), 10 mg/ml BSA (Bovine Serum Albumin), 4.5 mM $CaCl_2$, 0.1 M Citric acid, 6 mM Sephadex G-150 (cross-linked dextran gel), Sephadex G-25, SAAPFPNA (N-succinyl-alanine-alanine-phenylalanine-p-nitroanilide), 100 mM Sodium phosphate pH 7.2, 1 µg/µl Liquid rennet (Chymosin), Ammonium sulfate 60%, 1.45 mM Pepstatin A, 10 mM E-64 [(1S,2S)-2-(((S)-1-((4-Guanidinobutyl)amino)-4-methyl-1-oxopentan-2-yl)carbamoyl)cyclopropanecarboxylic acid)], 100 mM PMSF (phenyl-methyl-sulfonyl fluoride), 100 mM Cysteine, 0.5 µg/µl fluorescent casein.

2.2 Sample preparation

Flowers, leaves and receptacle of *Onopordum tauricum* were taken from the plant and stored at -20˚C. Different methods and extracting agents were used for protein extraction. Two methods were used for extraction, maceration and ultra-turrax, in combination with different extracting agents, including, water, citric acid 0.1 M pH 3, TRIS-HCl 25 mM pH 7.6, sodium acetate 0.1 M pH 5.5 and sodium citrate 0.1 M pH 3, followed by filtration and centrifugation. A comparison was done between the two methods and between extracting agents.

2.3 Protein concentration

Protein concentration was determined by using Bradford assay (Coomassie Brilliant Blue G-250). BSA was used as reference. Absorbance was measured at a wavelength of 595 nm and protein concentration was calculated using the equation obtained by measuring the absorbance of BSA in different concentration.

2.4 Milk clotting activity

Pasteurized cow milk was used to measure milk clotting activity. For this purpose, 100 µl aliquot of the sample was added in 1 ml of milk (after preheating at 45°C for 10 min) in the presence of 10 mM $CaCl_2$ and incubated at 45°C. The time between the mixing of the reagents and the initial appearance of the solid material was recorded and referred to the amount of proteins present in the sample.

2.5 Enzymatic activity

Proteolytic activity was measured using continuous fluorescent assay with casein-FITC (Figure 2-1). The reaction mixture consisted of 40 mM sodium acetate pH 5.5, 20 µg fluorescent casein, extract, and

water, in a final volume of 200 µl. Fluorescence was monitored continuously at 37°C, using a Synergy HT microplate reader equipped with 485 and 528 nm excitation and emission filters, respectively. One Unit of enzyme is defined as the amount of enzyme that produces an increase of 1 Unit of florescence per minute under assay conditions.

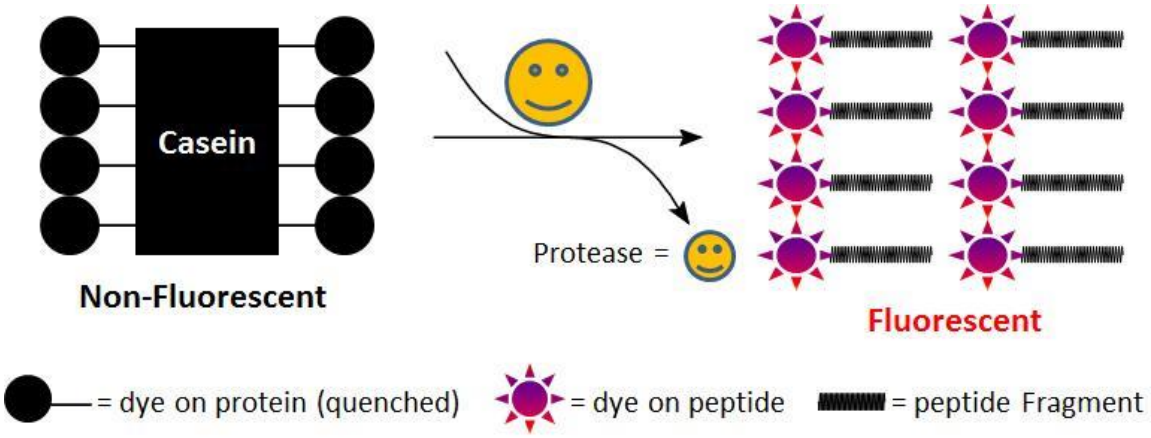

Figure 2-1: Continuous fluorescent assay with casein-FITC

Effect of inhibitors. To better understand the catalytic type of the protease/s present in the flowers of *O. tauricum*, proteolytic activity was also measured in the presence of inhibitors. Four inhibitors were used, one for each type of protease (serine, aspartic, cysteine, and metalloprotease): 1 mM PMSF, 0.1 mM Pepstatin A, 0.1 mM E-64, and 5 mM Cysteine, respectively.

2.6 Electrophoretic analysis (SDS-PAGE)

SDS-PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis) was performed in a BioRad Mini Protein electrophoresis apparatus according to the method developed by Laemmli (1970), using 15% acrylamide gel (Laemmli, 1970).

2.7 Purification

Ammonium sulfate fractionation. Ammonium sulfate 60% was slowly added to the extract while stirring it on ice (4°C) for 20 min. After 20 min, the extract was centrifuged at 15000 x g for 25 min at 4°C. The pellet was then resuspended in 0.1 M sodium acetate pH 5.5. However, this step removed only part of the pigment, thus a subsequent gel filtration chromatography was performed.

Gel filtration chromatography. This step was performed using a Sephadex G-150 resin column, equilibrated with sodium acetate 25 mM, pH 5.5. The eluted protein was collected in several fractions, where the protein concentration and proteolytic activity were measured. The active fractions were collected in a pool, which was subjected to ion-exchange chromatography.

Ion-exchange chromatography (FPLC). This step was performed in a FPLC instrument using an anion exchange chromatography column (Mono Q HR 5/5), equilibrated with sodium acetate 25 mM, pH 5.5. After equilibration, elution was performed with a linear gradient from 0 to 1 M NaCl in the presence of 25 mM sodium acetate pH 5.5. Elution profile was monitored at three wavelengths, 260 nm, 280 nm, and 340 nm.

Concentration through ultrafiltration. The concentration of the pool comprising the active fractions was performed using Amicon® Ultra 4 mL filters with a 10000 Da molecular weight cut-off. Following the addition of the pool, a centrifugation step was performed at 5 000 x g for a few minutes at 4°C.

Chapter 3
RESULTS AND DISCUSSION

3.1 Optimization of protease extraction from *O. tauricum*

Protease extraction is an essential step as it can affect all the other steps. Therefore, optimization of this process is very important. Two methods (maceration and ultra-turrax) and different extracting agents were used in this study with the main goal of increasing the extraction yield. Maceration was performed with water and citric acid, while homogenization with ultra-turrax was performed with TRIS-HCl, sodium acetate and sodium citrate buffers. For the first method, it was noted that maceration in water was better than citric acid as it extracted more protein, while for the homogenization method, sodium acetate proved to be better than the other extracting agents. The results of protein yield and milk clotting activity of different parts of *O. tauricum* plant treated with either water or sodium acetate pH 5.5 are presented in Table 3-1. As it can be seen from the table, the highest yield, both in terms of extracted protein (0.97 mg/g flowers) and milk clotting activity (21 sec/mg protein), was observed in the flowers extract prepared with sodium acetate buffer. This can be explained by the fact that the pH of sodium acetate extract was lower (pH 5.0) than the pH of aqueous extract (pH near neutral), which means that the protease is better extracted at slightly acidic pH.

Regarding the extraction method, homogenization with ultra-turrax was more efficient than maceration for both protein extraction and milk clotting activity. Thus, for our purpose it is the best method for protease extraction.

Table 3-1: Protein yield and milk clotting activity (MCA) of extracts from different parts of O. tauricum prepared in different extraction media

	Sodium acetate buffer		**Water**	
	Extracted protein [a]	MCA [b]	Extracted protein [a]	MCA [b]
Flowers	0.97	21	0.78	134
Leaves	0.48	> 2000	0.80	Nd
Receptacles	0.28	Nd	0.41	Nd

[a] mg protein/g fresh tissue
[b] seconds required for the curdle formation/mg protein
Nd: not detectable

3.2 Purification of proteases from *O. tauricum*

Plants are characterized by the presence of pigments of different colors, which in many cases can be extracted together with the other components of interest. The presence of these pigments in the crude extract can have negative effects in both caseinolytic activity of the protease and in the sensory properties of the final product, such as texture, taste, flavor, and color. From literature, we noted that these defects can be prevented by using purified extracts of plant coagulants (Chikpah, et al., 2014) (Amira, et al., 2017). Therefore, removal of these pigments from crude extracts of plant coagulants is an important step.

Flowers' crude extract of *O. tauricum* was characterized by a dark brown color. This can be due to high content of anthocyanins, which give these flowers their characteristic purple color. Three main steps of purification were used in this study to obtain a purified extract of the protease of interest (free from pigments and other impurities), namely ammonium sulfate 60% fractionation, gel chromatography and ion-exchange chromatography. The purification procedure started from 1.5 g flowers, with a final protein content of 30 µg and specific activity of 56 Units/mg protein, corresponding to a 14-fold purification. The results of all purification steps are summarized in Table 3-2.

Table 3-2: Purification of O. tauricum protease

STEP	Total protein (mg)	Caseinolytic activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification (-fold)
Extract [a]	9.30	37	3.98	100	
Ammonium sulfate	1.53	7	4.58	19	1.0
Sephadex G-150	0.60	19	31.67	51	8.0
Mono Q	0.03	1.67	55.67	5	14.0

[a] from 1.5 g flowers

The first step, ammonium sulfate (AS) fractionation, could only remove part of the pigment and the yield was quite low (19%). Gel chromatography (elution profile shown in Figure 3-1 and 3-3) on the other hand could remove a higher amount of pigment and the specific activity increased from 4.58 Units/mg after AS fractionation to 31.67 Units/mg after GC (Gel Chromatography), while the yield increased to 51%. This suggests that AS salt might inhibit the activity of the protease.

Gel chromatography was performed by using Sephadex G-150 matrix. Elution profile of the column (Figure 3-1) shows the absorbance values at 280 nm and 320 nm recorded for proteins and pigments, respectively, which are proportional to their concentration. As it can be seen from the graph, both lines follow the same trend and absorbance values are similar for both wavelengths. This indicates that the collected fractions have a similar concentration of proteins and pigments. However, caseinolytic activity was present in the fractions that have a low content of pigments.

To improve the purification, a gel chromatography with Sephadex G-25 resin was performed after the AS fractionation step. The results were very similar to the gel chromatography on the G-150 resin: the

absorbance values at 280 nm and 320 nm followed the same trend. in this gel filtration however the caseinolytic activity was present in the fractions that had high content of pigments, which corresponds to the first peak of absorbance (Figure 3-2).

After gel chromatography, the pool comprising the active fractions, was subjected to Mono Q chromatography (elution profile and proteolytic activity shown in Figure 3-3), which proved to be better than the previous steps in terms of pigments removal and specific activity. However, the yield (5%) in this final step was low when compared to gel chromatography and ammonium sulfate fractionation steps, mainly due to the loss of activity during the concentration step of the Mono Q pool.

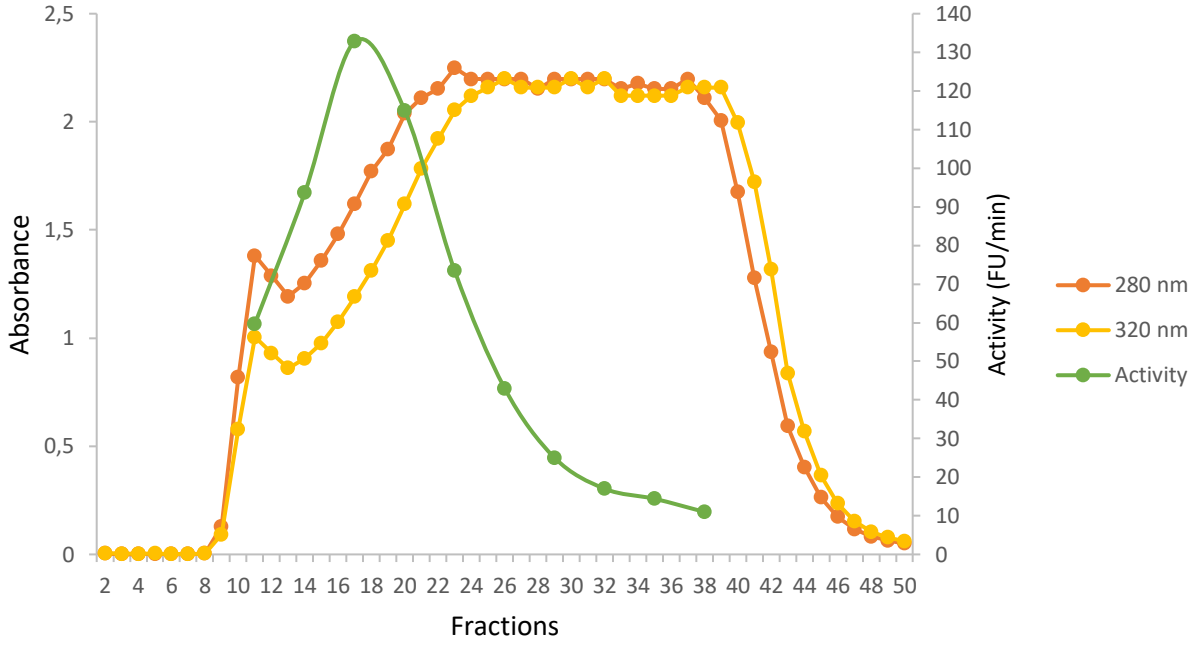

Figure 3-1: Sephadex G-150 chromatography – Elution profile of the column with absorbance values at 280 nm and 320 nm, and caseinolytic activity of proteases from O. tauricum flowers

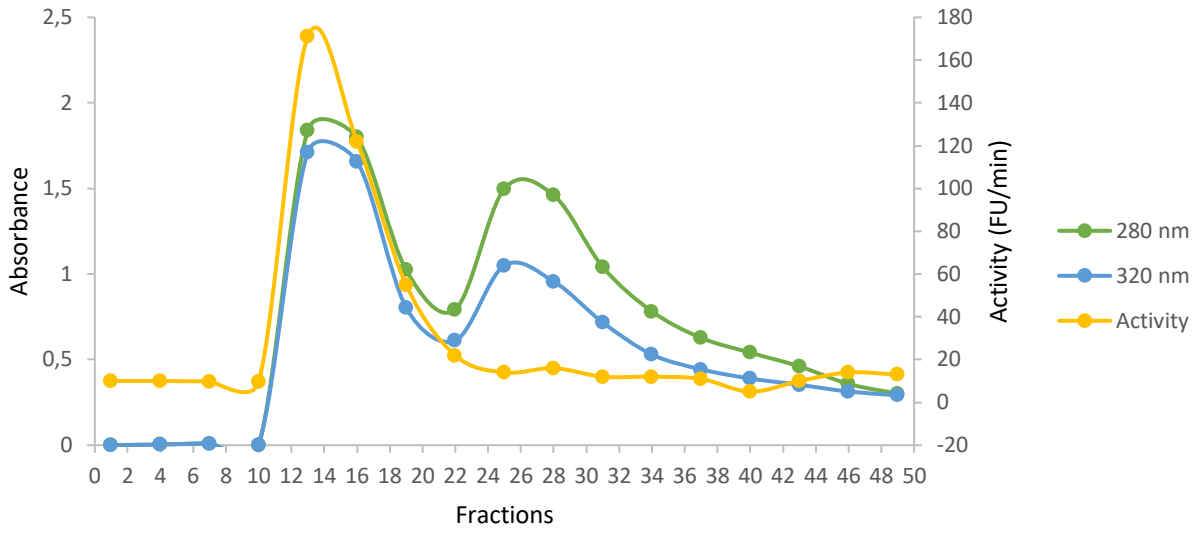

Figure 3-2: Gel chromatography (Sephadex G-25) – Elution profile of the column with absorbance values at 280 nm and 320 nm, and caseinolytic activity

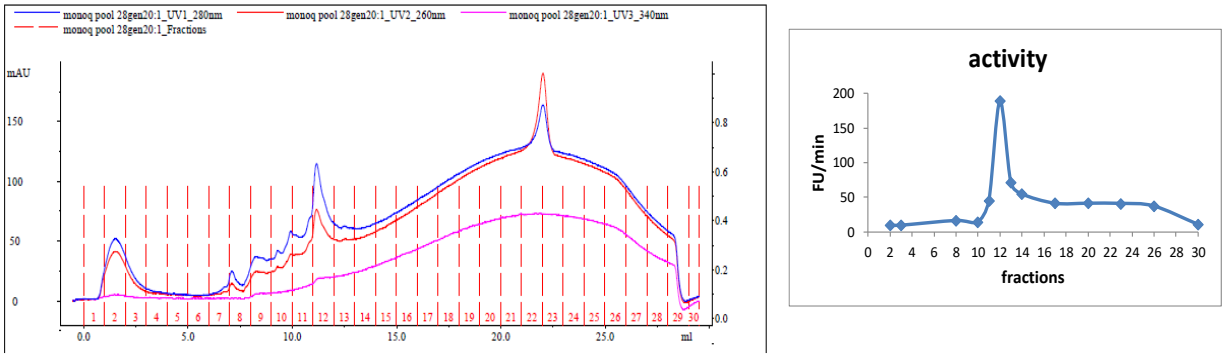

Figure 3-3: Mono Q chromatography – Elution profile of the column with absorbance values at 260 nm, 280 nm, and 340 nm (Left); Caseinolytic activity (Right)

Mono Q pool was subjected to SDS-PAGE analysis to determine the purity and the molecular weight of the *O. tauricum* protease. Results showed the presence of a major band migrating as a protein of about 30 000 Da under denaturing conditions (Figure 3-4).

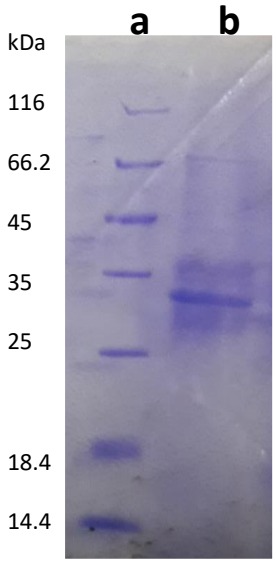

Figure 3-4: SDS-PAGE analysis – Lane a, molecular weight markers; lane b, Mono Q pool (4 µg)

After a three steps of purification procedure, even though the yield was very low, we could obtain a pure enzyme, free of pigments.

3.3 Effects of inhibitors

To determine the catalytic type (serine-, cysteine-, aspartyl-, and metalloprotease) of the protease extracted from *O. tauricum* flowers, the effect of four inhibitors (PMSF, E-64, Pepstatin A, and cysteine, respectively) in the caseinolytic activity of this protease was tested. The caseinolytic activity of this protease was only affected by Pepstatin A, which suggests that it is an aspartic protease (Table 3-3).

Table 3-3: Effect of four protease inhibitors in the caseinolytic activity of O. tauricum protease

Protease inhibitor	**Residual activity (%)**
No inhibitor	100
PMSF (1 mM)	89
Pepstatin A (0.1 mM)	11
E-64 (0.1 mM)	96
Cysteine (5.0 mM)	100

Activity was measured after pre-incubation of the enzyme for 30 min at 37°C in the presence of the indicated compound

CONCLUSION

Purification and characterization of the protease with milk clotting activity from *O. tauricum* flowers was successful. A three steps of purification procedure resulted in a pure enzyme, although the yield was very low.

SDS-PAGE analysis revealed the presence of only one major band of about 30 000 Da. This suggests that there is only one form of protease with milk-clotting activity, however further research needs to be done to confirm this assumption. The study of the effect of four inhibitors on caseinolytic activity of the purified enzyme showed that it was only affected by pepstatin A, indicating that it belongs to the class of aspartic proteases.

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