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Deliverable title	**D4.3 Pure and enzymatically active proteases**
Deliverable Lead:	UCAM (PARTY3)
Related Work Package:	**WP4 Characterization of thistle aqueous crude extracts (CEs)**
Related Task:	Task 4.4 Purification of proteases from CEs Task 4.5 Biochemical characterization of purified proteases
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Abstract	Different steps have been carried out to purify the active proteolytic fractions, including salting-out and ion exchange chromatography, according to **Llorente et al, 2004**. The effect of known inhibitors were tested to identify the type of purified proteases, following the procedure recently described by **Mohan et al. (2017)**. Caseinolytic specificity of purified proteases were assessed through Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis (SDS-PAGE) of hydrolyzed caseins, as previously described by **Anusha et al. (2014).** The structure of these proteases was defined through peptides mass fingerprinting, according to **Sarmento et al. (2009).**

Versioning and Contribution History

Version	Date	Modified by	Modification reason
v1.0	15.01.2023	Luis Tejada Portero	First version
V2.0	30.01.2023	Luis Tejada Portero	Comments after peer reviewing process

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1. Purification of proteases from CEs

Protease activity assays

Throughout the purification procedure, the proteolytic activity was determined by a continuous fluorescent assay adapted by Twining SS, 2014. Briefly, the reaction mixture consisted of 50 mM sodium acetate, pH 5.5 and 0.1 mg/ml casein-FITC type III from bovine milk (Sigma) in a final volume of 200 µl. Following the addition of the sample to be analyzed, the 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 fluorescence per minute under the assay conditions.

For determination of the milk clotting activity (MCA), a 100-µl aliquot of the sample was added to 1 ml of skim bovine milk in the presence of 10 mM $CaCl_2$ and incubated at 45 °C. The time elapsing between the mixing of the reagents and the initial appearance of solid material was recorded and referred to the amount of sample proteins.

Protease purification

Crude extract preparation

Optimization of protease extraction was carried out by analyzing the yield of proteins extracted and the MCA in different parts of the thistles, including leaves, flowers and receptacles. Deionized water or 50 mM sodium acetate buffer, pH 5.0 was added at a ratio sample (g): extraction media (ml) of 1:5. Samples in water were macerated at room temperature overnight, while samples in buffer were homogenized with an Ultra-Turrax homogenizer. After filtration, samples were centrifuged at 2500 x *g* for 10 min, and the supernatants were analyzed for protein content and MCA. Results are shown in Table 1.

Table 1. Protein yield and milk clotting activity (MCA) of extracts from different parts of the thistle prepared in different extraction media.

	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

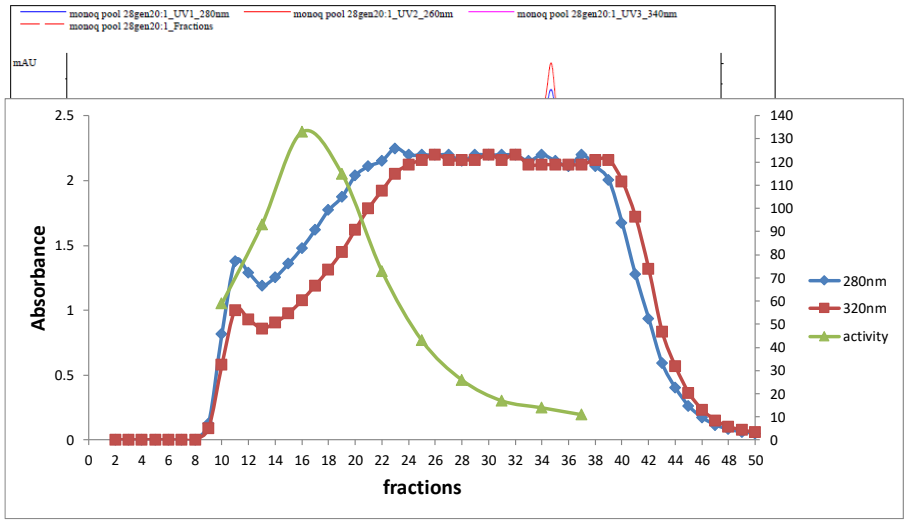
[a] mg protein/g fresh tissue
[b] seconds required for the curdle formation/mg of protein
nd: not detectable

The highest yield, both in terms of extracted proteins and clotting activity, was observed in the flowers extract prepared with acetate buffer.

Protease purification

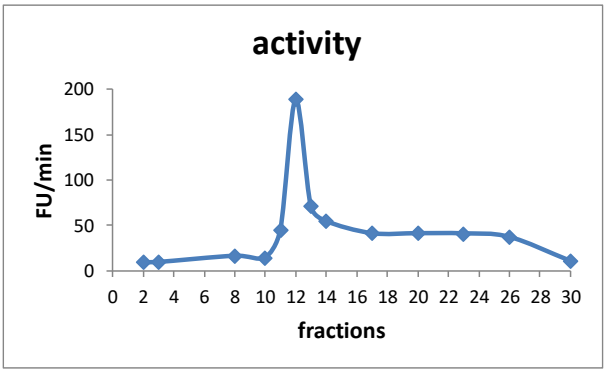
Due to the presence of strong purple pigments in the extract, a fractionated ammonium sulfate precipitation was carried out as the first purification step. After several trials, the best yield of activity was obtained with ammonium sulfate (AS) at 60% saturation. The salt was slowly added to the crude extract while stirring on ice. After 20 minutes stirring at 4°C, the extract was centrifuged at 15000 x g for 25 min at 4°C. The pellet was resuspended in 0.1M sodium acetate pH 5.5.
This step however only removed part of the pigments, therefore a subsequent gel filtration chromatography was performed. The AS sample was loaded into a column containing the resin Sephadex G150 (1.8 cm^2 x 13.2 cm), equilibrated with 25 mM sodium acetate pH 5.5. Elution was performed with the same buffer. The chromatographic profile of the column is shown in Figure 1.

Figure 1. Sephadex G150 chromatography. Elution profile of the column. Absorbance values at 280 and 320 nm, and the caseinolytic activity are shown.



The high absorbance at 320_{nm} measured in all fractions indicates the presence of colored pigments. A lower absorbance is however observed in the fractions containing the caseinolytic activity. These fractions were pooled, and the pool was desalted through a prepacked G25 column to be further subjected to an anionic exchange chromatography. To this end, a Mono Q column was used, equilibrated with 25 mM sodium acetate pH 5.5. After a washing step with the same buffer, elution was performed with a linear gradient from 0 to 1M NaCl in 25 mM sodium acetate pH 5.5. Figure 2 shows the elution profile of the column.

Figure 2. Mono Q chromatography. Elution profile of the column with absorbance values at 280nm, 260 nm and 340 nm (left panel) and caseinolytic activity (right panel).



A pool comprising the active fractions was concentrated through ultrafiltration by using a membrane with a cut-off 10.000 dalton. The final preparation was found to possess coagulating activity and its electrophoretic analysis showed the presence of a major band migrating as a protein of about 30.000 dalton under denaturating conditions (Figure 3).

Figure 3. SDS PAGE analysis of protease purification. Lane a, molecular weight markers; lane b, Mono Q pool (4 µg).

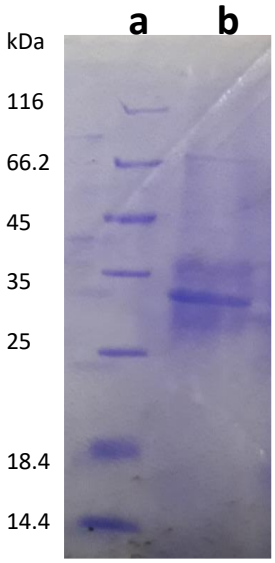


Table 2 summarizes the results of the purification procedure starting from 1.6 g of flowers. The final preparation contains 30 µg of proteins with a specific activity of 56 Units/mg, corresponding to a 14-fold purification. The final yield is only 5%; this low value is mainly due to the loss of activity during the concentration step of the MonoQ pool. The table shows that the 19% yield after the AS precipitation increases to 51% after the gel chromatography. This suggests that the ammonium sulfate salt might inhibit the enzymatic activity.

Table 2 – Purification of *O. tauricum* protease

STEP	total protein (mg)	caseinolytic activity (Units)	specific activity (Units/mg)	yield (%)	purification (-fold)
extract	9.30	37	3.98	100	
ammonium sulfate	1.53	7	4.58	19	1.1
Sephadex G150	0.60	19	31.67	51	8.0
desalting	0.60	13.2	22.00	36	5.5
MonoQ	0.03	1.67	55.67	5	14.0

To improve removal of pigments, we substituted the Sephadex G150 resin used for the gel filtration chromatography with the Sephadex G25. Elution conditions were as described above. Figure 4 shows the chromatographic profile of the column.

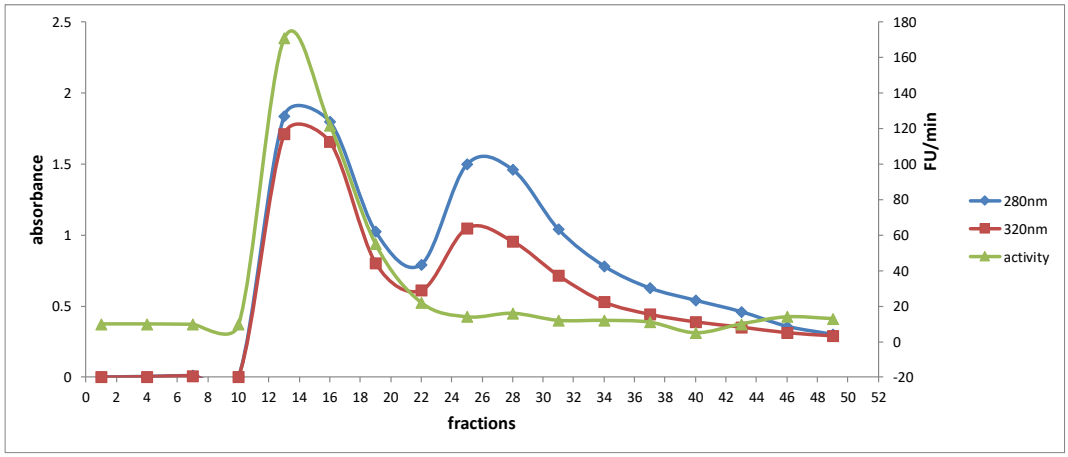


Figure 4. Sephadex G25 chromatography. Elution profile of the column with absorbance values at 280nm and 340 nm, and the caseinolytic activity

Pigments of low molecular weight were successfully removed from the enzyme, however some pigments still coeluted with the protease, suggesting the existence of a strong interaction between the pigments and the protein of interest. Therefore, alternative chromatographic steps should be optimized.

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2. Biochemical characterization of purified proteases

For the biochemical characterization of the protease, that we named tauricosin, the purification procedure was improved by performing the elution of the enzyme from the MonoQ column by using a NaCl gradient from 0 M to 0.5 M. A Tricine-SDS electrophoretic analysis of the fractions eluted from the MonoQ column showed the coelution of the proteolytic activity with two bands, indicating that the enzyme is formed by two subunits. Figure 1 shows the electrophoretic pattern of the final enzymatic preparation. The molecular weights of the two subunits (indicated by the red arrows in the figure) were calculated to be 32 kDa and 9.6 kDa.

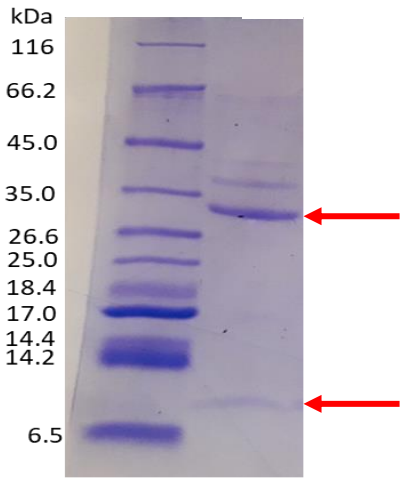

Figure 1. Tricine-SDS electrophoretic analysis of the final enzyme preparation.

Gel filtration analysis of the MonoQ pool resulted in a molecular weight of 39 kDa. Altogether, these results indicate that tauricosin is a heterodimeric protein, which is in line with the oligomeric structure of other thistle proteases (Verissimo et al, 1996; Sarmento et al, 2009, Llorente et al, 2004).

The characterization of the caseinolytic activity of purified tauricosin is shown in Fig. 2. The activity was determined by using the casein-FITC based assay described above or by using Na-caseinate as the substrate (Silva et al, 2005).

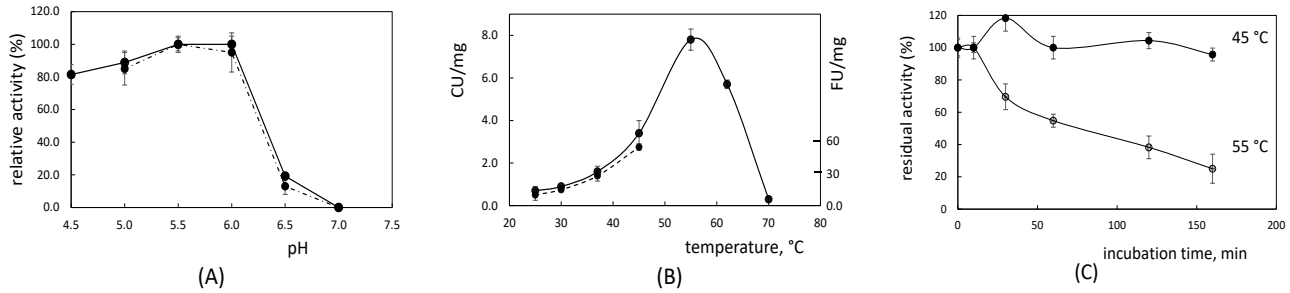

Fig. 2 Enzymatic properties of tauricosin. The proteolytic activity on bovine caseinate (continuous line) and bovine casein-FITC (dotted line) were determined at different pH values at 37°C (A), and at different temperatures, at pH 5.5 (B). Thermal stability was determined at pH 5.5 on bovine caseinate (C).

Optimal caseinolytic activity was achieved at pH values ranging from 4.5 to 6.0 (Fig. 2A), which was slightly lower than the optimum pH (5.7-6.0) exhibited by proteases isolated from *C. cardunculus* (Chen et al, 2003) and consistent with the proteolytic activity exhibited by reconstituted extracts on ewe's and goat's milk.
The caseinolytic activity showed an optimum temperature at 55°C and rapidly declined at higher temperatures (Figure 2B).
Regarding the thermal stability, after 2.5 h at 45°C, caseinolytic activity remained unchanged, while it rapidly declined during exposure of the purified enzyme at 55°C, with about 50% residual activity after 1 h. The marked thermal stability, as well as the high optimum temperature value displayed by tauricosin agreed with those reported for other thistle proteases (Sidrach et al, 2005; White et al 1999).
To get insight into the nature of the catalytic type of tauricosin, the effect of serine-, cysteine- and aspartic-inhibitors was tested on the caseinolytic activity of the purified enzyme. To this end, the enzyme was preincubated for 30 minutes at 37°C in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM pepstatin A or 0.1 mM E-64. The residual activity was measured with the casein-FITC assay at pH 5.5 and 37°C. Only pepstatin A was found to inhibit the enzyme, with 89% inhibition observed in the presence of 0.5 mM pepstatin A. These results confirmed that tauricosin was an aspartic acid protease.
To analyze the proteolytic activity of the pure enzyme on various caseins, commercial bovine α-, β- and κ-casein were dissolved in 0.1 M sodium phosphate buffer pH 6.2 and incubated with the purified enzyme or calf rennet as control, at 37°C for 60 minutes. The mixtures consisted of 0.7 mg/ml of each casein and 0.01 mg/ml of tauricosin or rennet. After 0 and 60 minutes of incubation, aliquots were subjected to SDS-PAGE on 15% acrylamide gel. As shown in Figure 3, tauricosin hydrolysed κ-casein similarly to rennet, while it showed a more intense proteolytic activity towards β-casein and α-casein, yielding peptides with lower molecular weight. The caseins degradation profile closely resembles that obtained with cynarases from *C. scolymus* (Chazarra et al., 2007).

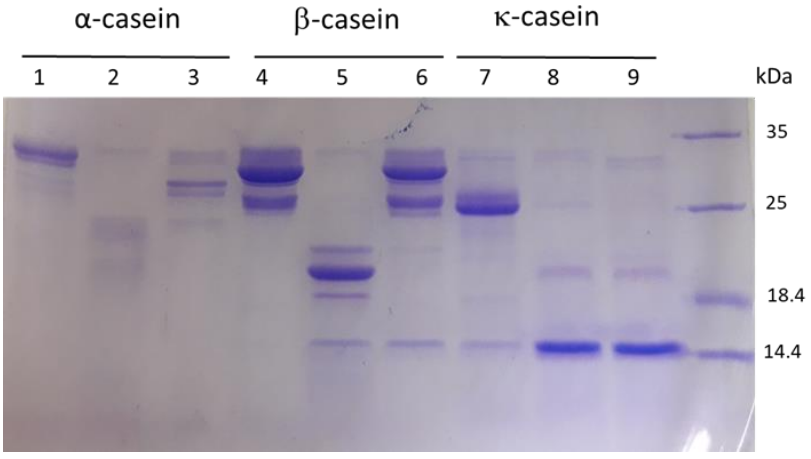

Fig. 3. Specificity of the caseinolytic activity. Hydrolysis of different caseins after 0 (lanes 1, 4, 7) and 30 min incubation with tauricosin (lanes 2, 5. 8) or rennin (3, 6, 9) was analysed by SDS-PAGE (D).

In summary, tauricosin is a heterodimeric enzyme, consisting of two subunits of 32 and 9.6 kDa. It is an aspartic-acid protease, with optimal activity at pH values lower than 6.0 and at 55°C. The purified enzyme hydrolyses κ-casein similarly to rennin, while it shows a more intense non-specific proteolytic activity towards β-casein and α-casein.

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