Potentialities of aqueous extract from cultivated Onopordum tauricum (Willd.) as milk clotting agent for cheesemaking

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Abstract:	In Western and Central Mediterranean countries proteases from wild herbaceous perennial plants commonly known as “thistles” have been used as milk coagulants in cheese-making for centuries. For the first time, the technological and biochemical traits of proteases from cultivated Onopordum tauricum Willd. (Taurian thistle, bull cottonthistle) were assessed. The optimal conditions for minimizing the clotting time and the non-specific proteolytic activity were estimated at the highest (T = 43-45 °C; [Ca2+] = 11-13 mM) and the lowest (T = 35-39 °C; [Ca2+] = 5 mM) temperature and calcium ion levels in the explored range respectively, thus highlighting the difficulty to set the best operative compromise in the first step of cheesemaking. In the conditions adopted in common cheesemaking practice (T = 37 °C; pH = 6.5) 1 mL of reconstituted extract from cultivated thistles coagulated 10 mL of ewe’s and goat’s milk in 114-146 and 129-167 seconds, respectively, and 1 mL of reconstituted extract from spontaneous thistles coagulated 10 mL of ewe’s and goat’s milk in 232-294 and 428-621 seconds, respectively, while no significant differences in the non-specific proteolytic activity between cultivated and spontaneous O. tauricum extracts were observed. The purified enzyme (tauricosin) was identified as an aspartic protease made up of two sub-units with molecular weights of 32 and 9.6 kDa, respectively. Experimental data encouraged the exploitation of O. tauricum as a new and sustainable non-food crop in marginal and rainfed lands of Mediterranean countries, thus reducing the potential biodiversity losses due to wild collection.

Cover Letter

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Dipartimento
di Scienze Agrarie,
Alimentari
ed Ambientali
D3A

Ancona, 12/04/2022

To the Editors of
"Food Research International"

Dear Editors,
would you please consider the manuscript titled "**Potentialities of aqueous extract from cultivated *Onopordum tauricum* (Willd.) as milk clotting agent for cheesemaking**" for publication in the Journal of *Food Research International*.
Plant extracts with milk clotting properties are traditionally used for cheese-making in several Mediterranean countries. Despite the increasing interest in the vegetable sources of milk-clotting enzymes, the use of vegetable rennets is geographically circumscribed and limited to few traditional products. Particularly, *Onopordum tauricum* was previously studied for its clotting properties, seed germinability and agronomic performance, as part of an international research programme aimed to assess the technological and socio-economic viability of the valorisation of spontaneous herbaceous plants in the Mediterranean area as traditional alternatives to animal rennet (https://veggiemedcheeses.com/). To close the circle and contribute to the reduction of potential biodiversity losses due to the wild plant collection, the technological performance of cultivated thistles (milk clotting activity, non-specific proteolytic activity) were assessed for the first time. Besides, the biochemical traits (molecular weight, inhibition, degradation patterns of caseins) of the purified enzyme (tauricosin) were elucidated for the first time. Experimental data encouraged the exploitation of *O. tauricum* as a new and sustainable non-food crop in marginal and rainfed lands of Mediterranean countries
For these reasons, we think that the proposed research fully meets the aims and scope of the journal.
Neither the manuscript nor any parts of its content are currently under consideration or published in another journal.

Thank you very much for your kind consideration.

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Response to Reviewers' comments

Reviewer #1

We would like to express our thanks to the Reviewer for the time he/she dedicated to our proposed paper, and we hope that his/her suggestions would be fully mirrored in the revised paper.

Abstract (Line 5). "...have been used..."
Response: the sentence was changed according to the Reviewer's suggestion.

Line 27 (pg 6). Please, cite Bradford article (Bradford, 1976)
Response: the original paper published by M.M. Bradford in 1976 was added to the References.

Line 49 (pg 7). "...O. tauricum extract..."
Response: the sentence was changed according to the Reviewer's suggestion.

Line 52 (pg 8). Another 0.025M acetate buffer, pH 5.5 was prepared with NaCl? What was the molarity of NaCl in the acetate buffer? What percentage of this NaCl-containing buffer did the enzyme elute?
Response: In the Materials and Methods section we have now clarified the use of 0.025 M acetate buffer, pH 5.5 (buffer B) in the Mono Q chromatography, by describing the buffer composition in terms of NaCl molarity in the different elution conditions. In the Result section, we have specified the percentage of NaCl the enzyme elutes.

Line 32 (pg 9). Why did the authors use different assays to quantify the proteolytic activity (casein-FITC and bovine milk casein)? I would like to see the functional biochemical characterization (effect of pH and temperature on enzyme activity) using both substrates, because these reactional enzyme properties may change according to the substrate
Response: We used the casein-FITC based assay because it is a fast and continuous assay that allows the simultaneous testing of many samples. It was especially useful for the RSM analysis and for monitoring the purification of the protease. For the determination of the optimum pH and optimum temperature of the purified enzyme we used the caseinate-assay in order to compare the proteolytic properties of the O. tauricum protease with those reported in the literature for other vegetable APs assayed with caseinate as the substrate.
We have now better described the use of the two assays in section 2.6 and moved in this section the description of the caseinate assay (present in section 2.8 in the previous version). In addition, according to the reviewer's request, we have tested the effect of pH and temperature on the proteolytic activity of the purified enzyme with the casein-FITC assay and included the results in Figures 2A and 2B.

Lines 34-39 (pg 11) "However, a univariate approach characterizes most of the literature data concerning the effect of independent variables on the clotting activity, thus disregarding the possible interactions among factors" It is confusing!
Response: we rewrote the sentence to better clarify what we meant.

Lines 49-51 (pg 12). "the clotting performance of cultivated thistles extract was 1.7 and 2.0 times better than wild thistles extract in ewe's and goat's milk, respectively". Please indicate that you are referring to the predicted MCT(sec) shown in table 3.
Response: the sentence was integrated as suggested by the Reviewer.

Lines 1-5 (pg 14) "Anyway, under the above conditions the predicted PA of wild thistle extract was 2.5 times higher than extract from cultivated plants". I could not see PA of wild extract 2.5 times higher than cultivated plants. In table 3, this difference is approximately 1.5 times. Please, clarify it! And cite the table in this sentence!
Response: we fully agree with the Reviewer, it was a mere mistake: the ratio between the PAs of spontaneous and cultivated thistle extracts was about 1.5. Moreover, a citation to Table 3 was added.

Line 7 (pg 14). "cheesemaking practice (table 4)"
Response: the sentence was changed according to the Reviewer's suggestion.

Lines 12-15 (pg 14). "By comparison, the optimal hydrolytic conditions for Cynara scolymus flower extract..." Please indicate which substrate was used for the C. scolymus protease assay.
Response: we specified the substrate used for the protease assay

Line 54 (pg 14). SDS-PAGE shows protein band with molecular mass greater than 35 kDa. There are not only two protein bands (32 and 9.6 kDa). So it would be better for the authors to refer to the partially purified protease
Response: We agree, and we have modified the text accordingly, also taking into account the concern raised by reviewer n. 4.

Line 2 (pg 15) "In particular, C. cardunculus cardosin A consists..."
Response: the mistake was fixed.

Line 12 (pg 15). "30 and 14.4 kDa..."
Response: the unit for molecular weights (kDa) was added.

Line 24 (pg 15). In fig. 2A, the first pH is 4.5
Response: the mistake has been fixed

Line 2 (pg 16) Did 0.5 M Pepstatin A inhibit only 11% of the enzyme activity? Generally, aspartic peptidases are very sensitive to Pepstatin A. I think it would be interesting to evaluate higher concentrations of Pepstatin A to see if there will be an increase in the rate of inhibition. I think the authors could run this experiment and display in a table the effect of these different concentrations of Pepstatin A and the effect of the other inhibitors at a single concentration
Response: We apologize for the mistake. We intended 11% residual activity, and not 11% inhibition. The mistake has been fixed. An 89% inhibition is an indication of an aspartic acid protease.

Lines 5-20. (pg 17). "The encouraging technological performance of O. tauricum extracts go hand in hand with botanical and agronomic data. Taurian thistle seeds showed a high germination rate (94%) when properly pre-treated (Zitti et al., 2021) and cultivated O. tauricum exhibited a significantly higher production of useful biomass (tubular flowers) per unit of surface than the most popular source of vegetable rennets Cynara cardunculu (Zenobi et al., 2021), thus confirming the potentialities of O. tauricum as an herbaceous crop for vegetable rennet production in the rainfed unfertilized Mediterranean environment." It is not useful in the conclusion, please remove it! And conclude by suggesting new studies to be done to improve and qualify the use of this enzyme in cheese making or use for other applications.
Response: We think the Conclusion section should highlight the link between the findings and the objectives of the research, as also suggested by the Reviewer #3, to "give a take-home message to the readers". Wild Onopordum tauricum was previously studied for its clotting properties, seed germinability and agronomic performance, as part of an international research programme aimed to assess the use of spontaneous herbaceous plants in the Mediterranean area as traditional alternatives to animal rennet (https://veggiemedcheeses.com/). However, massive wild plant collection could result in biodiversity losses. To close the circle, the technological performance of cultivated and wild thistles were compared to assess the exploitation of O. tauricum as a new and sustainable non-food crop in marginal and rainfed lands of Mediterranean countries. For these reasons, we think the lines 5-20 on page 17 should be kept in the text. As suggested by the Reviewer, we added a brief statement concerning the further studies needed for a full assessment of the use of O. tauricum proteases in cheesemaking.

All figures are not in good resolution. Please improve them!
Response: The original jpeg files look pretty good. Maybe some resolution got lost during the automated generation of the pdf file. Anyway, we uploaded new jpeg files together with the native ppt files.

Please, correct the yield in the table 5, instead 91% to G25 step, the correct is 97%: 98/101x100.
Please check all calculations and standardize approximation of decimal numbers

Response: Thank you for pointing out the mistake that was fixed.

Reviewer #3

In this study was investigated the potentialities of aqueous extract from cultivated Onopordum tauricum (Willd.) as milk clotting agent for cheesemaking.
I have checked the manuscript, overall, although the work has merits, the study is basic, and only the determination of the optimal conditions of pH, temperature and Ca+2, as well as purification data, are not enough for publication with the requirement criteria for FOODRES. Therefore, in the present form, the manuscript is not suitable for publication in this journal. In addition, some errors were found in the manuscript. Please, the MS should be checked for English grammar and usage.
Some suggestions for the improvement of the work:
Response: We fully agree with the Reviewer: the performance of *O. tauricum* proteases need to be assessed in a real cheesemaking process. In fact, a comparison between the physico-chemical, chemical, microbiological, and sensory characters (nutritional label, enumeration of hygiene indicator microorganisms and pro-technological bacteria, texture profile, colour, aroma, panel test, biogenic amines content, bioactive peptides) of ewes' milk cheeses made up with *O. tauricum* extract and conventional animal rennet is currently in progress. However, to our knowledge, this is the first study supporting the technological performance of a vegetable rennet obtained from cultivated herbaceous plants, and we intended to highlight this key aspect in the title of the paper. Moreover, the relationship between the non-specific proteolytic activity of *O. tauricum* extracts and the operative parameters (pH, T, Ca ion concentration), the purification yields, and the biochemical traits of the purified proteases (molecular weight, thermal stability, optimal pH, hydrolysis profile of caseins) were brand-new data. We would also highlight the multivariate approach for assessing the behaviour of the parameters (milk clotting time, non-specific proteolytic activity) as affected by the independent variables (pH, temperature, Ca ion concentration), while literature data concerning the technological performance of vegetable rennets just report univariate studies focused on one variable at a time while keeping constant all the others. Only Guiama et al. [Int. J. Food Eng. 2014, 10, 211–222] used a factorial experiment design, but with a different goal, i.e., optimizing the extraction parameters (fruit percentage, NaCl concentration, extraction temperature) to maximize the coagulant strength of Solanum aethiopicum fruit extract. For these reasons we think the presented data should deserve a specific and dedicated publication.
The manuscript was further checked for English spell, grammar, and usage. The following changes were made:
Page 2, line 5: "used"
Page 4, line 22: "which"
Page 4, line 29: "pH,"
Page 7, line 14: "thirteen"
Page 9, line 22: "one"
Page 11, lines 15-17: "could be attributed to the plant absorption during the maturation cycle"
Page 11, line 24: "conducted"
Page 13, line 5: "operative"
Page 13, line 17: "optimise"
Page 13, line 51: "showed"
Page 14, line 27: "sulphate"
Page 14, line 32: "which"
Page 15, line 2: "In particular, C. cardunculus …"
Page 15, line 17: "was"

Abstract: The Abstract should contain tangible and quantitative results. The quality of the information should be improved. Give specific results with statistical significance. The results need to be briefly discussed.
Response: the Abstract was revised according to the Reviewer's suggestions.

Mat. and Methods
Page 7 - Line 22-54: Add an official reference. Give the formula used to determine the milk-clotting activity (in IMCU.L of milk).

Response: a miscellaneous of different "unit of clotting activity" and different kind of substrates (milk species; reconstituted milk powder; crude, thermized or pasteurized milk) were used to describe the milk clotting performance of vegetable rennets of various origin, thus limiting the comparison among literature data, as also highlighted by Esteves et al. [Int. Dairy J. 2002, 12, 427–434]. Therefore, we chose to directly compare the clotting times, without converting them in any "units" of clotting activity.

Page 8 - Line 28-29: What are the conditions used in ultra-turrax?
Response: Conditions have been specified

Page 9 - Line 5-24: Why was the proteolytic activity of the purified enzyme performed differently than the crude extract? Why was bovine milk used in both methods and in other experiments milk from other species?
Response: A first attempt to analyse the proteolytic activity in the crude extract with the caseinate-based assay failed due to the interference of molecules (mainly polyphenols of vegetable origin) absorbing at 280 nm. In the revised version we now report the effect of pH and temperature on the proteolytic activity of the purified enzyme also with the casein-FITC assay that we used for the crude extract. The results are included in Figures 2A and 2B.
The two methods for assaying the proteolytic activity (*i.e.*, the casein-FITC assay and the caseinate assay) do not involve the use of any milk.
Since the major goal of the work was to characterize an aqueous extract of *O. tauricum* flowers to be used in cheesemaking with goat's and ewe's milks, we deeply characterized the milk clotting activity of the extract on these types of milk.

Other analyzes are fundamental, such as:
- Rheological evaluation of the coagulation process.
- Determination of gel consistency and fresh gel yield.
- Enzyme stability at pH, temperature and storage time.

Analysis of the specificity of the coagulant on the casein's fractions (i.e., in k-CN, is it in the 105-106 bond? and in the other fractions? and the impact on primary and secondary proteolysis? and the production of hydrophobic bitter-tasting peptides? HPLC analysis, capillary electrophoresis to evaluate proteolysis, volatile profile, organic acids..)
In parallel, cheese production and evaluation of the impact on yield and on the physico-chemical, rheological, structural, and sensory characteristics of the final products are essential for evaluating the full impact of the coagulant on the quality of the product.
Response: please see the response above.

Results and discussion
The authors speak of data published for the first time, but in the discussion of MCA results, they mention that data are consistent with those obtained by Mozzon et al., 2020. What is the originality/innovation?
Response: we referred to previously published data concerning spontaneously grown *O. tauricum* plants collected in July 2019. As pedoclimatic conditions could strongly affect the properties of the thistle extracts, we briefly compared the results of the RSM approach on the milk clotting performance of wild flowers harvested in two consecutive years. As previously explained, the originality/innovation of the paper was focused on the properties of the cultivated thistle extracts, to overcome the biodiversity losses due to massive wild plant collection and support the exploitation of *O. tauricum* as a new and sustainable non-food crop in marginal and rainfed lands of Mediterranean countries.

Conclusions
The conclusion needs to be rephrasing. The conclusion as it stands is too much a summary of the results. Conclusions in a manuscript are meant to highlight major findings, linked to your objectives and to give a take home message to the readers. Therefore, it should answer the following questions: What is new? Why is it important? What are the limitations and potential consequences?
Response: we think that all the issues stated by the Referee were addressed.
What is new? The technological performance and the biochemical traits of a novel aspartic-type endopeptidase from cultivated *Onopordum tauricum*.

Why is it important? Cultivation of thistles could both overcome the biodiversity losses due to massive wild plant collection and develop a new and sustainable non-food crop in marginal and rainfed lands of Mediterranean countries.
What are the limitations and potential consequences? The different conditions needed to both minimise the intense non-specific proteolytic activity towards β- and α-caseins and maximise the clotting activity could result in low curd yield and poor textural and sensory properties of cheeses. In fact, as previously reported to the attention of the Referee, cheesemaking trials are currently in progress. According to the suggestion of Reviewer #1, a brief statement concerning the further studies needed for a full assessment of the use of *O. tauricum* proteases in cheesemaking was added to the Conclusion section.

Reviewer #4

The study has some interesting information. It is recommended for publication after the following points are addressed by the authors.
We would like to thank the Reviewer for the precious comments and suggestions.

1. The study showed that the main use of this enzyme is for cheese processing, so it should be applied to cheese making to examine the application characteristics of the enzyme, not just for the rennet process.
Response: We fully agree with the Reviewer: the performance of O. tauricum proteases needs to be assessed in a real cheesemaking process. In fact, a full characterization (physico-chemical, chemical, microbiological, and sensory) of thistle-curdled ewes' milk cheeses, as well as the investigation of nutritionally valuable (phenolic components deriving from crude extracts, bioactive peptides) and hazardous (biogenic amines) substances in the final product, are currently in progress.

2. Highlights. The second point should be replaced.
Response: we rephrased the first and second points to avoid misunderstandings about the technological properties of thistle extracts (clotting vs proteolytic)

3. Page 4, Line 4 Did nine APs isolated from C. cardunculus have milk clotting activity?
Response: We specified the proteases with milk clotting activity and included the appropriate references.

4. Page 4, Line 24-25 Please specify the same condition. This is important for the determination of milk clotting activity.
Response: APs with milk clotting activity from *C. scolymus* are specified in the cited references.

5. Page 10 The authors need to explain this result. (Those values were much lower than previously observed in spontaneously grown plants collected in 2019 (Mozzon et al., 2020).)
Response: Pedoclimatic conditions could strongly affect the composition of plant tissues. For this reason, in the Result and Discussion section, we compare the results (total protein content, dry extract yields, clotting performance) obtained from wild flowers harvested in two consecutive years. Annual variations in the climatic conditions could significantly limit the application of wild thistles for cheesemaking, while agronomic practices could buffer and/or compensate for them, thus increasing the interest in the cultivation of Taurian thistles.

6. Page 13 In section 3.3, the authors want to control a balanced breakdown of caseins into small peptides and free amino acids. Just minimizing the PA is not enough, plasmin should also be considered.
Response: According to reviewer's suggestion, the contribution of milk proteases to proteolysis was briefly presented.

7. Page 14 In section 3.4, SDS-PAGE analysis of the purified protease is limited. Using mass spectrometry to identified the purified protease is required.
Response: We agree that the identification of the protease by the SDS PAGE analysis shown in Fig. 1 is limited. On the other hand, identification of protein using mass spectrometry might not be resolutive in our case since the sequence of this protease is still unknown, and to our knowledge, no proteases from other Onopordum spp have been deposited so far. Therefore, to confirm the identification of the purified protease, we have included in Figure 1 the SDS-PAGE analysis of several fractions eluted from the Mono Q column

(Figure 1B). The findings that i) the two bands indicated by the arrows coeluted and that ii) the proteolytic activity was detected only in the fractions with the two bands, suggest that the two bands represent the protease of interest. Bands at molecular weight higher than 35 kDa were also present in the first active fractions, but their elution profile did not match with the proteolytic activity. Unfortunately, these contaminants were still present in the active fractions deriving from the chromatography performed with a broader elution gradient (Figure 1C and 1D).

8. From Figure 2D, it can be seen that the degree of hydrolysis of α-casein and β-casein by this protease is much higher than that of rennet, and the excessive hydrolysis of α-and β-casein will cause problems such as poor curdling properties of milk and low yield of cheese, how to solve this problem?
Response: this point is a well-known issue, nevertheless several appreciated and PDO soft cheeses prepared with vegetable rennets are presently available on the EU market. We are currently evaluating the rheological, nutritional and sensory properties of cheeses prepared with ewe's milk and *O. tauricum* extract to assess the suitability of our vegetable rennet.

- Cultivated *Onopordum tauricum* showed better clotting ~~performance~~ activity than wild thistles
- ~~Proteolytic activity showed no differences between c~~Cultivated and wild thistles showed no differences in their proteolytic activities
- The purified tauricosin was identified as a heterodimeric aspartic-type protease
- *O. tauricum* showed good potential as herbaceous crop for vegetable rennet production

Potentialities of aqueous extract from cultivated *Onopordum tauricum* (Willd.) as milk clotting agent for cheesemaking

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Abstract

In Western and Central Mediterranean countries proteases from wild herbaceous perennial plants commonly known as "thistles" have been ~~using~~ used as milk coagulants in cheese-making for centuries. For the first time, the technological and biochemical traits of proteases from cultivated *Onopordum tauricum* Willd. (Taurian thistle, bull cottonthistle) were assessed. The optimal conditions for minimizing the clotting time and the non-specific proteolytic activity were estimated at the highest (T = 43-45 °C; [Ca^{2+}] = 11-13 mM) and the lowest (T = 35-39 °C; [Ca^{2+}] = 5 mM) temperature and calcium ion levels in the explored range respectively, thus highlighting the difficulty to set the best operative compromise in the first step of cheesemaking. ~~Anyway, the clotting performance of cultivated thistles extract was better than wild thistles extract in~~In the conditions adopted in common cheesemaking practice (T = 37 °C; pH = 6.5) 1 mL of reconstituted extract from cultivated thistles coagulated 10 mL of ewe's and goat's milk in 114-146 and 129-167 seconds, respectively, and 1 mL of reconstituted extract from spontaneous thistles coagulated 10 mL of ewe's and goat's milk in 232-294 and 428-621 seconds, respectively, while no significant differences in the non-specific proteolytic activity between cultivated and spontaneous *O. tauricum* extracts were observed. The purified enzyme (tauricosin) was identified as an aspartic protease made up of two sub-units with molecular weights of 32 and 9.6 kDa, respectively. Experimental data encouraged the exploitation of *O. tauricum* as a new and sustainable non-food crop in marginal and rainfed lands of Mediterranean countries, thus reducing the potential biodiversity losses due to wild collection.

Keywords: Vegetable coagulant; Milk clotting; Aqueous extract; Clotting activity; Protease; *Onopordum tauricum*; Taurian thistle.

1. Introduction[1]

The use of vegetable coagulants in cheesemaking takes utmost importance nowadays, due to the growing demand and the reduced availability of ruminant stomachs (Jacob; Jaros, & Rohm, 2011). Vegetable enzymes from different plant organs (leaves, flowers, stems, fruits) and products (latex) were extensively studied as potential coagulants in the production of cheese (de Farias et al., 2020, Colombo et al., 2018, Liburdi, Boselli, Giangolini, Amatiste, & Esti, 2019, Shah, Mir, & Paray, 2014, Mazorra-Manzano et al., 2013). Ewe's and goat's cheeses produced with vegetable rennet are widely popular in Spain and Portugal (Torta del Casar, Los Ibores, Serpa, Azeitão, Nisa, Évora, Casar de Cáceres, Serra de Estrela, Los Pedroches, La Serena, Castelo Branco, Flor de Guía) (Reis, & Malcata, 2011, Roseiro, Barbosa, Ames; & Wilbey, 2003, Tejada, & Fernández-Salguero, 2003, Fernández-Salguero, Tejada, & Gómez, 2003) and some of them gained the European Protected Designation of Origin (POD), thanks to their unique taste, aroma, and flavour (EUR-Lex. Council Regulation, 2006, Barracosa, Rosa, Barros, & Pires, 2018). "Caciofiore" is a small-batch cheese manufactured in Central Italy regions with raw ewe's milk, which represents a magnificent model of Mediterranean soft cheese made with vegetable coagulants (Cardinali et al., 2017, Cardinali et al., 2016).

Among the plant proteases, the enzymatic activities extracted from thistles belonging to the genus *Cynara* deserve a special mention, being the most significant plants to produce vegetable rennets (Tejada, Vioque, Gómez, & Fernández-Salguero, 2008b, Gomes et al., 2019, Ordiales et al., 2013, Silva, & Malcata, 2005). The milk clotting activity of thistle extracts is due to several types of aspartic proteases (APs). The most studied are APs from *C. cardunculus*, which produce cheeses with a creamy, soft texture and slightly piquant aroma (Alavi; & Momen, 2020). Cheese-making trials have demonstrated the suitability of these proteases for the

Abbreviations: MCT, milk clotting time; PA, proteolytic activity; AP, aspartic protease; casein-FITC, casein fluorescein isothiocyanate; PMSF, phenylmethylsulfonyl fluoride; E64, N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine

manufacture of cheeses without the defects (e.g., bitterness; excessive softness of the cheese paste) typically occurring in cheeses produced with plant-based rennet substitutes, due to an excessive non-specific proteolytic activity (Agboola; Chan; Zhao, & Rehman, 2009, Ben Amira, Besbes, Attia, & Blecker, 2017). To date, nine APs were isolated from *C. cardunculus* flowers, six cardosins (Veríssimo et al., 1996, Sarmento et al., 2009) and three cyprosins (Cordeiro, Pais, & Brodelius, 1994). Cyprosins and cardosins A and B were found to be endowed with milk clotting activity (Heimgartner, Pietrzak, Geertsen, Brodelius, Figueiredo, & Pais, 1990, Silva, & Malcata, 2005). ~~*They*~~ *C. cardunculus* APs are the products of a multigene family (Pimentel, Van Der Straeten, Pires, Faro, & Rodrigues-Pousada, 2007) and share a similar primary structure but different activities and substrate specificity. All of them are glycosylated heterodimers, exhibiting a marked microheterogeneity in terms of isoelectric point and molecular mass, ~~that~~which were ascribed to the proteolytic processing of the precursors during the activation process (Sarmento et al., 2009). APs with milk clotting activity were also purified from *C. scolymus*. They share a high degree of sequence homology with *C. cardunculus* proteases, the same optimum acidic ~~pH~~pH, and a similar proteolytic activity on the various milk caseins (Llorente, Brutti, & Caffini, 2004, Chazarra, Sidrach, López-Molina, & Rodríguez-López, 2007). Proteases with milk clotting activity were also characterized in other genera within the tribe *Cynareae*, like *Centaurea calcitrapa* (Domingos et al., 1998) and *Cirsium* vulgare (Lufrano et al., 2012).

The chemical, biochemical and technological traits of many other thistle extracts were studied. Particularly, *Onopordum* spp. contain, in their flower heads, aspartic proteases with high milk-clotting activity. A few studies reported the performance of the enzymes of *O. acanthium* L. and *O. turcicum* Danin (Brutti; Pardo, Caffini, & Natalucci, 2012, Tamer, & Mutlu, 1994, Tamer, 1993), but only one research investigated the clotting properties of *O. tauricum* Willd. (Taurian thistle, bull cottonthistle) on different milks (ewe, goat, and cow) and reported a milk

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clotting activity only 5-15 times lower than conventional calf rennet, when assayed in the same conditions (Mozzon et al., 2020). *O. tauricum* is a wild biennial or annual herb characterized by a high spiny stem, up to two meters tall, triangular-lobed leaves and a hemispherical inflorescence (4–6 cm in diameter) provided with pink-purple tubular flowers. Taurian thistles are well adapted to the xerothermic conditions of Mediterranean regions, thus suggesting their exploitation as cultivated non-food crop in marginal lands. In a recent paper, Zenobi et al. (2021) described the effect of different plant densities on the development and differentiation of cultivated *O. tauricum*: a significantly higher production of flower heads (7926-9555 *vs* 1734-2290 g/m^2) and tubular flowers (3500 *vs* 800 g/m^2) per unit of surface than *Cynara cardunculus* L. confirmed the potentialities of *O. tauricum* as herbaceous crop for vegetable rennet production in a Mediterranean environment with low inputs. The germination ecology of two different accessions of wild thistle seeds subjected to different pre-treatments (gibberellic acid, vernalization) was also investigated (Zitti, Di Cecco, Casavecchia; Martino, & Aquilanti, 2021).

Based on the above premises, in this study we compared the technological properties (milk clotting activity, non-specific proteolytic activity) and the mineral composition of crude extracts from spontaneous and cultivated *O. tauricum* plants and collected new data about the biochemical characterization of the purified protease, with the aim of implementing a new sustainable non-food cropping system in Mediterranean rainfed areas, thus contributing to the reduction of potential biodiversity losses due to the wild plant collection.

2. Material and methods

2.1. Vegetable material and crude extract preparation

Flower heads of wild *O. tauricum* plants were collected in July 2020, along the fringes of Monti Sibillini National Parks, Central Italy (municipality of Visso, Cupi hamlet;

42°59'56.8"N 13°06'53.5"E; 980 m a.s.l.). Thistle seeds collected in 2019 in the same place were sown in February 2020 in a greenhouse, transplanted in a silty-clay soil site located at the Pasquale Rosati experimental farm of the Università Politecnica delle Marche (municipality of Agugliano; 43°32' N 13°22' E; 100 m a.s.l.; slope gradient 10%) in February 2020, and harvested in July 2020, according to Zenobi et al (2021). Tubular flowers were manually separated from wild and cultivated thistle flower heads and macerated in demineralized water, according to the procedure described in Mozzon et al (2020). Aqueous extracts were then freeze-dried in a benchtop equipment (VirTis Advantage, Steroglass S.r.l., Perugia, Italy), stored at -20 °C and reconstituted with demineralized water 1:10 w/v at the time of use. Weights of fresh flowers and dried extracts were recorded to calculate the extract yields.

2.2. Protein content

The total protein content of the reconstituted extracts was determined according to the colorimetric method originally developed by Bradford (1976). Details are given in Mozzon et al (2020).

2.3. Mineral composition

Minerals (Ca, P, Na, K, Mg, Zn and Mn) of the aqueous crude extracts from wild and cultivated *O. tauricum* were determined according to AOAC (2002) method. Briefly, 0.3 g of samples were ashed in the furnace at 525 °C for 16 h. After mineralization, samples were solubilized in 1 mL HNO_3 65% and then to a final volume of 50 mL HNO_3 1% (v/v) with 0.1% (w/v) CsCl to avoid sodium and potassium ionisation and with $LaCl_3$ 0.1% (w/v) for Ca and Mg detection. Ca, Na, K, Mg, Zn and Mn were detected by an atomic absorption spectrometer AAnalyst 300 (Perkin Elmer, Norwalk, CT, USA), while P was measured at 400 nm by an UV-1800 spectrophotometer (Shimadzu Corporation, Tokyo, Japan).

2.4. Experimental design

The response surface methodology (RSM) approach was used to study the relationships between three independent factors (temperature, pH, Ca ions concentration) and the performance of reconstituted thistle extracts on raw milk of different origin (clotting activity) and on modified casein-based substrate (non-specific proteolytic activity). Each independent variable was selected at three levels, based on preliminary trials: 35, 40 and 45°C for temperature, 5.0, 5.5 and 6.0 for pH, and 5, 10 and 15 mM for Ca ions concentration. The D-optimal criterion was used to get the best compromise between the amount of useful information and the number of experiments. The software (see Statistical analysis paragraph) generated a total of ~~13~~thirteen runs that were carried out in double (Supplementary Tables A1 and A2). The order of the experiments was fully randomized to minimize the effects of unexplained variability.

2.5. Milk clotting activity assay

Crude ewe's and goat's milk were collected from a local farm and transferred to the laboratories of Università Politecnica delle Marche, where they were immediately skimmed by centrifugation (5000g, 35°C, 10 min) and freeze-dried. For clotting tests, milk powders were re-dissolved in buffer solutions (pH 5.0, 5.5, and 6.0), based on their dry matter contents which were 14 and 11% w/w for ewe's and goat's milk, respectively. The buffers were prepared from a sodium acetate 100 mM stock solution and adjusting the pH to the desired value by the addition of acetic acid. A test tube containing 5 mL of reconstituted milk was previously thermostated in a M20 model water bath (Lauda GmbH, Königshofen, Germany). A calcium chloride (Sigma-Aldrich, Milan, Italy) solution (500 g/L) was added to the substrate to achieve the desired final concentration without any significant changes in the whole substrate volume (5-15 µL). Finally, 500 µL of reconstituted *O. tauricum* extract were added to the mixture and the time needed for first flocks appeared (milk clotting time, MCT) on the tube wall was recorded.

2.6. Proteolytic activity assay

The proteolytic activity of the reconstituted *O. tauricum* extract was measured on casein-fluorescein isothiocyanate (casein-FITC) through a continuous fluorescent assay adapted from Twining (1984). The reaction mixture consisted of 40 mM sodium acetate buffers (pH 5.0, 5.5, and 6.0), 0.1 mg/ml casein-FITC type III from bovine milk (Sigma-Aldrich, St. Louis, MO, USA), and calcium chloride 5, 10 and 15 mM in a final volume of 200 µl. Following the addition of the sample (1 µg) to be analysed, the fluorescence was continuously monitored at 35, 40 and 45 °C, using a Synergy HT microplate reader equipped with 485 and 528 nm excitation and emission filters, respectively. One Fluorescence Unit (FU) ~~of proteolytic activity (PA)~~ was defined as the amount of enzyme that produces an increase of 1 Unit of fluorescence per minute under the assay conditions. The casein-FITC based assay was also used to monitor the purification of the protease and to characterize the purified enzyme. The effects of pH and temperature on the proteolytic activity of the purified enzyme were also assayed by using bovine milk Na-caseinate as substrate. To this end, the pure enzyme was incubated with 2% caseinate (w/v) in sodium phosphate buffer 0.1 M at the indicated pH values and temperatures. At different times aliquots were withdrawn and the reaction was stopped by adding 5% trichloroacetic acid at a 1:2 ratio (v/v) (Silva et al., 2005). After centrifugation at 16000 × g for 5 min, the absorbance of the supernatant was measured at 280 nm and the initial rate of the reaction was calculated. One Caseinolytic Unit (CU) was defined as the amount of enzyme that produces an increase of one absorbance unit per minute at 280 nm under the assay conditions.

2.7. Enzyme purification

About 15 g of fresh flowers manually separated from cultivated plants were homogenized with Ultra-turrax® equipment (IKA-Werke GmbH & Co., Staufen, Germany) in 0.1 M

sodium acetate buffer pH 5.5 (buffer A), at a 1:5 ratio (w/v), at 20000 rpm for 2 minutes four times, with 2 minutes intervals on ice. The homogenate was centrifuged at 11000 × g for 30 min at 4°C and the supernatant, referred as the crude extract, was made 60% saturated with ammonium sulphate. After stirring for 20 minutes at 4°C, the suspension was centrifuged as above. The pellet was resuspended in 6 ml of buffer A and, after centrifugation as above, the supernatant was desalted using a PD-10 column containing Sephadex G25 resin (GE Healthcare, Chicago, IL, USA) equilibrated and eluted with 0.025 M sodium acetate buffer pH 5.5 (buffer B). The sample (1 mL) was loaded onto a Mono Q column (GE Healthcare, Chicago, IL, USA) equilibrated with buffer B. After a washing step with the same buffer, elution was performed with a linear gradient of NaCl from 0 to 0.5 M in buffer B. In an alternative elution procedure, after a washing step with buffer B containing 0.15 M NaCl, elution was performed with a linear gradient of NaCl from 0.15 to 0.3 M in buffer B. The final preparation was stored at -20°C. SDS-PAGE and Tricine SDS-PAGE were performed according to Laemmli (1970) and Schägger (2006), respectively.

2.8. Molecular and enzymatic properties of purified protease

The molecular mass of the final preparation was estimated by gel filtration chromatography on a Superose 12 10/300 GL column (GE Healthcare, Chicago, IL, USA) equilibrated and eluted with 25 mM sodium acetate buffer pH 5.5 containing 0.3 M NaCl. The effect of pH and temperature on the proteolytic activity of the purified enzyme was ~~The proteolytic activity of the purified enzyme was determined using bovine milk Na-caseinate as substrate. The pure enzyme was incubated with 2% caseinate (w/v) in sodium acetate buffer 0.1 M pH 5.5 at 37°C. At different times aliquots were withdrawn and the reaction was stopped by adding 5% trichloroacetic acid at a 1:2 ratio (v/v) (Silva et al., 2005). After centrifugation at 16000 × g for 5 min, the absorbance of the supernatant was measured at 280 nm and the~~

~~initial rate of the reaction was calculated. One Caseinolytic Unit was defined as the amount of enzyme that produces an increase of 1one absorbance unit per minute at 280 nm under the assay conditions. The optimum pH was~~ determined ~~by monitoring the caseinolytic activity at 37°C in 0.1 M sodium phosphate buffer, at pH values ranging from 4.5 to 7.0.~~with bovine –caseinate and bovine casein-FITC as the substrates. For thermal stability, the purified protease was incubated at 45°C and 55°C. Residual activity was measured using the casein-FITC assay at pH 5.5 and 37°C. ~~The optimum temperature was determined by using the caseinolytic activity assay.~~ The effect of known proteases' inhibitors on the proteolytic activity was evaluated by preincubating the purified enzyme for 30 minutes at 37°C in 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. To analyse the proteolytic activity of the pure enzyme on various caseins, commercial bovine α-, β- and κ-casein (Sigma-Aldrich, St. Louis, MO, USA) 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 enzyme or rennet. After 0 and 60 minutes of incubation, aliquots were subjected to SDS-PAGE on 15% acrylamide gel.

2.9. Statistical analysis

The software JMP Version 11.0.0 (SAS Institute Inc., Cary, NC, USA) was used to both design the experimental plan and analyse the results of clotting and proteolytic assays. A second-order response surface was used to fit the milk clotting and proteolytic activities data matrices. The statistical significance of the model was assessed by analysis of variance (ANOVA).

3. Results and discussion

3.1. Characterization of aqueous extracts

The total protein content of the reconstituted extracts was 0.846 and 0.668 µg/µL for wild and cultivated flowers, respectively. Those values were much lower than previously observed in spontaneously grown plants collected in 2019 (Mozzon et al., 2020), even if the dry extract yields (4.60 and 4.36 g/100 g of fresh flowers for wild thistle population and cultivated plants, respectively) were of the same magnitude of 2019 data. Consequently, the experiments on extracts prepared from plants collected in the summer of 2020 were conducted with much higher substrate/enzyme ratios (697-883 and 426-539 for ewe's and goat's milk, respectively), based on the protein contents of the skimmed milks (5.9% w/w and 3.6% w/w for ewe's and goats' milk, respectively) and the volume of extracts used (500 µL). Annual variations in the climatic conditions could significantly limit the application of wild thistles for cheesemaking, while agronomic practices could buffer and/or compensate for them, thus increasing the interest in the cultivation of Taurian thistles.

The contents of macro and micro elements in aqueous extracts of spontaneous and cultivated *O. tauricum* were summarised in Table 1. According to these results, the aqueous extract from cultivated plants showed a higher content of macro elements ($p < 0.05$) than the wild thistles, while no differences could be detected for micro elements (Zn and Mn). The differences in mineral contents could be attributed to different growing systems, growing conditions as well as differences in plant age. Artichoke flower heads also represent valuable sources of minerals, showing in particular K and Ca (Ceccarelli et al., 2010), however, there are very few works about the mineral composition of cardoons' extracts. Biel, Witkowicz, Piątkowska, & Podsiadło (2020) in commercial leaf extracts of artichoke (*Cynara scolymus*) found the same order of magnitude for minerals with the only exception for K (506.3 mg/100 of dry weight), absorbed most by artichoke plants during their growing cycles. The plant parts are sources of minerals but there are differences

regarding their minerals content: in particular, leaves and heads are rich in K and Ca while stalks are rich in Na (Petropoulos, Pereira, Tzortzakis, Barros, & Ferreira, 2018). According to some authors (Ben Amira et al., 2018) the calcium abundance in flowers could ~~probably~~ be ~~due to the fact that~~attributed to the plant ~~has collected this~~absorption ~~element for its growth,~~ during the maturation cycle.

3.2. Milk clotting activity

The clotting tests were ~~carried out~~conducted on skimmed milks to reduce foaming and better catch the beginning of flocculation (Mozzon et al., 2020). The capability of proteases to specifically hydrolyse the Phe_{105}–Met_{106} bond of κ-casein, thus causing the destabilization of casein micelles, is a key aspect to assess a potential substitute of conventional animal rennets. ~~However, a univariate approach characterizes most of the literature data concerning the effect of independent variables on the clotting activity, thus disregarding the possible interactions among factors~~Despite the number of independent variables affecting the clotting performance of proteases, most of the published studies analysed the effect of one factor at a time while keeping constant all the others, thus disregarding the possible interactions among them (de Farias et al., 2020, Liburdi et al., 2019, Llorente et al., 2004, Chazarra et al., 2007). Therefore, ~~A~~a multivariate approach and a second order polynomial model equation ~~was~~ were used to reveal potential interactive effects among the experimental variables and to predict the optimal conditions for milk clotting and the best performance of crude extracts. A standard least square regression method was used to fit data from the experimental design and estimate the parameters of the mathematical models. The responses at each design point, the statistical significance of the regression coefficients, and the response surface 3D plots were provided in the Supporting Information (Supplementary Tables A3 and A4, Supplementary Figures A1-A3). The agreement between the predicted R^2 (measure of the reliability to predict the

response, i.e., MCT) and the adjusted R^2 (measure of the quality of data fitting) was used as a global quality indicator of the polynomial models: only the clotting of ewe's milk by aqueous extract from cultivated thistles showed a significant ($p < 0.05$) reliability (Table 2). The mutual interactions between each pair of factors were found to be not significant. However, the clotting properties of vegetable extracts were affected by both the kind of milk and the kind of thistles (wild *vs* cultivated): the clotting properties of *O. tauricum* extracts in goat's milk were significantly affected only by pH while the other two factors (temperature and Ca ion concentration) affected the response (MCT) of wild thistle extract; in ewe's milk, all the three linear factors showed a significant influence in the clotting performance of cultivated thistle extract, while the temperature was not significant in describing the behaviour of wild thistle extract. The independent factors were analysed by using the desirability function to estimate the best conditions for minimizing the clotting time (Table 3). In all the milk/coagulant systems, the optimal temperature for clotting was the highest (45°C) or close to the highest (43°C) in the range explored (35–45°C) and the optimal pH value (5.0–5.5) was the lowest in the actual range studied (5.0–6.0). These results were consistent with previously published data on *O. tauricum* aqueous extracts (Mozzon et al., 2020). The optimal Ca ion concentrations were 11-13 mM, higher than the Ca levels used in most of the published studies on the clotting performance of vegetable rennets (Mozzon et al., 2020), thus confirming the need of Ca fortification to improve the clotting performance of thistle proteases. In the optimal conditions (T = 43-45°C; pH = 5.0-5.5; $[Ca^{2+}]$ = 11-13 mM), the clotting performance (predicted MCTs shown in Table 3) of cultivated thistles extract was 1.7 and 2.0 times better than wild thistles extract in ewe's and goat's milk, respectively. Those data could be considered even more favourable for cultivated plants by considering the total protein content of the reconstituted extracts. The optimal factors to minimize the clotting time of thistle extracts were far from parameters

adopted in common cheesemaking practice. Therefore, in the prediction profiler all factors were selected as random (temperature T 37°C ± 1; [Ca^{2+}] 10 mM ± 0.4; pH 6.5 ± 0.1) and the predicted MCTs were extrapolated from 20 runs. As expected, all clotting times lengthened in the ~~real operative~~operative conditions in the cheese factory, but the clotting performance of extracts from wild thistles got worse than those of extracts from cultivated plants (Table 4).

3.3. Proteolytic activity

The measure of the proteolytic activity of vegetable rennets is of paramount importance to ~~optimize~~ optimise the textural and sensory properties of cheeses. In fact, the proper development of the sensory properties of cheese during ripening needs a balanced breakdown of caseins into small peptides and free amino acids, but an excessive non-specific proteolytic activity could result in low curd yields and sensory defects in cheese (bitter flavour, softness) (Tejada, Abellán, Cayuela, Martínez-Cacha, & Fernández-Salguero, 2008a). The involvement of additional proteases, like milk plasmin, might also significantly contribute to proteolysis during ripening and therefore should be taken into consideration (Bastian, & Brown, 1996). An aspartic protease (onopordosin) was previously characterized in *Onopordum acanthium* flowers, showing a milk clotting activity/proteolytic activity ratio better than protease from *C. cardunculus*, thus representing a promising plant coagulant (Brutti et al., 2012). The good agreement between R^2 and adjusted R^2 indicated the high adequacy of the models for describing the behaviour of the PA in different conditions of temperature, pH, and Ca ion level (Table 2). Unlike the clotting activity, the latter factor ([Ca^{2+}]) did not affect the proteolytic performance of both wild and cultivated thistle extracts. Temperature and pH exerted a strong (quadratic) effect on PA of reconstituted extract of cultivated thistles, while pH was the only factor that significantly affected the PA of the reconstituted extract of wild *O. tauricum* flowers. The

desirability function ~~identified~~showed the pH 5.0 as optimal for both the best clotting performance and the lowest non-specific proteolytic activity (Table 3). However, the optimal conditions for minimizing the PA were estimated at the lowest temperature and [Ca^{2+}] values in the explored range, thus highlighting the difficulty to set the best operative compromise in the first step of cheesemaking. Anyway, under the above conditions the predicted PA of wild thistle extract was approximately ~~2.5~~1.5 times higher than extract from cultivated plants, as shown in Table 3. By fixing factors to operative conditions adopted in common cheesemaking practice (Table 4), the PA of extract from cultivated plants increased by 14-20 times, while the PA of extract from wild plants increased by 9-13 times, leading to no significant differences in the PA at those specific constraints. By comparison, the optimal hydrolytic conditions for *Cynara scolymus* flower extract towards bovine casein were as follows: pH 6.2, temperature 50 º C, protein concentration 0.023 mg/ml. As a result, a K_m value of 5.66 mg/ml and a V_{max} of 8.47 mUAb/min were reported (Bueno-Gavilá et al., 2020).

3.4. Purification of protease and molecular properties

The protease responsible for the coagulating activity was purified through the procedure outlined in Table 5. It consisted of an ammonium ~~sulfate~~sulphate precipitation step that removed most of the pigments, at the same time allowing the concentration of the sample; a desalting step through gel filtration; an ionic exchange chromatographic step, ~~that~~which yielded ~~the~~ a highly purified enzyme preparation~~pure enzyme~~. In the last step, by performing the elution with a gradient of NaCl from 0 to 0.5 M in the equilibration buffer, only one peak with proteolytic and milk clotting activity was obtained that eluted at about 0.25 M NaCl (Figure 1A). In particular, we found that 0.1 ml of 0.01 mg/ml purified protease was able to coagulate 1 ml of bovine skimmed milk in the presence of 10 mM $CaCl_2$, at 45°C, in about 8 minutes. SDS-PAGE analysis of the fractions eluted from the

Mono Q column (Figure 1B) showed the coelution of the proteolytic activity with two bands (indicated by the arrows in the figure), suggesting that they might represent the two subunits of the protease of interest. When the ~~last~~ chromatographic step ~~of the purification~~ was performed with an elution gradient from 0.15 to 0.3 M NaCl, the protein peak appeared very broad, indicating a marked heterogeneity (Figure ~~1B~~1C). ~~However,~~ Tricine SDS-PAGE analysis of different fractions of the peak yielded ~~showed~~ the same protein pattern and ~~, revealing the presence of two bands with~~ molecular weights of 32 and 9.6 kDa, were calculated for the two bands of interest ~~respectively~~ (Figure ~~1C~~1D). Gel filtration analyses of the fractions also resulted in a similar molecular mass of about 39 kDa (Figure ~~1D~~1E). ~~This result, combined with the SDS-PAGE data, showed~~Altogether, these results suggested that the enzyme was a heterodimeric protein. This was in line with the general finding that thistle proteases are heterodimeric proteins. In particular, ~~in~~ *C. cardunculus* cardosin A consists of two subunits with MW of 31 and 15 kDa, whereas cardosin B consists of two subunits with MW of 34 and 14 kDa (Veríssimo et al., 1996). Other four cardosins (E, F, G and H) were also characterized, with 29 and 15 kDa subunits (Sarmento et al., 2009). In *C. scolymus*, five different cynarases with subunits of MW of 30 and 14.4 kDa were described (Llorente et al., 2004). The heterogeneity of the purified protease, as revealed by the ionic-chromatographic step, is a feature common to all thistle proteases so far characterized, that ~~has been~~was ascribed to the coexistence of molecular forms of different isoelectric point values (Sarmento et al., 2009).

3.5. Enzymatic properties of pure protease.

Optimal caseinolytic activity was achieved at pH values ranging from 4.~~0~~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, Zhao, & Agboola, 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). The enzyme thermal stability is shown in Figure 2C. 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 were in agreement with those reported for other thistle proteases, like cyprosins and cynarases (Sidrach, García-Cánovas, Tudela, & Neptuno Rodríguez-López, 2005, White, Cordeiro, Arnold, Brodelius, & Kay 1999). A markedly lower optimum temperature (40°C) was reported for the partially purified protease from *O. acanthium* (Benkahoul, Benchiheub, Bellil, Khelifi, & Maza, 2016). To get insight into the nature of the catalytic type of tauricosin, the effect of serine-, cysteine- and aspartic-inhibitors (PMSF, E-64, pepstatin A) was tested on the caseinolytic activity of the purified enzyme, as described in Materials and Methods. Only pepstatin A was found to inhibit the enzyme, with ~~11~~89% inhibition observed in the presence of 0.5 mM pepstatin A. These results confirmed that tauricosin was an aspartic acid protease. The degradation pattern of α-, β-, and κ-caseins after 30 min incubation with purified tauricosin was compared to that obtained with rennin after incubation in the same conditions. As shown in Figure 2D, tauricosin hydrolysed κ-casein similarly to rennin, 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).

4. Conclusion

In summary, this is the first study to report the technological performance and the biochemical traits of a novel aspartic-type endopeptidase from cultivated *Onopordum*

tauricum. The purified enzyme (tauricosin) was a heterodimeric protein made up of two sub-units with molecular weights of 32 and 9.6 kDa, respectively. The crude aqueous extracts prepared from tubular flowers of both spontaneous and cultivated plants showed optimal conditions for clotting ewe's and goat's milk (T = 43-45°C; pH = 5.0-5.5; [Ca^{2+}] = 11-13 mM) far from parameters adopted in common cheesemaking practice. However, in all conditions the cultivated plants provided extracts having better clotting performance than wild thistles. Tauricosin hydrolysed κ-casein similarly to rennin (clotting activity), while it showed a more intense non-specific proteolytic activity towards β- and α-caseins. Lower temperatures (35°C) and Ca ion levels (5 mM) were needed for minimizing the potentially undesired non-specific PA, thus highlighting the difficulty to set the best operative compromise in the early steps of cheesemaking (coagulation, curd cutting, whey separation). Furthermore, no significant differences in the PA at operative conditions adopted in common cheesemaking practice were observed between extracts of wild and cultivated thistles. The encouraging technological performance of *O. tauricum* extracts go hand in hand with botanical and agronomic data. Taurian thistle seeds showed a high germination rate (94%) when properly pre-treated (Zitti et al., 2021) and cultivated *O. tauricum* exhibited a significantly higher production of useful biomass (tubular flowers) per unit of surface than the most popular source of vegetable rennets *Cynara cardunculus* (Zenobi et al., 2021), thus confirming the potentialities of *O. tauricum* as an herbaceous crop for vegetable rennet production in the rainfed unfertilized Mediterranean environment. Further studies concerning the rheological properties of curd and cheese together with the chemical, microbiological, and sensory characterization of the final products are needed to provide a full assessment of *O. tauricum* proteases as vegetable rennet.

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CRediT authorship contribution statement

Roberta Foligni: Methodology, Validation, Formal analysis, Investigation, Writing-review and editing, Visualization. **Cinzia Mannozzi:** Methodology, Validation, Formal analysis, Investigation, Writing-review and editing, Visualization. **Massimiliano Gasparrini:** Methodology, Validation, Formal analysis, Investigation, Writing-review and editing, Visualization. **Nadia Raffaelli:** Conceptualization, Investigation, Resources, Writing–original draft preparation. **Federica Zamporlini:** Investigation. **Luis Tejada:** Writing-review and editing. **Cindy Bande-De León:** Investigation, Writing-review and editing. **Roberto Orsini:** Investigation. **Pamela Manzi:** Formal analysis, Investigation, Resources, Writing–original draft preparation. **Maria Gabriella Di Costanzo:** Investigation. **Mena Ritota:** Investigation. **Lucia Aquilanti:** Supervision, Project administration, Funding acquisition. **Massimo Mozzon:** Conceptualization**,** ~~Resouces~~Resources, Writing–original draft preparation.

Declaration of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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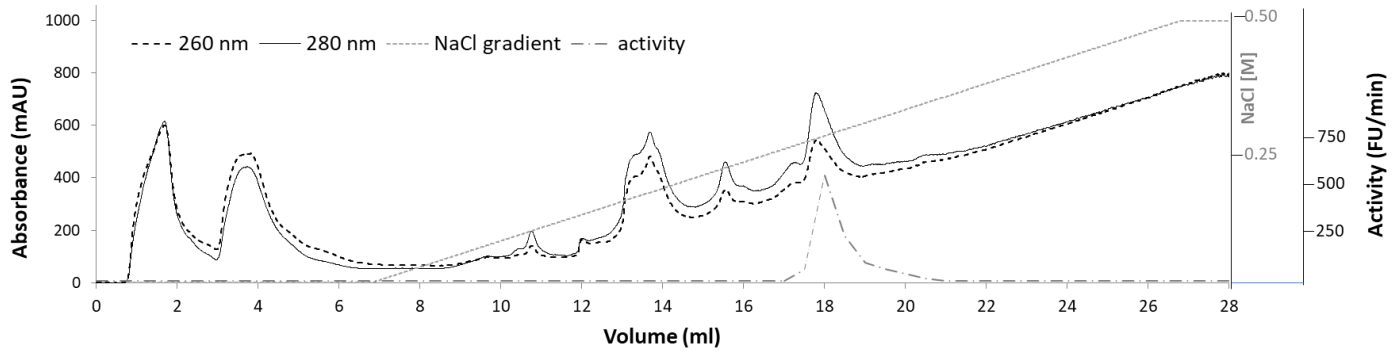
Figure captions

Figure 1. Purification and molecular properties of tauricosin. Anion-exchange chromatography on the Mono Q column eluted with a linear gradient of NaCl, from 0 to 0.5 M (A). Eluted fractions were analysed by SDS-PAGE, with a 15% polyacrylamide gel (B). Profile of the Mono Q column eluted with a NaCl gradient ~~and~~ from 0.15 to 0.3 ~~25~~M (~~B~~C). Fractions a and b were analysed by Tricine SDS-PAGE (~~C~~D) and gel filtration chromatography (~~D~~E) on a Superose 12 column calibrated with (1) dimeric (133 kDa), (2) monomeric (66 kDa) bovine serum albumin, (3) ovalbumin (44 kDa), and (4) carbonic anhydrase (29 kDa).

Figure 2. Enzymatic properties of tauricosin. The proteolytic ~~activity~~ activities on bovine caseinate (continuous line) and bovine casein-FITC ~~(~~(dotted line) ~~(U/mg, Caseinolytic Unit per mg of pure enzyme)~~ w~~as~~ere determined at different pH values at 37°C (A), and at different temperatures, at pH 5.5 (B). The casein-FITC assay was used at pH values ranging from 5.0 to 7.0 since it has a limited sensitivity at pH 4.5. Likewise, temperatures higher than 45°C were not tested because out of the temperature working range of the fluorimeter.

~~The effect of temperature on the caseinolytic activity (B) and t~~Thermal stability ~~(C)~~ was determined at pH 5.5 (C). 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).



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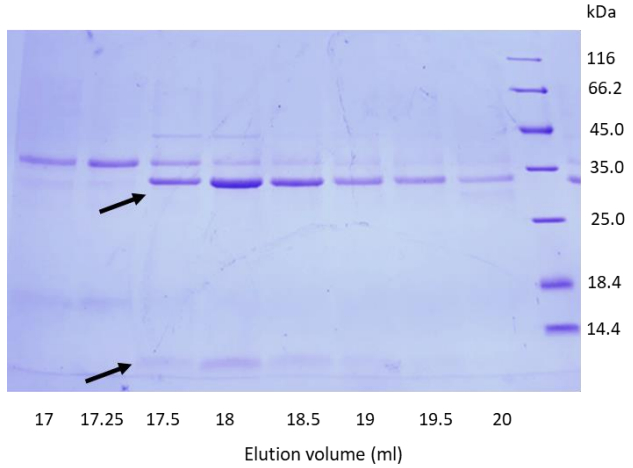

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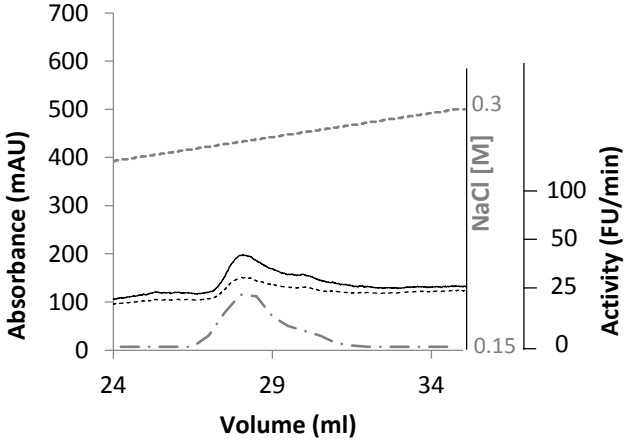

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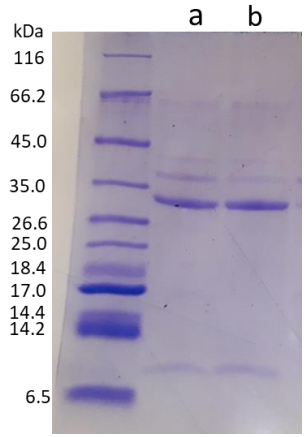

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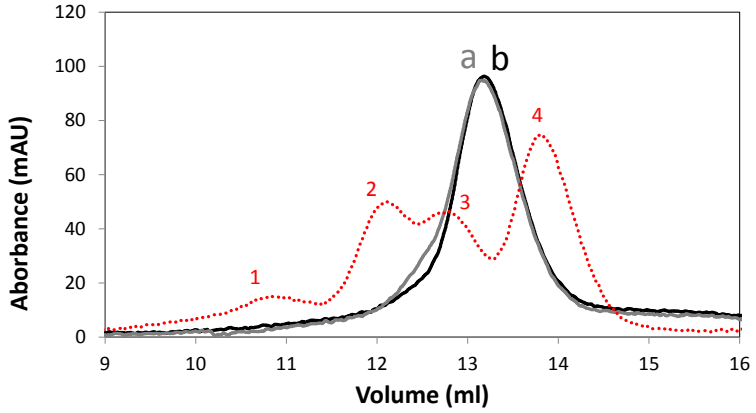

(E)

Figure 1.jpg

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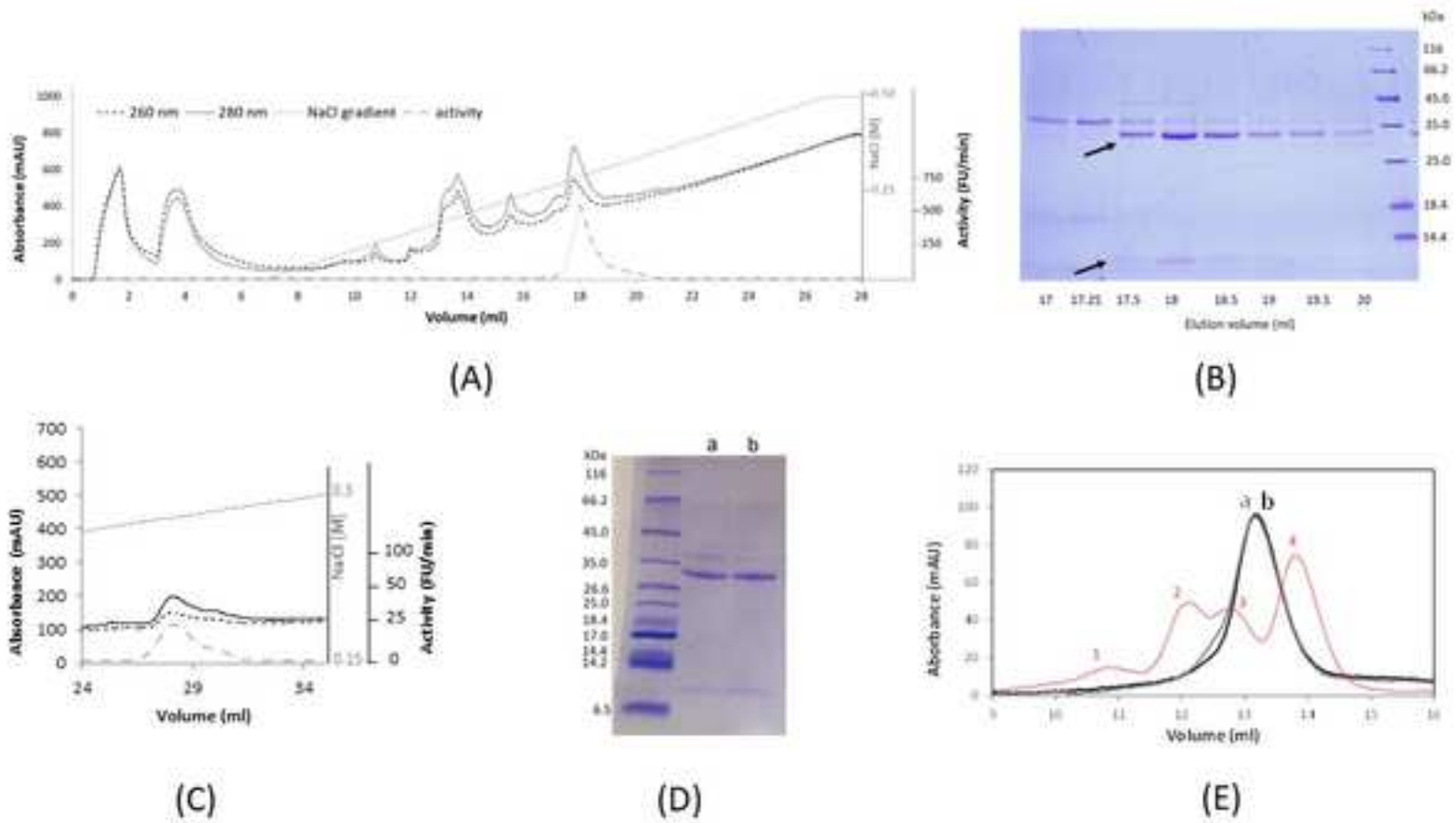

Figure 2.ppt

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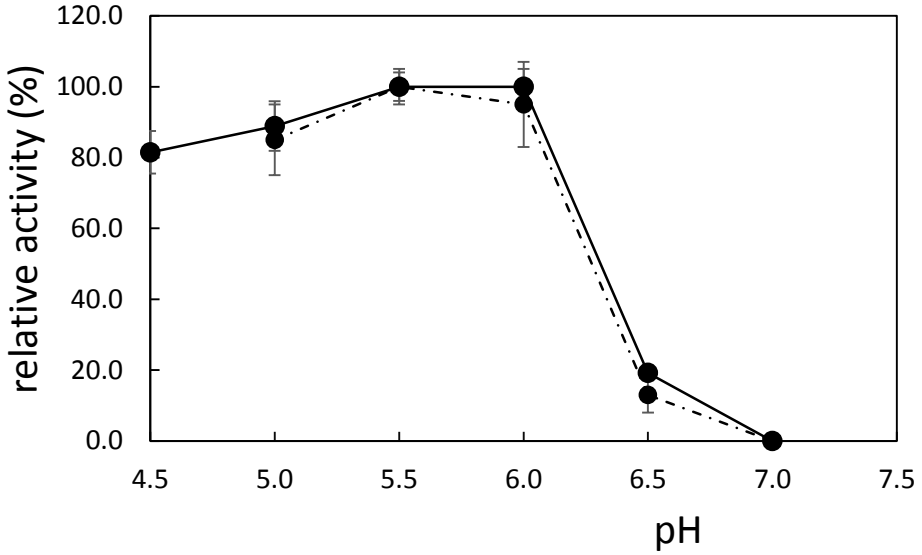
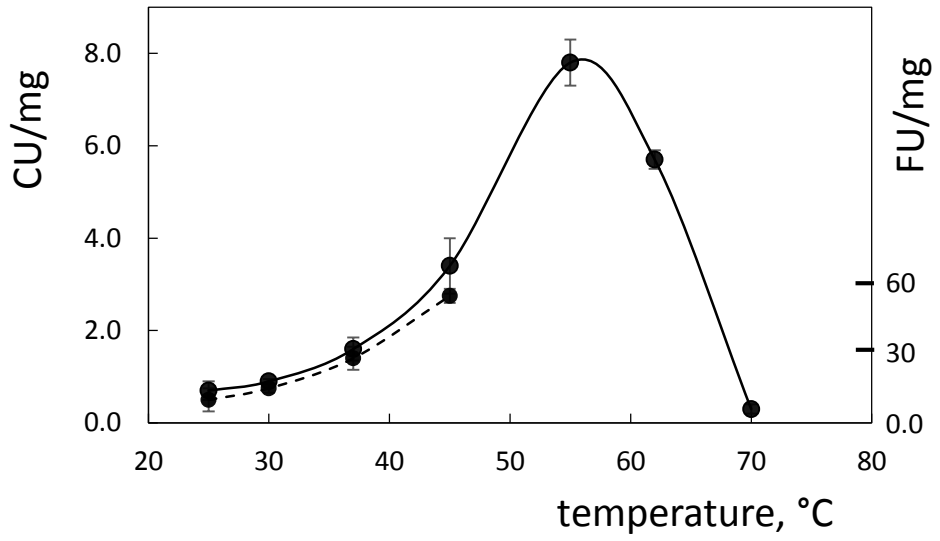


(A)



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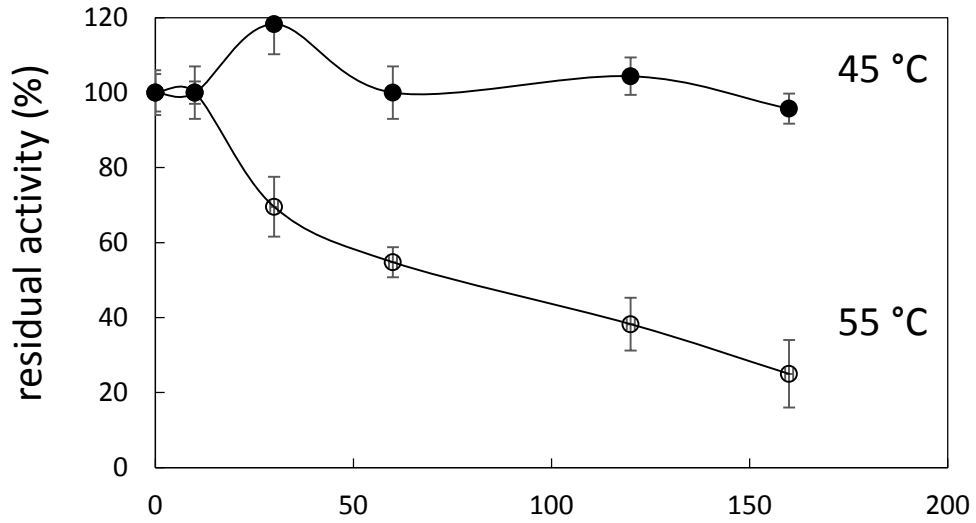

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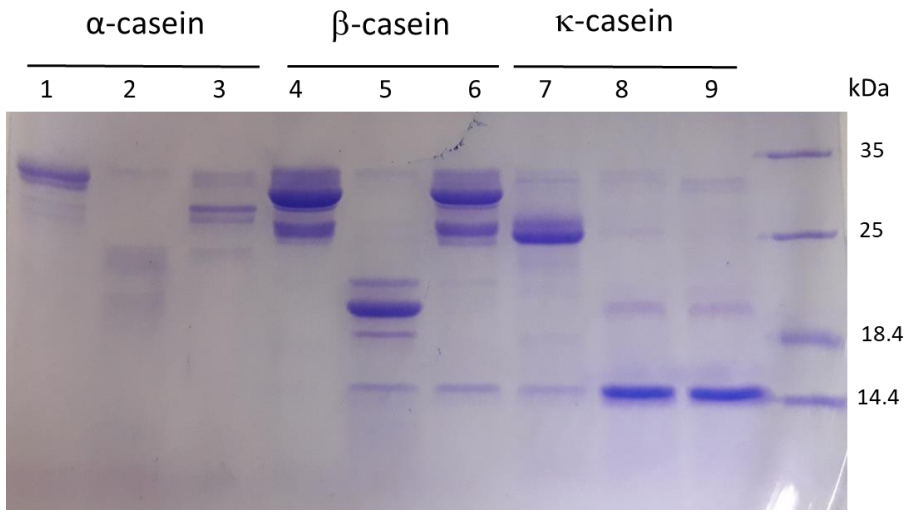

(D)

Figure 2.jpg

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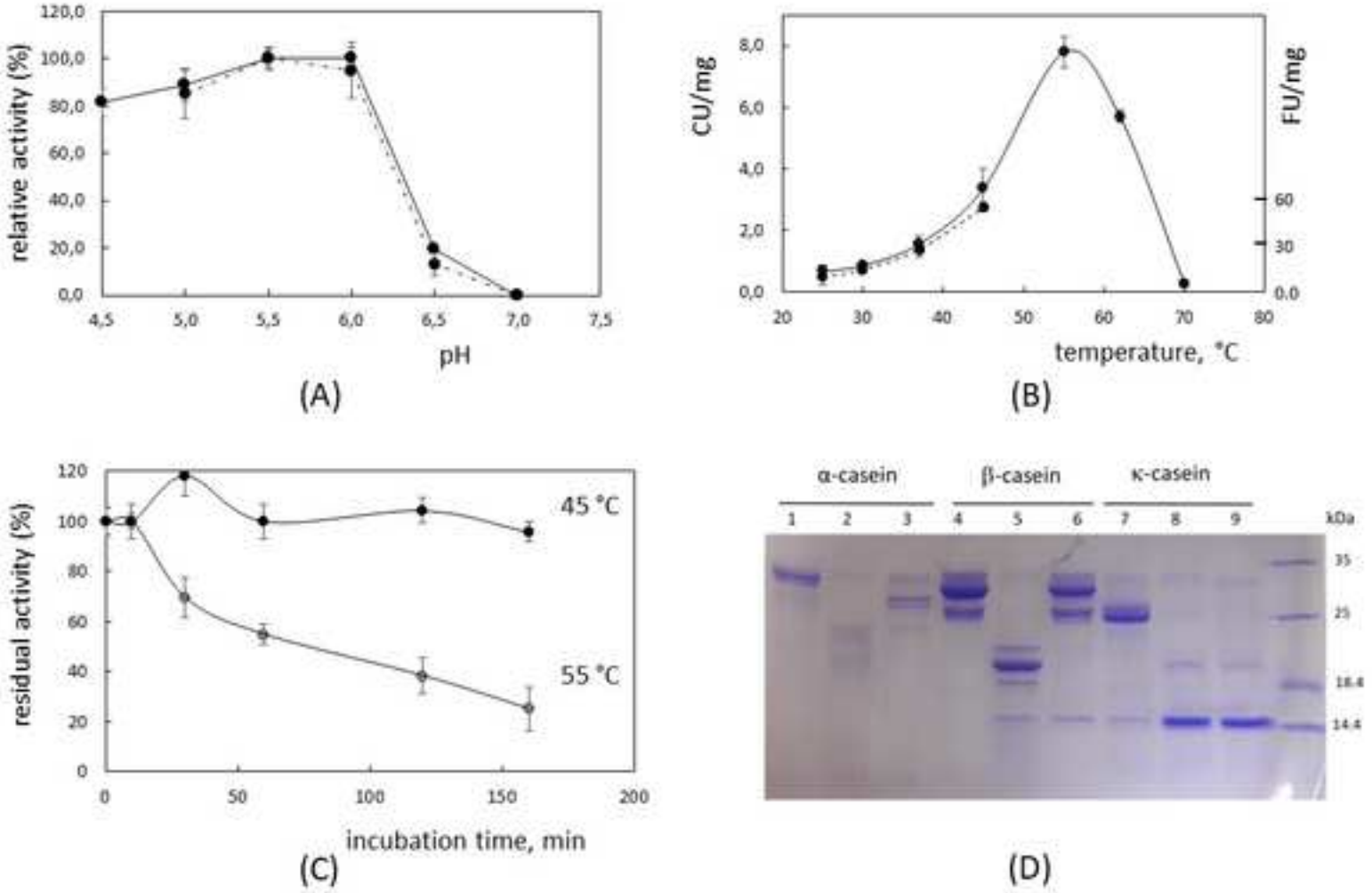


Table 1. Mineral composition (mg/100 g dry weight) of aqueous extract from cultivated and wild *Onopordum tauricum* (Willd.)

Minerals	Cultivated	Wild
Ca	220.2 ± 2.1[a]	190.5 ± 2.5[b]
P	844.5 ± 9.8[a]	761.3 ± 25.7[b]
Na	127.1 ± 1.0[a]	41.8 ± 2.3[b]
K	6871.2 ± 97.2[a]	5347.8 ± 192.8[b]
Mg	150.4 ± 2.3[a]	87.0 ± 1.2[b]
Zn	2.6 ± 0.2	2.9 ± 0.1
Mn	0.8 ± 0.0	0.8 ± 0.0

Different superscript letters mean significant differences ($p < 0.05$). Data are mean ± standard deviation of three experiments.

Table 2. Predicted model equations for the milk clotting time (MCT) and proteolytic activity (PA) responses.

Code	Model[1]	R^2	Adjusted R^2	F Ratio	*p*-Value
	Ewe's milk				
RE_st	MCT = 127.13 + 46.73 (pH) - 40.07 ([Ca^{2+}])	0.9556	0.8223	7.17	0.0662
RE_ct	MCT = 62.26 - 17.29 (T) + 17.83 (pH) − 13.19 ([Ca^{2+}])	0.9907	0.9629	35.57	0.0068*
	Goat's milk				
RE_st	MCT = 193.48 - 53.80 (T) - 52.43 ([Ca^{2+}])	0.9561	0.8245	7.26	0.0650
RE_ct	MCT = 75.73 + 24.18 (pH)	0.9345	0.7380	4.76	0.1132
	Casein fluorescein isothiocyanate				
RE_st	PA = 18.84 + 11.88 (pH)	0.9918	0.9673	40.47	0.0056*
RE_ct	PA = 14.76 + 11.29 (pH) + 3.59 $(T)^2$ + 2.77 $(pH)^2$	0.9968	0.9871	102.76	0.0014*

[1]Equations are reported using only the significant factors ($p < 0.05$). [Ca^{2+}], calcium chloride concentration. RE_st, reconstituted extract of wild *O. tauricum* flowers. RE_ct, reconstituted extract of cultivated thistles.

Table 3. Optimal conditions to minimize the milk clotting time (MCT) and the proteolytic activity (PA)

	T (°C)	[Ca^{2+}] (mM)	pH (Units)	Predicted	Desirability	Measured
				MCT (sec)		**MCT (sec)[1]**
Ewe's milk + RE_st	45	12	5.0	67	0.9158	70
Ewe's milk + RE_ct	45	12	5.0	39	0.9877	38
Goat's milk + RE_st	45	13	5.5	98	0.9863	95
Goat's milk + RE_ct	43	11	5.0	49	0.9871	50
				PA (FU/min)		**PA (FU/min)[1]**
Modified casein + RE_st	35	5	5.0	4.7	0.8680	4.6
Modified casein + RE_ct	39	5	5.0	2.9	0.9016	3.0

[1]average value of two replicates. [Ca2+], calcium chloride concentration. FU, fluorescence units. RE_st, reconstituted extract of *O. tauricum* flowers spontaneously grown up. RE_st, reconstituted extract of wild thistles. RE_ct, reconstituted extract of cultivated thistles.

Table 4. Predicted milk clotting time (MCT) and proteolytic activity (PA) at specific constraints.

	T (°C)	$[Ca^{2+}]$ (mM)	pH (Units)	Predicted variable
				MCT (sec)
Ewe's milk + RE_st	34 – 38	9.4 – 10.6	6.3 – 6.7	232 – 294
Ewe's milk + RE_ct	36 – 39	9.6 – 10.8	6.3 – 6.7	114 – 146
Goat's milk + RE_st	34 – 39	9.4 – 10.9	6.4 – 6.7	428 – 621
Goat's milk + RE_ct	36 - 38	9.7 - 10.6	6.3 - 6.7	129 – 167
				PA (FU/min)
Modified casein + RE_st	35 - 39	9.7 – 11.1	6.3 – 6.8	42.3 – 62.2
Modified casein + RE_ct	35 - 38	9.3 – 10.6	6.3 – 6.7	40.8 – 58.3

FU, fluorescence units

Table 5. Purification of tauricosine from cultivated *O. tauricum* flowers.

Step	**Total protein[a] (mg)**	**Total activity[b] (FU)**	**Specific activity (FU/mg)**	**Yield (%)**	**Purification factor**
Crude extract	45.8	101	2.2	100	-
AS fractionation	19.2	108	5.6	107	2.6
G25	13.3	98	7.4	~~91~~97	3.3
Mono Q	0.44	14	31.8	14	14.4

[a]starting from 15 g fresh flowers. [b]The enzymatic activity was assayed using casein-FITC as ~~the~~ substrate, at pH 5.5 and 37°C, as reported in Materials and Methods. FU, fluorescence units

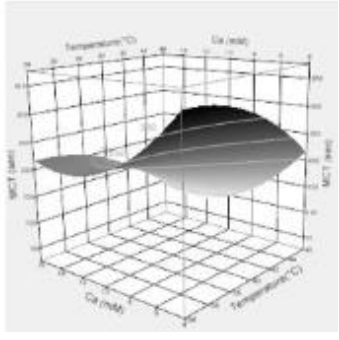
Graphical Abstract

Potentialities of aqueous extract from cultivated *Onopordum tauricum* (Willd.) as milk clotting agent for cheesemaking

Onopordum tauricum

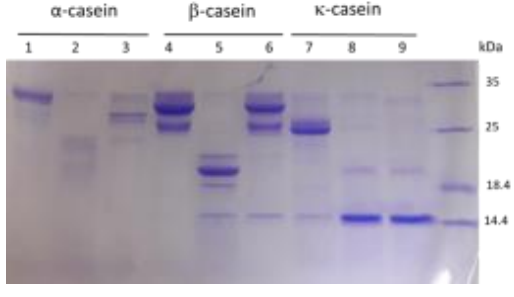
Wild vs cultivated

Milk Clotting activity



Purified tauricosin aspartic protease

Non-specific proteolytic activity



Optimal conditions:

Milk clotting activity: (43-45 °C; [Ca2+] = 11-13 mM)

Proteolytic activity: (35-39 °C; [Ca2+] = 5 mM)

CRediT authorship contribution statement

Roberta Foligni: Methodology, Validation, Formal analysis, Investigation, Writing-review and editing, Visualization. **Cinzia Mannozzi:** Methodology, Validation, Formal analysis, Investigation, Writing-review and editing, Visualization. **Massimiliano Gasparrini:** Methodology, Validation, Formal analysis, Investigation, Writing-review and editing, Visualization. **Nadia Raffaelli:** Conceptualization, Investigation, Resources, Writing–original draft preparation. **Federica Zamporlini:** Investigation. **Luis Tejada:** Writing-review and editing. **Cindy Bande-De León:** Investigation, Writing-review and editing. **Roberto Orsini:** Investigation. **Pamela Manzi:** Formal analysis, Investigation, Resources, Writing–original draft preparation. **Maria Gabriella Di Costanzo:** Investigation. **Mena Ritota:** Investigation. **Lucia Aquilanti:** Supervision, Project administration, Funding acquisition. **Massimo Mozzon:** Conceptualization**,** Resources, Writing–original draft preparation.

Conflict of Interest form

Ancona, 12.04.2022

To the Editor of

Food Research International

Dr., Massimiliano Gasparrini Ph.D.

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Dear Editor,

regarding the original manuscript titled "**Potentialities of aqueous extract from cultivated *Onopordum tauricum* (Willd.) as milk clotting agent for cheesemaking"** submitted for publication to Food Research International, on behalf of all the author,

I declare that **there is no conflict of interest**.

Kind regards

Massimiliano Gasparrini