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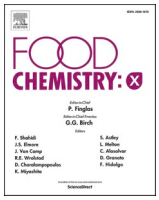

Hydrolysates and peptide fractions from pork and chicken skin collagen as pancreatic lipase inhibitors

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ABSTRACT

The objective of this work was to obtain hydrolysates and peptide fractions from pork (PSC) and chicken (CSC) skin collagen extracts and to evaluate their ability as pancreatic lipase inhibitors. Collagen extracts were hydrolyzed with collagenase or a protease from *Bacillus licheniformis* (MPRO NX®) at 6, 12, and 24 h. After 24 h incubation, the highest degree of hydrolysis of PSC ($p < 0.05$) was obtained with collagenase (72.58%), while in CSC was obtained with MPRO NX® (64.45%). Hydrolysates obtained at 24 h had the highest inhibitory activity of lipase ($p < 0.05$). CSC/collagenase hydrolysates (10 mg/mL) presented the highest inhibitory activity (75.53%) ($p < 0.05$). Ultrafiltrated fractions >5 kDa from CSC/collagenase and PSC/MPRO NX® hydrolysates were the most bioactive fractions (IC_{50}: 4.33 mg/mL). The highest were obtained by CSC peptides (IC_{50}s: 6.30 and 6.08 mg/mL). These results may be considered as a novel approach to use collagen hydrolysates, or their peptide fractions, as promising natural inhibitors of pancreatic lipase.

Introduction

Protein hydrolysates have acquired great relevance in recent years, since they have been attributed beneficial health properties, due to their content of low molecular weight bioactive peptides (Idowu, Benjakul, Sinthusamran, Sookchoo, & Kishimura, 2019). With the purpose to provide added-value, several studies have been performed in order to evaluate protein-rich animal by-products as potential sources of health beneficial protein hydrolysates (Toldrá, Mora, & Reig, 2016). Skin is one of the most abundant non-hazard protein-rich by-product generated in the meat industry, which has a high content of collagen. Several authors have reported obtaining protein hydrolysates with small peptides (<5 kDa) with bioactive properties such as antimicrobial, antioxidant, inhibitory activity of angiotensin converting enzyme of and α-amylase from the enzymatic hydrolysis of pig (PSC) and chicken skin collagen (CSC) (Kumar et al., 2019; Li, Chen, Wang, Ji, & Wu, 2007; Soladoye, Saldo, Peiro, Rovira, & Mor-Mur, 2015). All these bioactivities shown by skin collagen hydrolysates and/or peptides have been strongly related to several metabolic diseases.

Obesity, is a metabolic illness that has been considered a main public health problem, since it increases the risk of suffering chronic degenerative diseases (Brandt, Kleinert, Tschöp, & Müller, 2018). To prevent or reduce obesity, the main strategy is to have a well-balanced diet in combination with regular physical activity. However, changing activity and eating habits is very challenging, and very often leads into failure. Therefore, the implementation of metabolic treatments or supplements, as additional contributors, are often necessary to prevent or reduce obesity (Hu, Tao, Wang, Xiao, & Wang, 2016). Several protein hydrolysates and peptide fractions, from different protein sources, have shown anti-obesogenic properties by modifying dietary lipid metabolism and absorption.

Collagen hydrolysates from marine and bovine bone gelatin have shown anti-obesogenic activity in mice by reducing weight gain, serum lipids levels, as well as inhibiting lipogenesis and adipocytes differentiation in supplemented animals (Lee et al., 2017; Tometsuka, Funato, Mizuno, & Taga, 2021; Woo, Song, Kang, & Noh, 2018). However, the anti-obesogenic activity of collagen hydrolysates by modulating lipid digestibility and absorption has not been evaluated, yet. Dietary lipid digestion and absorption can be reduced by inhibiting the activity of pancreatic lipase (Hu et al., 2016; Rahim, Takahashi, & Yamaki, 2015).

Now days, there are commercial pharmaceutical drugs designed to inhibit pancreatic lipase. Blocking the ability of this lipase causes a

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reduction of fat absorption, promoting weight loss (Ballinger & Peikin, 2002). Recently, natural alternatives have been evaluated with the potential to have a similar effect than lipase-inhibiting commercial drugs (Fan, Cui, Zhang, & Zhang, 2018; Mudgil, Kamal, Yuen, & Maqsood, 2018). Peptides with low molecular weight (<5 kDa) isolated from hydrolysates of fish muscle, *Spirulina platensis* and also from fermented dairy products, have shown *in vitro* anti-obesogenic bioactivity by reducing the hydrolytic action of pancreatic lipase (Fan et al., 2018; Gil-Rodríguez & Beresford, 2019; Liu, Wang, Peng, & Wang, 2013; Mudgil et al., 2018). This activity has been attributed to the amino acid composition and structure of the peptides present in these hydrolysates. It has been reported that pancreatic inhibitory peptides possessed several residues of proline and glycine in their sequence, especially on their N- and C-terminal, which have been suggested were implicated in their bioactivity (Jakubczyk et al., 2019). It is worth noticing, that pork and chicken skin collagen is rich on these amino acids residues (Soladoye et al., 2015); hence, it can be hypothesized that enzymatic hydrolysis of these sources of collagen may have the potential to produce anti-obesogenic hydrolysates and peptide fractions. However, it is important to consider that composition and sequence of peptide fractions strongly depends on the hydrolytic enzyme used (Alemán, Gómez-Guillén, & Montero, 2013). Therefore, the objective of this work was to evaluate the ability of two enzymes, collagenase and proteases from *Bacillus licheniformis,* to produce hydrolysates, as well as peptide fractions, from pork and chicken skin collagen extracts with the ability to reduce the activity of pancreatic lipase.

Materials and methods

Reagents

Porcine pancreatic lipase (100 – 500 units/mg), porcine gastric mucosa pepsin (≥250 units/mg), sodium hydroxide, sodium monobasic phosphate, sodium dibasic phosphate, acetic acid, butyl alcohol, o-phthaldialdehyde, cyclohexane, cupric acetate and orlistat were purchased from Sigma Aldrish (St. Louis, MO, USA). Collagenase type I from *Clostridium histolyticum* (300 units/mg) was purchased from Worthinton (Lakewood, NJ, USA), MPRO NX® protease from *Bacillus licheniformis* (180 unit/mg) was purchased from ENMEX (Mexico City, Mexico) and sodium chloride was purchased from Merck (Mexico City, Mexico).

Skin preparation

Fresh pork and chicken skin were obtained from local abattoirs. Skins were cut into small pieces and stored at –18 °C. Skin pieces were defrosted at 4 °C, and grounded three times in a meat grinder (Hobart Dayton, model 4152, Ohio, USA). The first time, skin pieces were passed through a disk of 0.635 cm was, and then twice through a disk of 0.476 cm.

Collagen extraction

Collagen extraction was carried out following the method reported by Nalinanon, Benjakul, Visessanguan, and Kishimura (2007). The complete extraction process was performed at 4 °C. The non-collagenous protein of the skin was removed with a 0.1 M NaOH solution at a ratio of 1:10 (w/v) and stirred for 6 h. Then, samples were washed with water until neutral pH was obtained. Skin from both sources were then defatted adding 10% butyl alcohol at ratio of 1:10 (w/v) and stirred for 18 h. Defatted skins were washed three times with cold water and then lyophilized.

Afterwards, lyophilized defatted skins were soaked in 0.5 M acetic acid at a ratio of 1:10 (w/v) with agitation for 24 h. Next, samples were centrifuged at 20,000 g for 20 min at 4 °C, and then supernatants were collected (acid-soluble collagen). The non-soluble fractions of defatted skin were soaked in 0.5 M acetic acid pH of 2.5 at a ratio 1:10 (w/v) with 0.1% of pepsin for 24 h. Samples were then submerged in an ice bath to inactivate the enzyme and centrifuged at 20,000 g for 20 min at 4 °C and the supernatants were collected (soluble collagen in acetic acid + pepsin).

Collected samples (acid soluble collagen and acid-pepsin soluble collagen) were combined and precipitated by adding NaCl (until a concentration of 2 M NaCl was reached) and centrifuged at 20,000*g* for 1 h. The resulting pellet (collagen extract) was dissolved in 0.5 M acetic acid and dialyzed for 24 h with 0.1 M acetic acid, and then with water for another 2 two days. Dialyzed sample was lyophilized.

Proximal analysis

Quantification of moisture, fat and protein content in skin and collagen extracts, were performed using the methods of the Association of Official Analytical Chemists (AOAC, 1990).

Collagen quantification

Hydroxyproline content of resulting pellets was determinate by the method of Bergman and Loxley (1963). For quantification of collagen, ten milligrams of extracts were added into tubes of 50 mL and hydrolyzed with 6 N HCl for 24 h at 110 °C in oil bath. Subsequently, samples were placed in a 100 mL volumetric flask, aforated with HPLC water and filtrated through a Whatman paper No. 41. The pH of filtrated samples was adjusted to neutral with NaOH.

One milliliter of sample was pipetted in a tube and 1 mL of citrate buffer pH 6 was added and mixed. After, 1 mL of chloramine T solution was added, mixed and let stand for 4 min. Subsequently, 3 mL of 1.8 M perchloric acid were added and mixed, and finally 2 mL of 5% solution of 4-dimetilamine benzaldehyde were added to each tube and heated to 60 °C for 25 min in a water bath. The samples were cooled in running water for 2 min, and the hydroxyproline content was determined based on the absorbance (at 558 nm) obtained using a hydroxyproline standard curve. Content of total and insoluble collagen (reported as μg collagen/100 μg protein of extracts) was determined based on the content of hydroxyproline and multiply by the conversion factor of 7.7 (Wu et al., 2017).

Enzymatic hydrolysis

Soluble collagen extracts were dissolved (0.01 g protein/mL) in 4 mL of 0.1 M sodium–phosphate buffer pH 7.5 for collagenase hydrolysis and pH 7 for MPRO NX® hydrolysis. Enzymes were added in a ratio of 1:100 enzyme:substrate. Hydrolysis process was performed with agitation at 37 °C for collagenase and 50 °C for MPRO NX®. Samples were taken at 6, 12 and 24 h of incubation. Enzyme inactivation was achieved by heating samples to 90 °C for 15 min.

Degree of hydrolysis

Degree of hydrolysis (DH) was measured by the o-phthaldialdehyde (OPA) method (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003; Zhang, Olsen, Grossi, & Otte, 2013). A sample aliquot of 155 μL was added to 3 mL of OPA reagent (this solution was prepared the same day of measuring as described by Church, Swaisgood, Porter, and Catignani (1983), and incubated for 2 min at 25 °C. Then, the absorbance was measured in a spectrophotometer (Agilent Technologies, Cary 60 UV–vis, St. Clara, C.A., USA) at 340 nm in a quartz cuvette. DH was calculated by the formula:

$$\mathrm{DH}(\%) = \frac{n}{N} x100$$

where **n** is the average number of peptide bonds hydrolyzed, and **N** is the total number peptide bonds (the number of 4287 peptide bonds of collagen reported by Zhang et al. (2013) was used). **n** was calculated

from the absorbance measurements according to the formula:

$$n = \frac{\Delta AbsMd}{\varepsilon c}$$

where **ΔAbs** is the absorbance at 340 nm of the hydrolyzed sample – unhydrolyzed sample; **M** is the protein molecular mass (Da); **d** the dilution factor; **ε** the molar extinction coefficient at 340 nm (6020 M^{-1} cm^{-1}); and **c** the protein concentration (g/L) (10 g/L).

Ultrafiltration of collagen hydrolysates

Samples of collagen hydrolysates were fractionated based on their molecular weight by ultrafiltration using membranes of 5, 3 and 1 kDa (Millipore Co., USA). Hydrolysates were first ultrafiltrated through a membrane with a molecular weight cut–off (MWCO) of 5 kDa. This fraction was subsequently passed through a 3 kDa MWCO membrane, and the new ultrafiltrate was consequently filtered through a 1 kDa MWCO. The different peptides fractions with theoretically weight were F1: higher than 5 kDa; F2: between 5 and 3 kDa; F3: between 3 and 1 kDa and F4: lower than 1 kDa, were stored at –40 °C, until tested. Ultrafiltration yield was estimated and reported is Fig. S5.

Electrophoretic pattern

Molecular weight (MW) of collagen extracts and their different hydrolysates was determined by sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS–PAGE) analysis (Laemmli, 1970), using a 4% and 7% stacking and resolving gel, respectively. High MW marker standard (10 – 250 kDa) (Sigma-Aldrich, St. Louis, MO, USA) was used to estimate the MW of proteins. Gels were stained with Coomassie blue R-250.

Size exclusion chromatography

In order to characterize the peptide profile of ultrafiltrated fractions, (as well as their respective hydrolysates), gel filtration was performed in a FPLC in ÄKTA pure equipment (GE Healthcare, Piscataway, NJ, USA), using Superdex 75 10/300 GL column for collagen extracts and hydrolysates, and Superdex Peptide 10/300 GL column for peptide fractions. Samples (100 μL) were eluted using 50 mM phosphate buffer (pH 7.0) and 150 mM NaCl with a flow rate of 0.80 mL/min for 31 min. BSA (67 kDa), ovalbumin (43 kDa), ribonuclease (13.7 kDa), aprotinin (6.512 kDa) and vitamin B_{12} (1.355 kDa) were used as MW standards for collagen extracts and hydrolysates. Cytochrome (13.6 kDa), aprotinin, vitamin B12 and glycine (0.188 kDa) were used as MW standards for peptide fractions. Detection was performed at 280 nm (Rendon-Rosales et al., 2019).

Enzymatic activity of pancreatic lipase

The reduction of pancreatic lipase activity was measured by the method reported by Slanc et al. (2009), with slight modifications. Pancreatic lipase solution at a concentration of 10 mg/mL in Tris-HCl buffer (75 mM, pH 8.5) was prepared the same day of measuring. A mixture of 162 μL of 75 mM Tris-HCl buffer pH 8.5, 12 μL of enzyme solution, and 16 μL of protein hydrolysates or non-hydrolyzed collagen extract (10 mg/mL), or ultrafiltrated peptide fractions (2.5 to 7.5 mg protein/mL to establish IC_{50}) were incubated at 37 °C for 25 min. After, a solution of 10 μL of 3.3 mM *p*-nitrophenylpalmitate in ethanol was added, and incubated for 15 min at 37 °C. For control sample, the protein fraction was substituted with 16 μL of 0.1 M sodium phosphate buffer pH 7.5. A sample without enzyme as sample blank was also prepared and measured. Orlistat was used as a positive inhibitor control (10 μg/mL). The absorbance was measured at 405 nm in microtiter plate well. The inhibition percentage of pancreatic lipase activity for non-hydrolyzed skin collagen extracts and their different hydrolysates was evaluated using the equation reported by Xiang et al. (2020).

Pancreatic lipase activity inhibition %: $(1 - \left(\frac{AbsSample - AbsBlanksample)}{AbsControl - AbsBlankControl}\right))x100$

IC_{50} (concentration required to achieve a 50% inhibition) for ultrafiltrated peptide fractions was calculated by curves percentage of inhibition versus concentration treatments in mg/mL. The equation of this curve allowed to calculate the IC_{50}.

Statistical analysis

The data of proximal analysis, yield extraction, collagen content were analyzed by a one-way analysis of variance. On the other hand, the percentage DH and pancreatic lipase inhibitory activity (PLIA) were measured by a general linear model analysis of variance; analyzed factors on DH were enzyme type and hydrolysis time. For PLIA of hydrolysates the variation factors were treatments and hydrolysis time, while for PLIA of peptide fractions the variation factors were treatments and molecular weight. Data were repeated in triplicate and presented as mean ± their standard error. Significances were estimated at a 0.05 probability level. Means comparison was performed by Tukey-Krammer. All data were processed using the statistical package NCSS 2011.

Results and discussion

Proximal composition

Pork and chicken skin had the same amount of moisture ($p > 0.05$). However, significant differences in the content of fat and protein were found between pork and chicken skin. Chicken skin had a greater percentage ($p < 0.05$) of fat with 38.76% compared to pork skin with 29.98% (Table S1). In contrast, protein content in pork skin (28.32%) was greater than chicken skin (15.21%). Similar composition of chicken and pork skin have been reported by other authors (Ajayi & Akomolafe, 2016; Choi et al., 2016).

Yield and collagen content of extracts

Extraction yield (w/w) of lyophilized soluble collagen based on total protein content in pork skin was greater than for chicken skin ($p < 0.05$) with 10.82% compared to 7.74%, respectively (Table 1). Protein content and soluble collagen content (μg collagen/100 μg protein) of lyophilized collagen extracts of pork and chicken are also shown in Table 1. PSC extract resulted with a higher protein and soluble collagen content than CSC extract ($p < 0.05$), with a 99.61 vs 73.12% of protein and 95.19 vs 87.21% of collagen, respectively. Insoluble collagen was not registered since all collagen of the extracts were soluble. Protein profile by size exclusion chromatography, as well as the electrophoretic pattern, confirmed the composition of pork and chicken skin collagen extracts (Fig. S1).

Soluble collagen extraction yield results on the present study were

Table 1
Yield, protein, and collagen content of lyophilized extracts of pork and chicken skin.

	Pork	Chicken
Yield (%)	10.82 ± 0.50^a	7.86 ± 0.80^b
Protein (%)	99.61 ± 0.47^a	73.12 ± 0.34^b
Fat (%)	ND**	25.02 ± 0.34
Soluble Collagen (%) *	95.19 ± 2.10^a	87.21 ± 2.90^b

Yield of soluble collagen (percentage base on defatted lyophilized sample).
* μg soluble collagen/100 μg protein.
** ND: No determinate.
Different literals within rows indicate significant differences between means $p < 0.05$.

similar to yields reported by Kittiphattanabawon, Benjakul, Visessanguan, Nagai, and Tanaka (2005) for bigeye snapper skin of 10.9%. However, our yield results were lower than those reported in feet chicken (22.94%) and pork and chicken skin (24.3%) by Hashim, Ridzwan, and Bakar (2014) and Gojkovic, Marova, Matouskova, Obruca, and Miloslav (2014), respectively. Nevertheless in the present study, protein and collagen content were higher than those reported Gojkovic et al. (2014) for protein (29.9 and 27%) and collagen (25 and 40.6%) in soluble collagen extracts of pork and chicken skin respectively.

Degree of hydrolysis (DH)

The hydrolytic behavior of soluble collagen extract from pork skin (Fig. 1) was affected by the interaction between hydrolysis time and enzyme type ($p < 0.05$). Initially, PSC extract had a degree hydrolysis of 3.76 %, which was increased during incubation with collagenase ($p < 0.05$) to 57.83% and 65.60% at 6 and 12 h, respectively, reaching a DH of 72.5% at the end of incubation time. Whereas MPRO NX® was able to increase DH to 55% at 6 h of hydrolysis, and remained stable until 12 h of incubation; thereafter, a significant increase of DH was achieved at the end of incubation reaching 62.47%. Collagenase had a higher hydrolytic effect ($p < 0.05$) than MPRO NX® since it was able to attain a faster and higher DH ($p < 0.05$) than MPRO NX®. Electrophoretic pattern of PSC hydrolysates obtained at the different incubation times is presented in Fig. S2.

The greater hydrolytic effect of collagenase compared with protease of *Bacillus licheniformis* may be due to its specificity for collagen. This enzyme recognizes the cleavage site of X-Gly bond (where X is most often a neutral amino acid) in the peptide sequence Pro-X-Gly-Pro (Haralson & Hassell, 1995). Notwithstanding, proteases from *Bacillus licheniformis*, such as MPRO NX®, have been used successfully for the hydrolysis of collagen, even when these enzymes have a wide spectrum of cleavage sites, with no specificity to collagen sequence (Toldrá et al., 2016; Zhang et al., 2013).

A similar hydrolytic behavior for collagenase has been reported for other animal sources of collagen. For Milkfish skin collagen a DH of 79% was reached using collagenase incubation for 1.5 h (Baehaki, Suhartono, Sukarno, & D., & Setyahadi, S., 2016). However, Kumar, Shakila, Jeyasekaran, and Sciences (2019) reported a lower DH (7.6%) in unicorn leatherjackt fish skin after incubation of 6 h. This difference can be explained by the temperature of the hydrolysis used in this study (35 °C), which it was lower than the used in the leatherjackt fish collagen study (50 °C). Regarding the hydrolytic ability of MPRO NX® found in the present study, it was higher than one reported (15%) for insoluble collagen from bovine tendon hydrolyzed using alcalase from *Bacillus licheniformis* incubated during 4 h (Zhang et al., 2013). However, Baehaki et al. (2016), reported a similar DH of 51.85% for collagen hydrolysates from fish skin produced by the protease of *Bacillus licheniformis*.

Contrary to our results in PSC hydrolysis, degree of hydrolysis in CSC hydrolysates (Fig. 2) was not affected by the type of enzyme ($p > 0.05$). A possible explanation of the differences may be due to the difference between the purity of collagen extracts. PSC extract had a higher content of collagen in relation to total protein, than CSC extract. Therefore, other non-protein compounds, such as fat, in CSC extract may affect the enzyme-substrate interaction, decreasing the hydrolytic behavior. However, incubation time, with both enzymes, significantly increased % DH. Electrophoretic profile of CSC hydrolysates attained at different incubation periods is presented in Fig. S3.

Initially CSC extract had a DH of 8.38% and was increased during hydrolysis ($p < 0.05$) reaching 53.75, 58.39 and 63.32% at 6, 12 and 24 h, respectively. A higher DH of 79% was reported for chicken collagen hydrolysates obtained by alcalase from *Bacillus licheniformis* incubated during 24 h (Onuh, Girgih, Aluko, & Aliani, 2013). This slight difference between our results may be explained due to the differences of enzyme concentration used for incubation. In the present study MPRO NX® was added at a lower concentration (1% vs 4% used by the referenced study). Nevertheless, our results were higher than those reported by Soladoye et al. (2015) in chicken collagen hydrolysates obtained after incubation with Flavourzyme® and Alcalase ® during 5 h with a DH of 26% and 20%, respectively.

Inactivation of pancreatic lipase

Fig. 3 shows the ability of the different pork and chicken hydrolysates and non-hydrolyzed collagen extracts (10 mg/mL) to inhibit the activity of pancreatic lipase. This bioactivity was affected by the

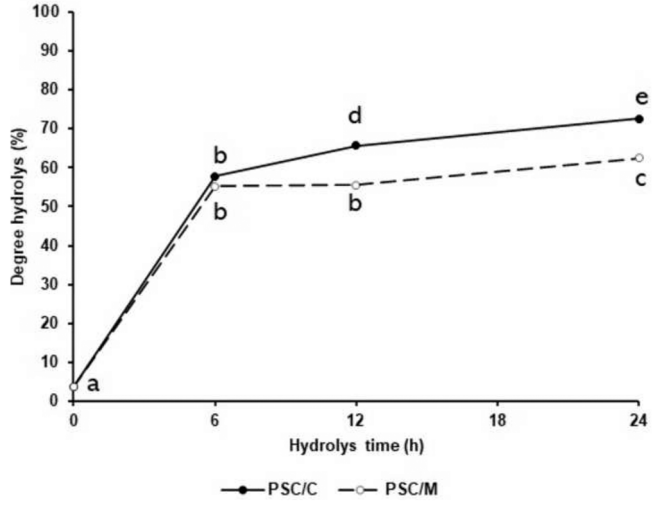

Fig. 1. Hydrolytic effect of collagenase and MPRO NX® in pork collagen at of different incubation times. MPRO NX®: protease of *Bacillus licheniformis*. PSC/C: pork skin collagen hydrolysate obtained by collagenase; PSC/M: pork skin collagen hydrolysate obtained with MPRO NX®. Error bars represent standard deviations from triplicate samples. Different literals indicate significant differences between means by effect of the interaction enzyme type and hydrolysis time ($p < 0.05$).

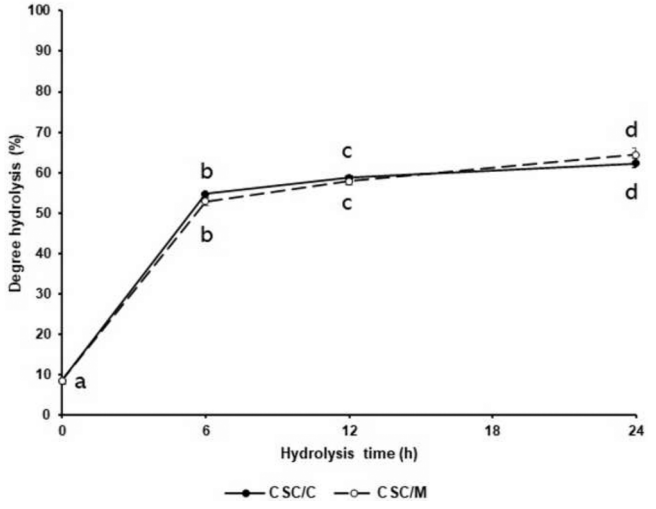

Fig. 2. Hydrolytic effect of collagenase and MPRO NX® in chicken collagen at of different incubation times. MPRO NX®: protease of *Bacillus licheniformis*. CSC/C: chicken skin collagen hydrolysate by collagenase; CSC/M: chicken skin collagen hydrolysate obtained by MPRO NX®. Error bars represent standard deviations from triplicate samples. Different literals indicate significant differences between means by effect of the interaction enzyme type and hydrolysis time ($p < 0.05$).

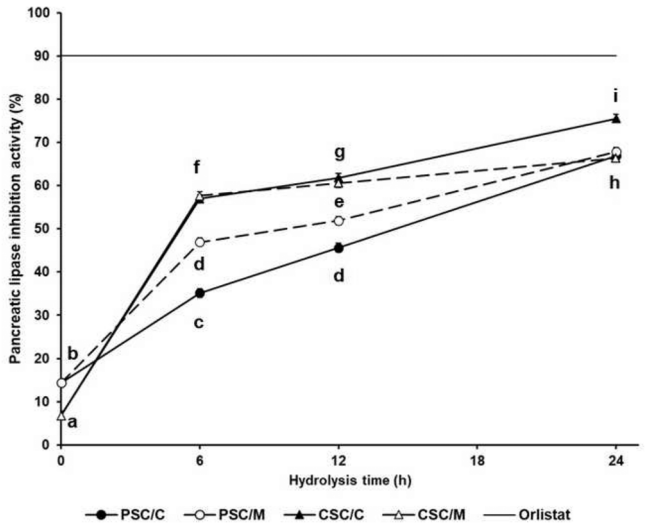

Fig. 3. Pancreatic lipase inhibitory effect of collagen extracts and hydrolysates (10 mg/mL) produced with collagenase and MPRO NX® through of hydrolysis time. MPRO NX®: protease of *Bacillus licheniformis*. PSC/C: pork skin collagen hydrolysate obtained by collagenase; PSC/M: pork skin collagen hydrolysate obtained with MPRO NX®; CSC/C: chicken skin collagen hydrolysate by collagenase; CSC/M: chicken skin collagen hydrolysate obtained by MPRO NX®. Error bars represent standard deviations from triplicate samples. Different literals indicate significant differences between means from collagen extracts and hydrolysates by effect of the interaction between hydrolysate (collagen extract-enzyme type) and incubation time ($p < 0.05$).

interaction between treatment vs incubation time ($p < 0.05$). It is worth noticing how lipase inhibition was increased ($p < 0.05$) by hydrolysis regardless of the type of enzyme used.

Non-hydrolyzed PSC extract showed a pancreatic lipase inhibitory activity (PLIA) of 12.84%. PLIA of hydrolysates obtained from this source by using either hydrolytic enzyme, was higher in comparison to non-hydrolyzed collagen extract ($p < 0.05$), reaching a 55% increase on PLIA. Pork skin collagen hydrolysates obtained by MPRO NX® (PSC/M) at 6 and 12 h of hydrolysis had a higher PLIA (46.94% and 51.90%, respectively) ($p < 0.05$) than pork skin collagen hydrolysates obtained by collagenase (PSC/C) (35.15% and 45.63%, respectively) at the same hydrolysis time. However, the inhibitory effect was similar ($p > 0.05$) between extracts hydrolyzed by MPRO NX® and collagenase (67.87% and 66.88%, respectively) during 24 h.

Regarding to non-hydrolyzed chicken skin collagen extract it showed a PLIA of 7.43%, however, hydrolysates obtained with both enzymes had a higher PLIA ($p < 0.05$). The inhibitory ability of hydrolysates obtained after 24 h incubation was 68% higher than non-hydrolyzed CSC extracts. PLIA of chicken collagen hydrolysates obtained at 6 and 12 h was not affected by enzyme type ($p > 0.05$) (57% and 61%, respectively). However, a significant increase of PLIA ($p < 0.05$) was achieved by extracts hydrolyzed by collagenase (CSC/C) and MPRO NX® (CSC/M) during 24 h incubation reaching 75.53% and 66.36% PLIA, respectively.

Comparing PLIA between PSC and CSC samples, non-hydrolyzed CSC extract had a lower PLIA than PSC ($p < 0.05$). However, the inhibitory activity was greater ($p < 0.05$) in CSC hydrolysates than PSC hydrolysates incubated during 6 and 12 h with either collagenase or MPRO NX®. CSC/C hydrolysate obtained at 24 h was the most effective ($p < 0.05$) to inhibit the activity of pancreatic lipase.

The difference in the lipase inhibitory activity between the different hydrolysates tested in this study can be due to the most likely differences of amino acid composition, sequence and length of the peptides that were produced after hydrolysis depending on the enzyme and the source of skin collagen. The inhibitory ability shown by hydrolysate samples were closely related to their degree hydrolysis, since this parameter, as it was discussed in the previous section, significantly increased during incubation. A similar behavior was reported by Liu et al. (2013) describing a directly proportional association between the inhibitory activity on lipase and the degree hydrolysis of fish muscle hydrolysates obtained with papain and protamex. This behavior can be related to the presence of small peptides in hydrolysates with high DH, since it has been reported that as the DH increases, the presence of small peptides also is increased (Morais et al., 2013).

Based on difference in the specificity of cleavage site between both enzymes, collagenase and MPRO NX® most likely generated different type of peptides in spite of acting in the same protein source. Other authors have also reported different abilities to inhibit pancreatic lipase when using hydrolysates obtained from the same protein source and produced by different enzyme types (Awosika & Aluko, 2019; Fan et al., 2018). Liu et al. (2013) reported for fish muscle hydrolysates (0.22% w/v fish water-soluble protein) a pancreatic lipase inhibition between 30 and 45 % depending on the enzyme type used (alkaline protease, neutral protease, protamex and papain).

Nevertheless, none of the skin collagen hydrolysates obtained in this study were more effective than orlistat, which had a 90% of inhibition by using only 10 μg/mL. The greater inhibitory efficiency of orlistat is attributed to its highly selective interaction with the active site of pancreatic lipase, specifically with its serine residue (Ballinger & Peikin, 2002). However, due to the several negative side effects caused by this synthetic drug, protein hydrolysates and peptide fractions are promising natural alternatives to inhibit the activity of pancreatic lipase, with the potential of no side-effects (Fan et al., 2018; Gil-Rodriguez et al., 2019, Xiang et al., 2020; Liu et al., 2013).

Comparing with hydrolysates of other source proteins, % PLIA of 10 mg/mL of skin collagen hydrolysates obtained at 24 h of incubation were higher than those reported for 10 mg/mL of *Spirulina platensis* hydrolysates obtained with papain, pepsin, alcalase and trypsin (27.24, 50.61, 51.29 and 30.65%, respectively) (Fan et al., 2018). Also, our hydrolysates had a percentage of inhibition of 16 to 25% higher than 9.5 mg/ml of faba bean seeds hydrolysates fermented by *L. plantarum* 299v incubated at 22 °C (Jakubczyk et al., 2019). In contrast, hydrolysates obtained in the present study were less bioactive than camel milk protein hydrolysates obtained by alcalase, bromelain and papain, since was necessary a higher concentration of our hydrolysates (5 mg/mL) than camel milk protein hydrolysates (lower at 0.1 mg/mL) to reach an 50% inhibition of the activity of pancreatic lipase Mudgil et al. (2018).

Since hydrolysates obtained at 24 h of incubation had a higher lipase inhibition activity, they were fractionated by ultrafiltration, in order to describe if their PLIA was due to the size of their peptide fractions and to find if any of these peptide fractions possessed a greater bioactivity. Peptide profile confirming the separation efficacy of ultrafiltration and the composition of each peptide fraction obtained from each hydrolysate is presented in Table S2.

Lipase inhibitory activity of peptide fractions (5 mg/mL) was affected ($p < 0.05$) by the interaction between hydrolysate (skin collagen type-enzyme) and peptide fraction's molecular weight (Fig. S4). Generally, from Fig. S4 it was possible to conclude that fractions with a MW > 5 kDa and <1 kDa from CSC and PSC hydrolyzed with either enzyme exhibited the highest bioactivity in comparison to the other ultrafiltrated fractions. Additionally, the bioactivity of skin collagen peptide fractions <1 kDa was similar to their respective hydrolysates. Therefore, the IC_{50}s of these samples were determined and are presented in Table 2.

PSC samples' IC_{50}s variated from 4.33 to 5.71 mg/mL. Non-fractionated hydrolysates and their respective peptide fractions had similar IC_{50}s ($p > 0.05$). However, IC_{50} of F1 from PSC/M hydrolysate was lower ($p < 0.05$) in comparison to fractions obtained from PSC/C hydrolysate. The concentration needed to reach a 50% inhibition using CSC samples were in between 4.33 and 6.30 mg/mL. Peptide fraction F4 from CSC/C hydrolysate was less effective ($p < 0.05$) in comparison to both non-fractionated CSC hydrolysates and their respective F1

Table 2
Concentration to reach an 50% inhibition of pancreatic lipase (IC_{50} mg/mL) of pork and chicken skin collagen hydrolysate and their ultrafiltrated peptide fractions.

Treatment	Hydrolysate	F1*	F4*
Pork			
Collaganese	5.04 ± 0.018 ^{abc}	5.71 ± 0.12 ^{cde}	5.24 ± 0.32 ^{bc}
MPRO NX®	4.94 ± 0.22 ^{abc}	4.33 ± 0.32 ^{a}	4.93 ± 0.29 ^{abc}
Chicken			
Collagenase	4.65 ± 0.029 ^{ab}	4.33 ± 0.06 ^{a}	6.30 ± 0.48 ^{e}
MPRO NX®	5.33 ± 0.18 ^{bcd}	5.07 ± 0.22 ^{abc}	6.08 ± 0.21 ^{de}

*F1: peptide fraction > 5 kDa; F4: peptide fraction < 1 kDa.
Different literals indicate significant differences between means $p < 0.05$.

fractions. IC_{50}s of non-fractionated hydrolysates from both, pork and chicken were similar, regardless on the enzyme used to obtain them. In general, the most effective peptide fractions were F1 from PSC/M and CSC/C and the less bioactive were F4 from both CSC hydrolysates.

Form these results, it can be inferred that bioactivity of PSC and CSC hydrolysates obtained by either collagenase or MPRO NX®, can be attributed to the inhibitory activity of peptides in fractions F1 and F4, since these fractions had the highest yield (Fig. S5). Bechaux, Gatellier, Le Page, Drillet, and Sante-Lhoutellier (2019) described that the bioactivity of a hydrolysate or a peptide mixture can be attributed to the presence of a peptide with a greater bioactivity, or various moderately bioactive peptides in high concentrations.

Several studies have reported that ultrafiltrated fractions, especially those with MW < 3 kDa, possessed a significantly higher bioactivity than their hydrolysates (Alemán et al., 2013; Jakubczyk et al., 2019; Soladoye et al., 2015), contrasting to what we found in our study. Nevertheless Gil-Rodríguez and Beresford (2019) and Awosika and Aluko (2019), also reported that fractionation by MW of fermented milk and yellow field pea protein hydrolysates (respectively) reduced their lipase inhibition activity.

The IC_{50} values for unfractionated PSC and CSC hydrolysates were similar to those reported by Awosika and Aluko (2019) for yellow field pea hydrolyzed with chymotrypsin or pepsin (4 – 5 mg/mL). However, our values were lower than those reported faba bean seeds hydrolysates (5.61 – 9.52 mg/mL) (Jakubczyk et al., 2019).

Generally, in comparison with other peptide fractions obtained from different protein sources, PSC and CSC peptide fractions possessed a similar o higher ability to inhibit pancreatic lipase. For example, IC_{50} of F1 fractions (4.33 – 5.7 mg/mL) were similar to those concentrations reported for fractions with MW 10 – 5 kDa from yellow field pea hydrolysates obtained by different enzymes (Awosika & Aluko, 2019). However, F4 fractions obtained in our study, regardless of the source, were more effective to inhibit the activity of pancreatic lipase than the fraction with MW < 3 kDa obtained from *Spirulina plantasis* hydrolysates, which had only a 37.8% inhibition by using approximately a 2-fold higher concentration (Fan et al., 2018). Also, all PSC and CSC peptide fractions had lower IC_{50}s than fraction <1 kDa from yellow field pea protein hydrolysate, with an IC_{50} of 8 mg/mL (Awosika & Aluko, 2019). In contrast, F4 fractions in the present study had a lower bioactivity than peptides of same MW reported by Jakubczyk et al. (2019) from fermented faba beans seeds obtained at different times and temperature of fermentation. Peptide fractions from faba beans had IC_{50}s 2 to 5-fold lower.

Conclusion

Enzymatic hydrolysis of CSC and PSC with collagenase or MPRO NX® generated hydrolysates, which their ability to inhibit lipase pancreatic activity increased with degree of hydrolysis. However, the ultrafiltered process of the hydrolysates by MWCO was not able to produce peptide fractions with a higher effectivity to inhibit pancreatic lipase than their respective hydrolysates. Nevertheless, something to highlight is that peptides fraction <1 kDa (F4) from skin collagen possessed a good *in vitro* pancreatic lipase inhibitory activity. This is important, since small molecular weight peptide fractions may have the potential to exert a better bioavailability and bioaccessibility. Therefore, future studies to evaluate these characteristics during and after simulated gastrointestinal digestion tests, should be performed. Nevertheless, results obtained in this study can be considered a first approach to explore the potential to use chicken or pork skin collagen hydrolysates or their peptide fractions as an adjuvant option for obesity treatment.

Declaration of Competing 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100247.

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