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RESEARCH

Milk-Clotting and Proteolytic Properties of a Partially Purified Pepsin from Yellowfin Tuna (*Thunnus albacares*) and its Potential for Cheesemaking

Idalia Osuna-Ruíz[1] · Reyna Tiznado-Garzón[2] · Jesús Aarón Salazar-Leyva[1] · María de Lourdes García-Magaña[3] · Israel Benítez-García[1] · Jesús Martín Moreno-Hernández[4] · Isaura Bañuelos-Vargas[5] · Crisantema Hernández[6] · Emmanuel Martínez-Montaño[1,7]

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Abstract

The dairy industry traditionally uses aspartic proteases from bovine and microbial sources for cheese processing, but there is interest in alternative enzyme sources to supply the large global demand for rennet. This study investigated the biochemical characterization of a partially purified pepsin of yellowfin tuna with milk-clotting activity (MCA), in addition to evaluating its potential use in the manufacture of fresh cheese through texturometric and organoleptic characterization. The molecular weight of tuna pepsin was 36 kDa. The optimal temperature of MCA was recorded at 60 °C. Caseinolytic activity and MCA of tuna pepsin peaked at pH 6. A high MCA was recorded when 0.018% $CaCl_2$ was added to the reaction mixture. The electrophoretic profile of casein hydrolyzed by tuna pepsin showed a 14.8 kDa fragment, a molecular weight like that of para-κ-casein. Cheeses made from commercial chymosin and tuna pepsin showed a similar protein content (11% of total wet weight); however, cheese produced with pepsin had a higher fat content. The cohesiveness and chewiness of cheese made with partially purified pepsin were significantly higher than those made with chymosin. There were no differences in the sensory analysis between cheese made with both clotting agents; however, 60% of panelists preferred cheese made with chymosin. Pepsin extracted from yellowfin tuna stomach showed interesting milk-clotting properties with potential biotechnological use, which would contribute to the reusing and revalorization of byproducts derived from the tuna processing industry.

Keywords Milk-clotting activity · Fish pepsin · Rennet · Protease · Cheesemaking

Introduction

The key stage in cheese production is milk clotting. Animal rennin is commonly used as a milk-clotting agent, with bovine rennin as the agent traditionally used. Rennin is an enzymatic extract of the abomasum of lactating calves and its main component is chymosin (EC 3.4.23.4), an aspartic protease that specifically cleaves the Phe (105)-Met (106) peptide bond in the κ-casein chain and releases a glycomacropeptide and para-κ-casein, resulting in milk clotting (Nicosia et al., 2022).

✉ Emmanuel Martínez-Montaño
emartinez@upsin.edu.mx

1 Maestría en Ciencias Aplicadas, Unidad Académica de Ingeniería en Biotecnología, Universidad Politécnica de Sinaloa, 82199 Mazatlán, Sinaloa, Mexico

2 Ingeniería en Biotecnología, Unidad Académica de Ingeniería en Biotecnología, Universidad Politécnica de Sinaloa, 82199 Mazatlán, Sinaloa, Mexico

3 Laboratorio de Integral de Investigación de Alimentos, Tecnológico Nacional de México, Instituto Tecnológico de Tepic, 63175 Tepic, Nayarit, Mexico

4 Instituto Nacional de Investigaciones Forestales, Campo Experimental Valle de Culiacán, Agrícolas y Pecuarias, Sinaloa, Culiacán 80000, Mexico

5 Facultad de Ciencias del Mar, Universidad Autónoma de Sinaloa, 82000 Mazatlán, Sinaloa, Mexico

6 Centro de Investigación en Alimentación y Desarrollo, Av. Sábalo Cerritos S/N, A. C., Mazatlán, Sinaloa 82112, Mexico

7 Consejo Nacional de Ciencia y Tecnología, CONACYT, Ciudad de Mexico, México

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Extensive research has been conducted to date to develop enzyme agents as substitutes for chymosin due to the large global demand for milk-clotting agents, the limited access to bovine tissues in some geographic regions, and the search for clotting agents from non-animal sources due to cultural, religious, or health issues (Simmons et al., 2018). Rennet substitutes as recombinant chymosin and microbial milk coagulants have been the most extensively used milk-clotting agents in the dairy industry (Uniacke-Lowe & Fox, 2017); however, the use of microbial milk-clotting agents may involve disadvantages such as high levels of non-specific proteases, that hydrolyze others peptide bonds than Phe_{105}-Met_{106} bond, which may confer a bitter taste to cheese after storage (Ismail et al., 2019). Moreover, special attention has been paid to the use of proteolytic extracts from different parts of plants (e.g., seeds, latex, rhizomes, leaves, flowers, fruits) for cheese production (Ben Amira et al., 2017), given the good results obtained in terms of high yield of curd and good texture and taste of cheese (Mazorra-Manzano et al., 2018a).

Natural clotting agents other than calf rennin are proteases (mainly aspartic endopeptidases) obtained from marine animals such as fish and crustaceans, which have interesting catalytic properties including their capacity to function efficiently at low temperatures (Homaei et al., 2016; Osuna-Ruiz et al., 2019). These properties make them potentially suitable for use in the dairy industry because processes involving low temperatures are associated with the reduction of bacterial contamination and undesirable chemical reactions (Pereira & Fernández-Gimenez, 2017a). Despite the above, studies are scarce even with the catalytic advantages associated with proteases from marine animal species. Only proteases with milk-clotting activity have been reported for marine mammals such as the harp seal (*Pagophilus groenlandicus*; Shamsuzzaman & Haard, 1983), crustaceans such as the pelagic red crab (*Pleuroncodes planipes*; Pereira & Fernández-Giménez, 2017b), Argentinian red shrimp (*Pleoticus muelleri*; Pereira & Fernández-Giménez, 2017b), *Munida* prawns (Rossano et al., 2011), and teleost fish such as the Atlantic cod (*Gadus morhua;* Brewer et al., 1984), dogfish (*Scyliorhinus canicula*; Guerard & Le Gal, 1987), bigeye tuna (*Thunnus obesus*; Tavares et al., 1997), and Chilhuil sea catfish (*Bagre panamensis*; Osuna-Ruiz et al., 2019).

One of the main concerns when novel processes or non-conventional ingredients are considered for use in cheese production are the potential rheological and sensory properties of products (Soodam et al., 2014). Thus, cheeses made with plant coagulants using raw bovine, ovine, and/or caprine milk are very appreciated worldwide due to their textures and organoleptic characteristics (Feijoo-Siota & Villa, 2011). Moreover, some of the plant proteases that have been tested in cheese production result in yield, textures, and flavors that markedly differ from the controls made with traditional clotting agents, which is due to excessive proteolytic activity that produces bitter peptides (Mazorra-Manzano et al., 2013a, b; Nájera-Domínguez et al., 2022); however, as the taste preferences vary among individuals from different societies or cultures, the use of alternative milk-clotting agents such as plant proteases (Nicosia et al., 2022) or marine-origin proteases (Pereira & Fernández-Gimenez, 2017a) in the manufacture of cheese could offer a greater diversity of commercially interesting products. But, despite the interesting catalytic properties reported for enzymes from marine organisms, only few studies have reported texture and sensory analyses of cheese made with enzymes of marine sources (Tavares et al., 1997; Rossano et al., 2011; Pereira & Fernández-Giménez, 2017b). Moreover, the use of non-conventional enzyme sources (e.g., proteases from marine organisms) to elaborate cheese may impart them different tastes or textures as well as improve their functional properties (Sun-Waterhouse et al., 2014). Cheeses produced with enzyme extracts from the Argentinian prawn *P. muelleri* were considered very acceptable by tasters for texture in mouth and smell-taste (Pereira & Fernández-Giménez, 2017b). In contrast, cheese made with gastric enzymes of the bigeye tuna (*T. obesus*) were less preferred based on taste, texture, and overall preference than cheese made with rennet (Tavares et al., 1997). The role of texture in cheese acceptance is difficult to define because the taste cannot be decoupled from texture when consumers evaluate cheese; similarly, visual appearance affects the perception of both taste and texture (Foegeding & Drake, 2007). However, it has been reported that the sensory quality of cheese made with milk-clotting enzymes of marine origin is similar to the one of cheese manufactured with chymosin from calf and microbial sources (Shamsuzzaman & Haard, 1983; Tavares et al., 1997), in spite of the changes in the micro-structure and the generation of hydrolysis products (Rossano et al., 2005).

The objective of this study was to characterize the milk-clotting properties of pepsin from yellowfin tuna (*Thunnus albacares*) and assess their potential for use in the elaboration of miniature fresh cheese through the texturometric and organoleptic characterization of processed cheeses.

Material and Methods

Biological Samples

A sample of ten stomachs of juvenile yellowfin tuna *Thunnus albacares* were kindly donated by a fishery process plant (Ingeniería Industrial del Pacífico, S.A.) and transported to the laboratory, where it was stored at −20 °C to be used the next day.

Partial Purification of Stomach Proteolytic Enzymes

Enzyme extraction and partial purification were performed according to Osuna-Ruiz et al. (2019). In brief, frozen stomachs were cleaned of solid content (if any), cut in small pieces, and immediately homogenized with cold distilled water using a 1:3 (w/w) mass/water ratio. The homogenate was centrifuged at 8000 × g for 20 min at 3 °C and the supernatant was collected and pooled. Then, the supernatant (enzyme crude extract) was precipitated sequentially using ammonium sulfate $(NH_4)_2SO_4$ to reach 20–70% saturation (Burgess, 2009). Afterward, and with the aim of activating stomach pepsinogens (Brewer et al., 1984), the precipitate recovered from ammonium sulfate precipitation was resuspended with 10 mM Gly-HCl buffer at pH 3.0 and dialyzed for 24 h against two changes of 2 L of acid buffer at 4 °C using a dialysis membrane for 12–14 kDa molecular weight cut-off (MWCO; Fisherbrand™, Fisher Scientific). The dialyzed fraction (partially purified pepsin) was aliquoted and stored at −20 °C until used for enzyme analysis and miniature cheese elaboration.

Enzyme Assays and Soluble Protein Determination

Caseinolytic activity was determined using 1% casein from bovine milk dissolved in 0.01 M Tris–HCl, pH 6.5, at 30 °C, as substrate (Walter, 1984). In brief, 500 µL of protein substrate was mixed with 20 µL of enzyme extract and incubated for 10 min at 30 °C. After incubation, the reaction was stopped by the addition of 500 µL of 30% trichloroacetic acid. Then, the mixture was centrifuged at 16,000 × g for 5 min at 4 °C. Finally, the absorbance of the supernatant was measured at 280 nm. Specific enzyme activity for caseinolytic activity was expressed as µmol tyrosine formed per minute calculated with the following formula: enzyme activity (U/mg soluble protein) = [ΔAbs × final reaction volume (mL)]/[MEC × time (min) × enzyme volume (mL) × mg soluble protein], where ΔAbs is the increment in absorbance at 280 nm and MEC is the molar extinction coefficient for tyrosine (0.005 mL μg^{-1} cm^{-1}).

Soluble protein concentration (in mg/mL) in both enzyme sources was determined according to the modified method of Bradford (1976) using bovine serum albumin as protein standard.

Milk-clotting Activity Assay

Milk-clotting activity (MCA) was determined according to the method described by Mazorra-Manzano et al. (2013a) and modified by Osuna-Ruiz et al. (2019). Briefly, 10 mL of pasteurized low-fat milk (< 1% total fat) containing 0.022% $CaCl_2$ was mixed with 500 µL of the enzyme source using an enzyme/substrate (E/S) ratio of 5% and incubated at a constant temperature of 30 °C. Afterward, the time measured between the addition of the enzyme source to milk and the initial appearance of visible signs of coagulation was recorded. One milk-clotting unit (Soxhlet unit) was defined as the amount of enzyme source (mg soluble protein) required for clotting 1 mL of milk in 40 min under the assay conditions, calculated with the following equation: Soxhlet unit (SU)/mg protein = (M × 2400)/(E × t), where M is substrate volume (mL), E is the amount of enzyme (mg soluble protein), and t is clotting time (s).

The effect of temperature on the MCA of partially purified pepsin was determined by incubating the reaction mixture at different temperatures (25–70 °C). The effect of pH on MCA of partially purified pepsin was evaluated by adjusting the milk pH to 6.0, 6.5, and 7.0 with 0.1 mM NaOH or 0.1 mM HCl solution, as needed (Belenkaya et al., 2018; Slamani et al., 2018). Also, the effect of calcium chloride ($CaCl_2$) diluted in milk (0.011, 0.015, 0.018, and 0.022%) on the MCA was also evaluated.

Electrophoretic Analysis

The molecular weight of partially purified pepsin was determined with sodium dodecyl sulfate–polyacrylamide electrophoresis gel (SDS-PAGE) under polyacrylamide (PAA) reducing conditions (Laemmli, 1970) and using a staking gel with 5% PAA and a resolving gel with 10% SDS and 12% PAA. For protein-band analysis, a volume of enzyme extract was mixed with sample buffer (0.5 M Tris–HCl at pH 6.8, 10% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.05% bromophenol blue), heated at 95 °C for 5 min, then cooled and loaded into the gel (Ríos-Herrera et al., 2019). Samples loaded (80 µg of protein per well) into the gel were subjected to electrophoretic separation applying 70 V and 120 V for running of samples in staking and resolving gels, respectively. After electrophoresis, gels were stained by immersing them in a staining solution containing 0.1% Coomassie brilliant blue R-250, 50% methanol, and 10% acetic acid. Finally, stained gels were washed with destaining solution (50% methanol and 10% acetic acid) for 4 h.

Also, the proteolytic effect of partially purified pepsin on casein was analyzed by SDS-PAGE according to Mazorra-Manzano et al. (2013a). In brief, 0.45 mL of 1% casein from bovine milk diluted in 100 mm sodium phosphate buffer (pH 6.8) was incubated with 0.05 mL of diluted partially purified pepsin or a microbial rennet (Cuamix™, CHR-HANSEN A/S, Denmark) at 35 °C at different times over 60 min. After incubation, the enzyme reaction was stopped by adding 0.5 mL of sample buffer and immediately heating at 95 °C for 10 min; finally, the mixture was analyzed by SDS-PAGE as described above.

Elaboration of Miniature Fresh Cheese

Miniature fresh cheese was prepared using a microbial rennet (Cuamix™, CHR-HANSEN A/S, Denmark; ~ 280 international milk clotting units (IMCU)/mL) as positive control and yellowfin tuna partially purified pepsin extract as coagulants. A volume of 200 mL of milk was poured in a beaker and pasteurized using the standard holder method by heating the milk at 63 °C for 30 min (Jay, 1998); then, the milk was cooled to 30 °C, added with 0.022% $CaCl_2$, and mixed. The pH value registered in milk was 6.5. Each coagulant was separately added to milk in triplicate; the mixture was stirred for 2 min and incubated at room temperature. In the case of partially purified pepsin, an E/S ratio of 1% was used; for commercial rennet, an E/S ratio of 0.0125% was used, as recommended by the manufacturer. Once the milk was completely coagulated, it was cut in small squares of approximately 9 cm^2 and left at room temperature for 20 min to allow the serum to drain. Curd portions were transferred to 2-inch polyvinyl chloride molds and kept at 22 °C. After 24 h, these masses of miniature fresh cheese were used to calculate cheese yield as follows: Cheese yield (%) = (initial milk mass/fresh cheese mass) × 100. Finally, the cheese was stored at −20 °C until used for subsequent analysis.

Proximate Analysis

Fresh cheeses were analyzed for moisture, protein, fat, and ash contents following the official methods of AOAC (2010). Moisture content was determined gravimetrically by drying the sample in an oven at 105 °C for 12 h. A sample of fresh cheese was previously freeze-dried to determine protein content (micro-Kjeldahl method; N × 6.25), fat content (micro Foss Soxtec Avanti 2050 Automatic System), and ash content (calcination at 550 °C for 12 h).

Texture Profile Analysis of Miniature Cheese

The texture profile was determined according to Torres-González et al. (2015), with some modifications. Hardness, adhesiveness, cohesiveness, and chewiness were assessed using a texturometer (TA.XT plus C, Stable Micro Systems, UK) with the "Volodkevich bite jaws" attachment with double compression at 95%. Hardness was recorded as the maximum force during the first compression cycle. Cohesiveness.

was defined as the ratio of positive force area under the second and first compression cycles; adhesiveness is the negative area observed between the first and second cycles; finally, chewiness was calculated as the product of hardness × cohesiveness × springiness.

Sensory Analysis of Miniature Cheese

An affective sensory analysis was applied as recommended by Pedrero and Pangborn (1989), using an evaluation form with an unstructured 0-to-13 scale, with the participation of 30 untrained judges; aroma, color, flavor, and texture were evaluated; in addition, the judges were asked to indicate which type of cheese they prefer.

Statistical Analysis

Data are reported as mean ± standard deviation. All percent data were transformed to arcsine of the square root prior to the statistical analysis. The significance of the differences in the proximate composition of miniature cheese elaborated with microbial rennet or partially purified pepsin was assessed with a *t*-Student test. The data set was tested for normality and homogeneity of variance using the Kolmogorov–Smirnov and Levene tests, respectively. The effects of the E/S ratio and $CaCl_2$ concentration on the MCA of partially purified pepsin were separately analyzed using a one-way ANOVA. When significant differences were found, a Tukey's honestly significant difference (HSD) test was performed. Statistical differences were considered significant at $P < 0.05$. All statistical analyzes were performed using the SigmaPlot software, version 12.0 (Systat Software, Inc.; Erkrath, Germany).

Results and Discussions

Partial Purification of Stomach Proteolytic Enzymes

A protein fraction with a molecular weight of approximately 36 kDa (Fig. 1, lane 5) was partially purified from yellowfin tuna stomach tissue. This molecular weight is similar to the one reported for active pepsin purified from the stomach of albacore *Thunnus alalunga* (MW ≈ 36.8 kDa; Nalinanon et al., 2010a) and skipjack tuna *Katsuwonus pelamis* (MW ≈ 33 kDa; Nalinanon et al., 2010b). As observed from the SDS-PAGE, the partial purification process was efficient despite not having used any chromatographic method, as most of the protein fractions greater than 36 kDa disappeared. This may have been resulted from the activation of pepsinogens to pepsin after the pH was adjusted to 3.0 during the dialysis process, with the consequent hydrolytic activity of pepsin on other high-molecular-weight proteins (autolysis). Then, the dialysis process removed part of the protein fractions of molecular weights below 12 kDa, reflected in the lower soluble-protein concentration from 5.8 to 1.1 mg/mL recorded for the crude and partially purified extract, respectively. Besides, the milk-clotting activity showed a 14.1-fold increase, after the partial purification process (Table 1). Furthermore, the milk-clotting activity/

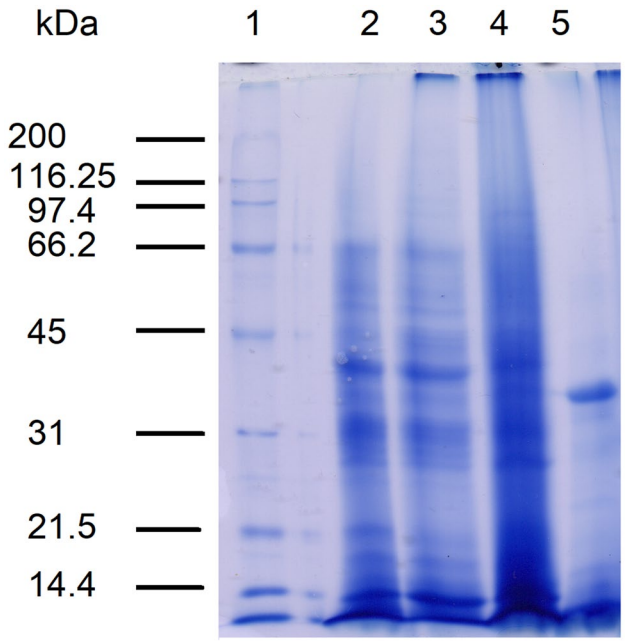

Fig. 1 SDS-PAGE of partially purified proteases of *Thunnus albacares*. (1) Molecular weight marker; (2) enzyme crude extract; (3) supernatant from 20% saturation; (4) precipitated fraction after 70% saturation; and (5) partially purified pepsin after dialysis processes

specific caseinolytic activity (PA) ratio (MCA/PA ratio) increased from 0.11 to 0.99 was observed when the tuna stomach crude extract was subjected to partial purification. An improvement of MCA/PA ratio after an enzyme purification process is the result of a high enzyme recovery with high specificity to hydrolyze κ casein in the purified fraction (Uniacke-Lowe & Fox, 2017). Non-specific enzymes show excessive proteolytic activity, mainly caseinolytic, which is considered an extremely negative factor in cheesemaking because proteolysis products are loss in whey (Belenkaya et al., 2018). Thus, a high MCA/PA ratio is consistent with desirable curd characteristics such as high yield and good texture (firmness) and flavor (not bitter) of cheese (Mazorra-Manzano et al., 2018b). In the present study, MCA/PA ratio of tuna pepsin (0.99) was low compared to MCA/PA ratio of 209 determined for microbial rennet (Cuamix™), but similar to MCA/PA ratios of chymosins reported for different mammal species which vary between 0.9 and 243 (Belenkaya et al., 2018; Nicosia et al., 2022). Reports of MCA/PA ratio values for proteases from aquatic organisms are too scarce. For example, a crude preparation of gastric proteases from harp seal (*P. groenlandicus*) registered a low MCA/PA ratio of 0.26; even so, a cheddar cheese was prepared using such gastric extract with a yield of cheese like those using conventional milk-clotting enzymes (Shamsuzzaman & Haard, 1983). Also, a pepsin extracted from the gastric mucosa of dogfish (*S. canicula*) stomach registered a low MCA/PA ratio of 3.57 compared to 64.18 obtained with chymosin (Guerard & Le Gal, 1987); however, thermal properties (optimum temperature for MCA is around 35 °C), as well as the response to pH variations (MCA is maintained at a fairly constant level at pH as high as 6.8) of dogfish enzyme, make it reasonably suitable to being a rennet substitute in the food industry (Guerard & Le Gal, 1987). Even so, aspartic pepsins from marine mammals have been resulted suitable rennet substitutes for cheese production (Brewer et al., 1984; Guerard & Le Gal, 1987; Tavares et al., 1997) despite their MCA/PA ratios being lower than chymosin or microbial rennet.

The enzymatic activity of milk-clotting agents from marine animal tissues such as teleost fish (Osuna-Ruiz et al., 2019) has been associated with aspartic proteases, mainly pepsin, which possess catalytic properties different from those of mammalian pepsin, including higher optimal pH and Michaelis constants (Km) (Guerard & Le Gal, 1987), as well as lower optimal temperatures and thermal

Table 1 Caseinolytic and milk-clotting activities of crude extracts and partially purified pepsin from *Thunnus albacares*

Purification steps for caseinolytic activity	Total volume (mL)	Soluble protein (mg protein/mL)	Specific activity (U/mg protein)	Total activity (U[a])	Purity (Fold)	Recovery (%)
Crude extracts	206.0	5.8	158.0	187,987	1.0	100
Partially purified pepsin	40.5	1.1	250.7	10,686	1.6	5.7

Purification steps for milk-clotting activity (MCA)	Total volume (mL)	Soluble protein (mg protein/mL)	MCA (SU/mg protein)	Total MCA (SU[b])	Purity (Fold)	Recovery (%)	MCA/PA[c] ratio
Crude extracts	206.0	5.8	17.7	21,054	1.0	100	0.112
Partially purified pepsin	40.5	1.1	249.5	10,636	14.1	50.5	0.995

[a]one unit of enzyme activity was expressed as μmol of tyrosine formed per minute using casein (pH 6.5) as protein substrate

[b]one unit of milk clotting activity expressed as the amount (mg) of crude extract or partially purified pepsin needed to coagulate 1 mL of milk at 30 °C

[c]Proteolytic activity (PA) = Specific caseinolytic activity (U/mg protein)

stability (Osuna-Ruiz et al., 2019). Despite the satisfactory results obtained with the partial purification process in terms of increased milk-clotting activity and the MCA/PA ratio, for future studies, we strongly recommend a chromatographic purification process and the subsequent characterization of pepsin and pepsin isoforms in stomach tissues of *T. albacares*.

Optimal MCA Temperature

The optimal MCA temperature recorded for partially purified tuna pepsin was 60 °C (Fig. 2a), which is higher than the optimal temperatures reported for the hemoglobinolytic activity of pepsin from this and other tuna species (De La Parra et al., 2007; Norris & Mathies, 1953) of 45 °C, approximately. This difference may be due to the addition of calcium chloride to the milk used in the MCA tests, which may have improved pepsin thermostability. There are previous reports of a positive relationship of the calcium concentration added (mainly as $CaCl_2$) and process temperature with the clotting rate (equivalent to an increase in MCA) and curd firmness during cheesemaking with commercial rennet (Nájera et al., 2003), as well as a negative relationship with the increase in pH. Apparently, the tuna pepsin used in our study display a similar behavior.

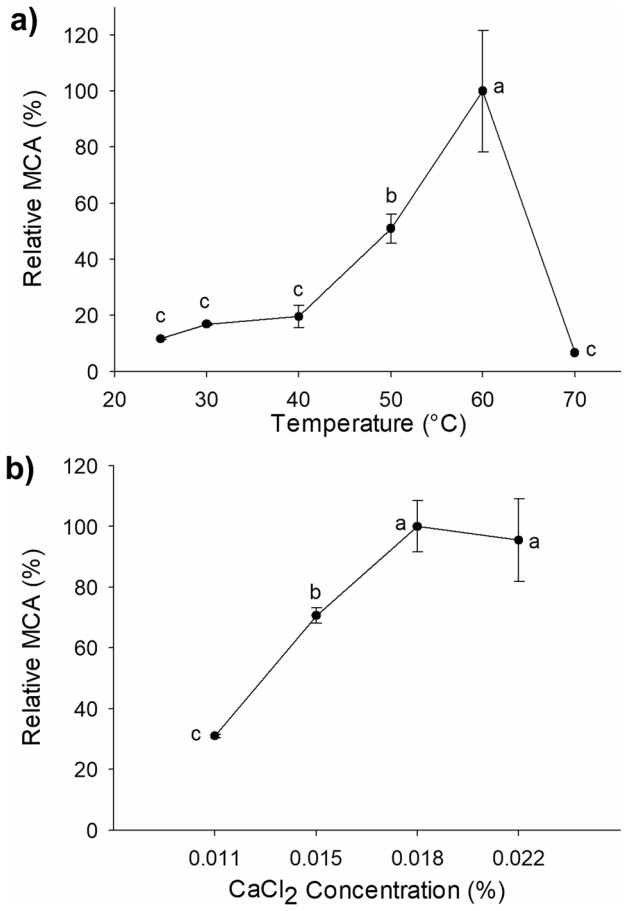

Fig. 2 Effect of **a** temperature (in °C) and **b** concentration of $CaCl_2$ on the relative milk clotting activity (MCA; mean ± SD) of partially purified pepsin of *T. albacares*. Values with different letter are significantly different ($P < 0.05$)

On the other hand, unlike pepsin isolated from the stomachs of terrestrial mammals, those extracted from fish show broad variations in catalytic properties. Thus, the optimal temperature and thermal stability of the proteolytic activity largely depend on the ambient temperature at which fish thrive because fish are ectothermic organisms, i.e., unable to regulate their body temperature. For the above, the use of pepsin from fish living in temperate and cold water as chymosin substitutes for cheese elaboration is appealing, as these pepsins can coagulate milk at low temperatures with a higher efficiency relative to calf chymosin (Osuna-Ruiz et al., 2019; Zhao et al., 2011). In the present study, the optimum temperature of tuna pepsin resulted higher than others aspartic proteases extracted from the digestive tissues of marine fish or crustaceans (Tavares et al., 1997; Rosanno et al., 2011; Osuna-Ruiz et al., 2019). Nevertheless, high optimum temperature or thermal resistance of milk-clotting enzymes may enhance functional properties including texture development and melting behavior in cheese, especially scalded varieties (Hayaloglu et al., 2014). In addition, the milk-clotting activity of tuna pepsin resulted similar between 25 and 40 °C (Fig. 2b), this property is rare in aspartic proteases from other marine fish in which milk-clotting activity decay significantly in temperatures below 40 °C (Guerard & Le Gal, 1987; Osuna-Ruiz et al., 2019). Therefore, in further studies, elaboration of fresh cheese using tuna pepsin at a temperature below 35 °C could be assayed. The latter because when chymosin or plant proteases have been used to coagulate milk at low or mild temperatures, benefits such as a better efficiency of the gelation process and a reduction of non-specific proteolysis were observed (Ben Amira et al., 2017).

Effect of Added Calcium Chloride Concentration on MCA

The addition of $CaCl_2$ at ~ 0.2 g/L (~ 1.8 mM Ca) to milk is common commercial practice, especially if cheese milk displays poor rennet coagulation and curd-forming characteristics (Guinee, 2007). The poor coagulation of milk is due to various factors related to milk production and handling, which can reduce ionic or micellar calcium levels that increase the dissociation of casein from micelles to serum, making casein susceptible to hydrolysis by milk proteinases. Consequently, any soluble peptides produced will not contribute to curd formation, thus lowering curd yield and affecting cheese firmness (Guinee, 2007).

Besides, some industrial milk-processing processes (e.g., pasteurization) adversely affect the solubility and cause the irreversible precipitation of milk calcium ions and salts (Belenkaya et al., 2018); thus, the addition of $CaCl_2$ to compensate for these effects is recommended. Therefore, the addition of calcium ions promotes and improves enzymatic milk clotting, so its inclusion in the cheese making process is important. For the above, the present study assessed the effect of $CaCl_2$ at concentrations ranging from 0.011 to 0.022% (0.99 to 1.98 mM) and found that the addition of 0.018% $CaCl_2$ promotes a higher milk-clotting activity when using tuna pepsin as a coagulant agent (Fig. 2b).

Optimal MCA pH

Both caseinolytic and milk-clotting activity (Fig. 3) decrease significantly when the pH of the substrate increases from 6 to 7. However, milk-clotting activity is still recorded at pH 7 (accounting for 5% of the MCA measured at pH 6); this is interesting when compared with the activity reported for pepsin from sheep stomach, which failed to clot milk at pH 6.8 (Slamani et al., 2018). Tavares et al. (1997) reported that pepsin from the bigeye tuna *Thunnus obesus* and calf rennet display a similar activity over a pH range from 5.5 to 6.3; however, unlike calf rennet, *T. obesus* pepsin is less prone to losing its milk-clotting activity at pH values above 6.4. Also, for pepsin from the dogfish *Scyliorhinus canicula*, the milk-clotting activity remains relatively constant even at pH 6.8 (Guerard & Le Gal, 1987), demonstrating the stability of the clotting activity of teleost fish pepsin even at near-neutral pH.

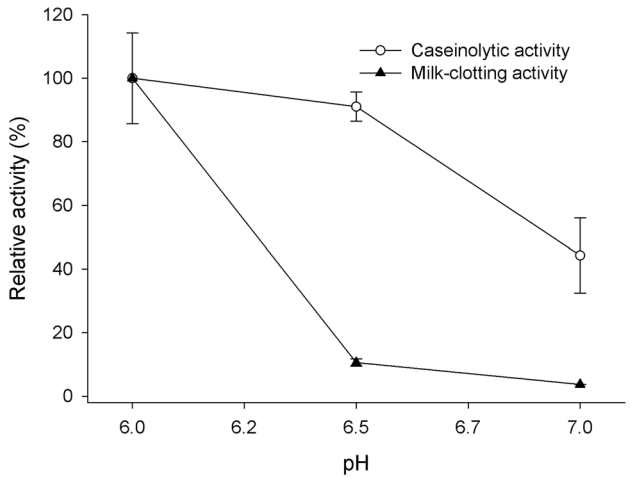

Fig. 3 Effect of pH on relative enzyme activity (mean ± SD) of partially purified pepsin of *T. albacares* using casein (caseinolytic activity) and low-fat milk (milk clotting activity) as substrates. Temperature of reaction mixture was 30 °C

Electrophoretic Monitoring of Milk-clotting Activity of Partially Purified Pepsin

Figure 4 shows the electrophoretic analysis of the protein fractions in a 1% non-hydrolyzed casein solution dissolved in a phosphate buffer (0 min) and every 10 min after incubation together with partially purified tuna pepsin (Fig. 4a) or with a microbial rennet (Cuamix™; Fig. 4b). Non-hydrolyzed casein shows several protein fractions with molecular weights in the range of 27 to 41 kDa. According to González-Velázquez et al. (2021), the molecular weight

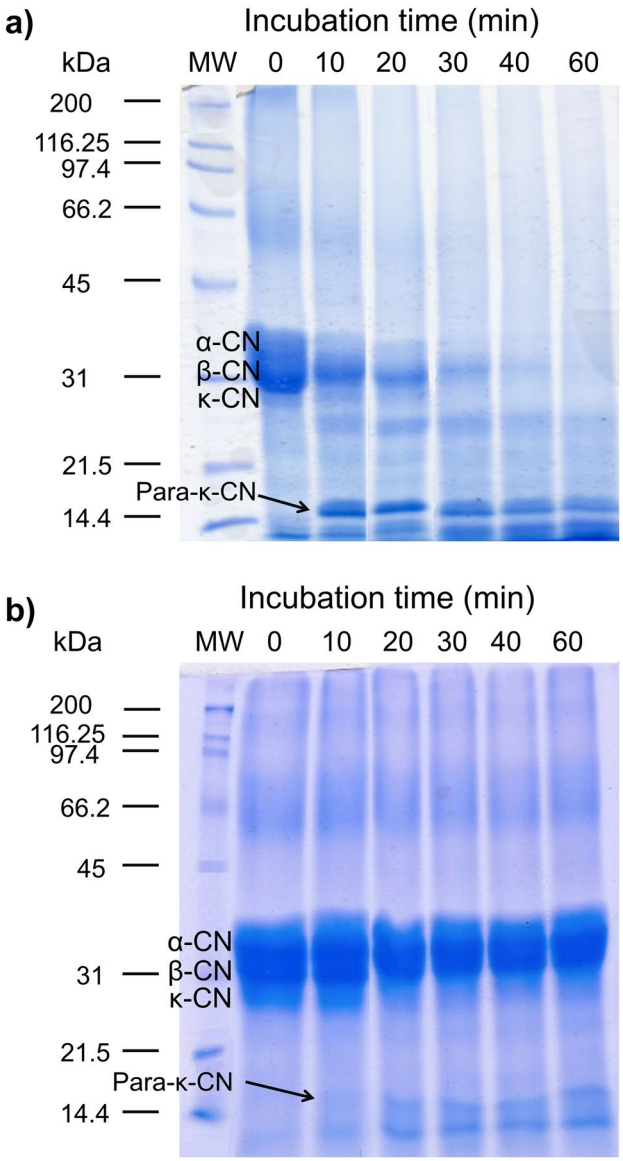

Fig. 4 SDS-PAGE analysis of hydrolysis of casein (pH 6.8, 35 °C) using partially purified pepsin of *T. albacares* (**a**) or a microbial rennet (Cuamix™) (**b**) at 0-, 10-, 20-, 30-, 40- and 60-min. MW, molecular weight marker; α-CN, α-casein; β-CN, β-casein; κ-CN, κ-casein; Para-κ-CN, para-κ-casein

Table 2 Yield and proximate composition of miniature cheese produced with microbial rennet and partially purified pepsin from *T. albacares*

Coagulant	Cheese yield (%)	Proximate composition of miniature cheese (%)				
		Moisture	Protein	Fat	Ash	NFE
Microbial rennet	15.1 ± 0.8	69.3 ± 1.5[a]	11.0 ± 0.4	13.3 ± 0.6[b]	1.7 ± 0.1[b]	5.0 ± 0.6
Partially purified pepsin	14.2 ± 1.4	66.3 ± 0.3[b]	10.8 ± 0.3	15.4 ± 0.2[a]	1.9 ± 0.1[a]	5.3 ± 0.2

Values with different superscript letters within a same column are significantly different ($P < 0.05$)

of α-, β-, and κ-caseins is approximately 33 kDa, 28 kDa, and 26 kDa, respectively, matching with some of the bands recorded for non-hydrolyzed casein.

When the casein was incubated with microbial rennet (Cuamix™) only an evident hydrolysis of κ-casein, as expected (Sato et al., 2018), into low molecular weight products like para-κ-casein was observed (Fig. 4b). The non-extensive hydrolysis of caseinates is the result of the high specificity of microbial rennet to hydrolyze κ-casein mainly at the Phe_{105}-Met_{105} position and the realizing of para-κ-casein (Moreno-Hernández et al., 2017). Thus, the high MCA/PA ratio of microbial rennet found in the present study is associated to its high specificity of hydrolyzed κ-casein. Instead, the incubation of diluted casein with partially purified tuna pepsin after 10 min (Fig. 4a) produces a rapid hydrolysis of casein fractions, predominantly α- and κ-caseins and to a lesser extent β-caseins, and the appearance of three protein fractions with a molecular weight of approximately 24 kDa, 14.8 kDa, and < 14.4 kDa, being predominant that with 14.8 kDa. The 14.8 kDa peptide fraction probably corresponds to para-κ-casein produced from the enzymatic hydrolysis of κ-casein (Egito et al., 2007). The same susceptibility to hydrolyze both α- and κ-caseins has been reported for pepsin of adult sheep (Slamani et al., 2018). Then, after 40 min of incubation, α-, β-, and κ-caseins were extensively hydrolyzed by tuna pepsin. Therefore, some strategies to diminish or inactivate the proteolysis achieved by pepsin after 10–20 min of reaction, could increase the MCA/PA ratio of tuna pepsin and improve its yield of curd or cheese.

Proximal Composition and Yield of Miniature Fresh Cheese

Fresh cheese is the most popular and widely sold type of cheese in Mexico and some Latin American countries. This is an unripe cheese, with a short shelf life and that can be produced with whole, partially skimmed, or skim milk (González-Córdova et al., 2016; Tunick & Van Hekken, 2010), characterized by a high moisture content (46–67%) and a low-fat content (18–29%) (Gutiérrez-Méndez et al., 2013; Tunick & Van Hekken, 2010). On the other hand, it has been reported that the protein and ash content of Mexican fresh cheese varies from 15 to 21%, and from 1 to 3%, respectively (González-Córdova et al., 2016; Tunick & Van Hekken, 2010). In general, the proximal composition of the cheese produced in the present study using microbial rennet and tuna pepsin lies within the values commonly reported for artisanal fresh cheese, except for protein content, which was slightly low (~ 11%) (Table 2). A higher fat content was observed in cheese made with tuna pepsin (15.4%) than in cheese made with microbial rennet (13.3%), which may be related to the lower moisture content in cheese made with tuna pepsin (66.3%) compared with cheese made with microbial rennet (69.3%). The protein and fat content of fresh cheese made in the present study were lower than the contents of protein (17%) and fat (16%) reported for similar fresh-type miniature cheeses (Gutiérrez-Méndez et al., 2013). Such differences derived mainly from the method to recover the curd. Thus, whereas Gutiérrez-Méndez et al.,

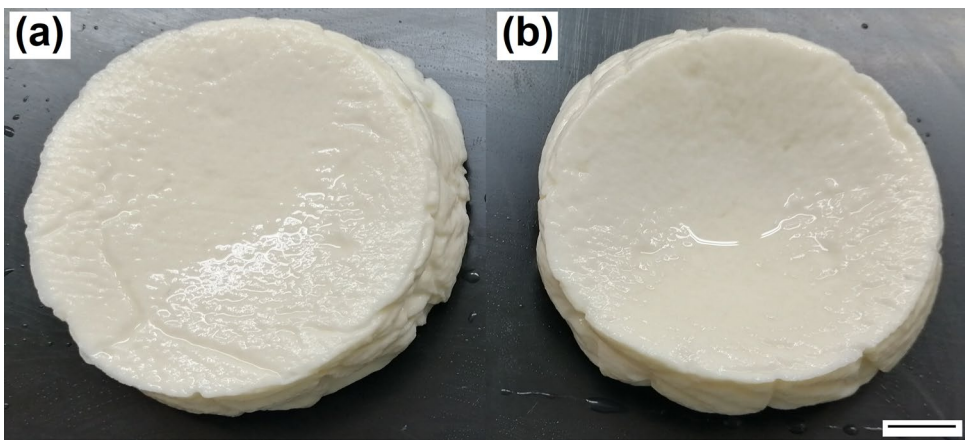

Fig. 5 Miniature cheese manufactured with **a** a microbial rennet (Cuamix™) and **b** partially purified pepsin of *T. albacares*. The bar indicates 1 cm in length

Table 3 Texture profile analysis of cheese produced with microbial rennet and partially purified pepsin from *T. albacares*

Treatment	Hardness (N)	Adhesiveness (N mm)	Cohesiveness	Chewiness (mJ)
Microbial rennet	5.57 ± 2.54	0.52 ± 0.28	1.36 ± 0.72[a]	67.2 ± 22.6[a]
Partially purified pepsin	4.33 ± 0.85	2.04 ± 1.25	0.84 ± 0.18[b]	37.2 ± 13.6[b]

The values are presented with their mean ± standard deviation ($n = 3$). Different letters indicate a significant difference ($\alpha = 0.05$) for sample

(2013) recovered the curd by centrifugation, in the present study the recovering of curd was realized in the traditional way by expelling the whey through drainage. The yield values recorded in this study for cheese made with microbial rennet and tuna pepsin were similar to those reported for fresh cheese made with vegetal rennet from *C. procera* (Abebe & Emire, 2020) (Fig. 5).

Texture and Sensory Evaluation of Miniature Fresh Cheese

Vast information concerning to effect of physical (e.g., high-pressure processing; Picon et al., 2013, Calzada et al., 2015) and chemical treatments (e.g., acidification; Dagostin et al., 2013) on the texture and flavor of fresh cheeses has been reported; however, only a few studies have analyzed the effect of the use of marine protease as alternative rennet (Tavares et al., 1997; Rossano et al., 2011) on texturometric and organoleptic characteristics.

Milk clotting with tuna pepsin affects cheese texture, producing less hardness, cohesion, and chewiness relative to cheese made with microbial rennet. However, these differences are not statistically significant, except for cohesiveness and chewiness (Table 3), where the values for cheese made with microbial rennet are higher than those reported by other authors (Caro et al., 2014; Shan et al., 2020). The hardness recorded for cheese made with tuna pepsin was almost half the hardness reported by Shan et al. (2020) for their cheese (cheese A: cheese B; 2:1) and, when compared with the hardness of the Mexican cheeses analyzed by Caro et al., 2014, the one showing similar hardness levels is the Oaxaca cheese. Regarding adhesiveness, the value observed in the present study is similar to that of the Mexican *morral* cheese (Caro et al., 2014), while cohesiveness is generally consistent with all the values reported for the Mexican cheeses studied (Caro et al., 2014) and the cheeses analyzed by Shan et al. (2020); the same occurs in the case of chewiness (Shan et al., 2020). The differences observed may be related to the moisture content of cheese (Khanal et al., 2018, 2019).

The differences observed between the texture profile of cheeses were not perceived by the sensory panel since similar scores were obtained for texture attributes (Table 4). There is an interaction between the mechanical-sensory properties (texture, color, aroma, flavor) and the composition and structure of cheese, which are perceived approximately at the same time, allowing to estimate the acceptance level by a potential consumer (Drake & Delahunty, 2017). Although the use of tuna pepsin conferred slight changes in the sensory characteristics, the differences in the evaluated parameters did not reach statistical significance (Table 4). However, in the overall evaluation of the cheeses, 60% of the panelists preferred the cheese made using microbial rennet, likely due to the slight perception of an aftertaste in the cheese produced with tuna pepsin. This behavior or sensory perception has been described as a slight "peculiar" flavor in cheeses made with alternative rennets of both vegetables (Abebe & Emire, 2020; Ben Amira et al., 2017) and marine sources (Tavares et al., 1997; Rossano et al., 2011). Differences in sensory perception can be influenced primarily by casein hydrolysis products related to the catalytic characteristics of clotting enzymes, their source (Rossano et al., 2011; García-Gómez et al., 2020), and concentration (Abebe & Emire, 2020).

Table 4 Sensorial analysis of cheese produced with microbial rennet and partially purified pepsin from *T. albacares*

Treatment	Color	Aroma	Flavor	Texture	Overall preference (%)
Microbial rennet	11.7 ± 1.4[a]	9.5 ± 3.0[a]	8.5 ± 2.8[a]	10.7 ± 1.9[a]	60
Partially purified pepsin	11.6 ± 1.6[a]	9.8 ± 3.0[a]	7.3 ± 3.5[a]	9.5 ± 3.1[a]	16.7

The values are presented with their mean ± standard deviation. Different letters indicate a significant difference ($\alpha = 0.05$) for sample

Conclusions

The results obtained in the present study confirm that tuna pepsin can be used to make fresh cheese with characteristics similar to those obtained using microbial rennet. The mini cheeses made with tuna pepsin had similar yields to those of the cheese made with a commercial coagulant. Although differences were found in some textural properties, these were not perceived in sensory evaluations. An aspect shared by alternative rennet sources is the generation of some protein hydrolysis peptides that confer peculiar flavors to cheese, which were perceived by the sensory panel. Future studies should focus on strategies to increase the acceptance of cheese made with tuna pepsin by consumers, mainly to reduce the perception of an aftertaste which could be modified by ripening cheese as a possible strategy.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval Ethical approval was not required for this research.

Consent to Participate All authors agreed to participate.

Consent for Publication Not applicable. The manuscript does not contain any individual person's data.

Competing Interests The authors declare no competing interests.

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