Isolation and characterisation of ovine collagen hydrolysates following thermal and different enzymatic treatments

[1]Ángeles-Santos, C., [2]Aguirre-Cruz, G., [1]Jiménez-Alvarado, R., [1]León-López, A. and [1,2]*Aguirre-Álvarez, G.

[1]*Universidad Autónoma del Estado de Hidalgo, Instituto de Ciencias Agropecuarias, Av. Universidad Km. 1. C.P. 43600, Tulancingo, Hidalgo, México*

[2]*Uni-Collagen S.A. de C.V. Arnulfo González No. 203, El Paraíso. C.P. 43684, Tulancingo, Hidalgo, México*

Abstract

Ovine collagen was hydrolysed by thermal treatment, followed by treatment with seven different commercial enzymes namely Heliozym (He), Propain 100 (Pro), Curtizyme (Cur), Cocktail (Ck), Fungal (Fu), Rohapon (Roh), and Polizym (Po), and compared with two control treatments (with and without thermal treatment at 50°C). The viscosity, hydroxyproline content, isoelectric point, and molecular weight were measured to establish the optimum conditions for enzymatic hydrolysis. Collagen hydrolysis was conducted at 50°C for 24 h. Treatment with Po resulted in the lowest viscosity (0.23 mPa*s) and yielded low molecular weight collagen fractions of around 20 kDa. The highest yield of hydroxyproline from collagen was reported for the Fu treatment at 12.65 mg/mL. The isoelectric point (pI) values differed significantly ($p < 0.05$) between the control treatments and the other treatments. The pI shifted from 8.5 (native collagen) to 2.0 in most of the hydrolysed collagens.

Introduction

Collagen is the most important structural protein in a wide variety of connective tissues such as ligaments, bones, and skin (Kim *et al.,* 2016). Partial acidic or enzymatic hydrolysis of this biopolymer produces small peptide molecules; however, complete hydrolysis would release the amino acids that form this protein. The degree of hydrolysis depends on a number of parameters such as enzyme concentration, initial substrate, pH, and temperature (Camacho *et al.,* 1998). Denaturation of native collagen occurs above 40°C, and produces three α-chains of similar molecular weight with a random coiled form.

After denaturation, each α-chain can be attacked by proteolytic enzymes (alcalase, papain, flavourzyme, and others) based on broad and simultaneous bond rupture reactions. Hydrolysis begins with the formation of an enzyme-substrate complex, which then undergoes cleavage of amidic bonds by nucleophilic attack by water molecules to form peptides that are separate from the enzyme. This process could then be repeated on the new peptides; however, complete collagen hydrolysis requires a linkage between the substrate and hydrolytic enzymes (Huo and Zhao, 2009).

The hydrolysis of collagen produces small peptides with low molecular weight (Mw) (3 - 6 kDa) (Cheung and Li-Chan, 2017; Hong *et al.,* 2017; Ketnawa *et al.,* 2017; Thuanthong *et al.,* 2017). The peptides produced by degradation of proteins are called natural peptides or hydrolysed proteins (Oshimura and Sakamoto, 2017). Hydrolysed collagen (HC) is obtained by the hydrolysis of gelatine by enzymatic treatment under acidic conditions followed by sterilisation, and a drying process. Enzymatic proteolysis of collagen has been demonstrated as the best method for solubilisation, emulsion, and release of biologically active peptides. However, HC and its native form differ significantly. For example, native collagen has a high Mw (300 kDa) as compared to its hydrolysates (3 - 6 kDa). This creates viscosity differences in the two collagen preparations.

Viscosity is of importance when HC is applied to food formulations. The addition of HC to soup is known to affect its viscosity and functional properties. Similarly, HC added to orange juice creates a product with improved functional properties with low viscosity and high-water solubility. These effects of HC addition to food formulations depend on the isoelectric point (pI). Collagen hydrolysates bind calcium ions, thus improving calcium bioavailability in the creation of functional foods for mineral deficiencies. HC also lowers syneresis when combined with modified starch

and guar gum in ham processing. In beverages, HC has good clarification effects and flocculation capability due to its synergistic effect on electrical charges.

HC has been studied in detail due to its natural properties, and it now has extensive applications in the food, biomedical, and cosmetic industries (León-López *et al.,* 2019b). It is used as an active component due to its excellent biocompatibility, penetrability (Zhuang and Zhao, 2009), and strong antioxidant properties (León-López *et al.,* 2019a). HC is absorbed well following oral administration, and readily distributes into cartilage tissues due to its biological activity and low molecular weight. HC shows good compatibility with ingredients used in health and beauty products for skin and hair as well as in a wide variety of food products (Denis *et al.,* 2008; Bilek and Bayram, 2015).

These benefits and properties of HC have revealed a need for more research to discover alternative methodologies, reagents, and raw materials for HC extraction. The present work thus describes the isolation and characterisation of HC from sheepskin following different enzyme treatments. The molecular, physico-chemical, and electrostatic properties of the extracted HC are also described.

Materials and methods

Materials

The raw material was a salted sheepskin with 48% water content and preserved in good condition with no degradation by microorganisms (no hair slip). The skin was shaved to remove most of the hair, and then soaked for 24 h at 20°C in water containing a degreaser and detergent, each at 1 g/L. Unnecessary fragments of tissue such as muscle and fat were then removed, and the skin was cut into small pieces of about 1 × 1 cm.

Extraction of collagen

Skin samples were placed in an aqueous solution of 0.5 M acetic acid (1:30, w/v) for 3 h. After swelling of the skin, gastric pepsin (porcine gastric mucosa; Sigma Aldrich, USA) was added at 1 g/L, and the mixture was continuously stirred for 48 h at 20°C. The resulting solution was filtered to remove undissolved solids. The collagen in the solution was precipitated with 2.6 M sodium chloride, and weighed to calculate the yield. The isolated collagen was re-solubilised in 0.5 M acetic acid at a ratio of 1:30 (w/v).

Hydrolysis of collagen

The re-solubilised collagen was adjusted to pH 8.0 with the addition of 0.1 M NaOH. Two control treatments were included in this experiment. The first control consisted of native collagen solution without thermal treatment (NTT). The second control was native collagen subjected to thermal treatment (TT) at 50°C for 24 h.

For enzymatic treatments, 1 g of a commercial enzyme was added to 50 mL of collagen solution (1:50, w/v). The enzymes were Heliozym (He; a pancreatic enzyme), Polizym (Po; KRKA, Novo Mesto, Slovenia; containing amylase, protease, B-glucanase, B-glucosidase, and cellulase), Curtizyme (Cur; ENMEX, CDMX, MX; a bacterial protease), Propain 100 (Pro; ENMEX, CDMX, MX; papain enzyme), Rohapon OO (Roh; TFL, León, Gto MX; a pancreatic enzyme), and Fungal (Fu; Thermo Fisher, CDMX, MX; a fungal serine protease).

The hydrolysis was carried out in a water bath (model A50630D Scorpion Scientific. USA) at 50°C for 24 h. Samples were cooled to room temperature for 30 min, and stored at 4°C. The samples were then frozen at -20°C for 2 h, and dried by water sublimation for 48 h under vacuum conditions (0.039 mbar) in a lyophiliser (LABCONCO Freezone, USA) at -30°C, with a collector temperature around -50°C.

Viscosity of collagen

The viscosity of HC samples was measured as described by Yu *et al.* (2014), with some modifications. A 200 mL sample of HC at 4°C was measured in a viscometer (Brookfield model RTVnt AR200, USA) with spindle number 2 at 100 rpm. Results were expressed in mPa*s.

Yield of collagen

The yield of HC samples was calculated based on the weight of the dry skin, and the following equation (Sinthusamran *et al.,* 2013):

$$\text{Yield (\%)} = \frac{\text{lyophilised collagen weight (g)}}{\text{initial dried skin weigh (g)}} \times 100$$

(Eq. 1)

Isoelectric point of collagen

The isoelectric point of HC samples was measured as described by Zhang *et al.* (2006), with slight modifications. Briefly, aqueous hydrolysed collagen solutions were titrated with 1 M NaOH or 1.0 M HCl in a Zetasizer instrument (Malvern Zetaweight Nano ZS, USA). The test was run at 25°C with decreasing pH intervals of about 0.5 units. The isoelectric point of the sample was interpreted as the pH value where the zeta potential was zero.

Content of collagen

The content of collagen was calculated from 0.2 g of lyophilised sample as described by Neuman and Logan (1950), with slight modifications. The sample was placed in a digestion tube with 5 mL 50% HCl, and heated in an oven at 100°C for 16 h. The digested solution was diluted to 100 mL in distilled water with gentle mixing. A 2 mL volume of that solution was placed in a 25 mL volumetric flask, followed by 2 mL 0.05 mol/L copper sulphate II (CuSO$_4$), 2 mL 1.25 mol/L NaOH, and 2 mL 6% hydrogen peroxide; it was gently mixed for 5 min, and then placed in a water bath at 40°C for 15 min. After cooling, 10 mL 0.9 mol/L sulphuric acid was added, followed by 5 mL 5% dimethylaminobenzaldehyde, and the sample was heated again in a water bath at 70°C for 30 min. The sample was cooled to room temperature, and absorbance was determined at 55 nm with a spectrophotometer. A calibration curve was prepared with hydroxyproline (Sigma Aldrich, USA) at 0, 5, 10, 15, and 20 mg.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weights were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) using a 4% polyacrylamide running gel and 12% polyacrylamide stacking gel. Samples were denatured in a water bath at 90°C for 3 min, and then a 20 μL sample was loaded per path. Proteins were separated at 136 V (ENDURO 300 V, Labnet International, Inc., USA). After electrophoresis, the gels were fixed and stained for 3 h in 0.05% Coomassie blue R-250 in methanol:acetic acid:water (50:10:40, v/v), followed by destaining in methanol:acetic acid:water (7.5:5:87.5, v/v). A molecular weight marker (BenchMark TM) from 10 to 220 kDa was also run in a lane on the gel.

Statistical analysis

Analysis of variance (ANOVA) was conducted on the experimental data followed by Tukey's test ($p \leq 0.05$). The data were analysed using IBM SPSS v25.0 software (SPSS Inc., Chicago, IL, USA). Three replicates per treatment were run in this experiment.

Results and discussion

Viscosity of collagen

Viscosity is a physical behaviour of molecules characterised by a resist to flow when a force or stress is applied. The shifting of viscosity to lower values is a process that depends on several factors such as the duration of hydrolysis as shown in Figure 1a with He treatment. The HC viscosity was significantly reduced from 14.3 to 10.5 mPa*s when the thermal and enzymatic treatment was extended up to 10 h. After 10 h of hydrolysis, no further significant differences were observed in the viscosity. For this reason, the hydrolysis time was set at 10 h for all subsequent treatments.

Figure 1b shows the viscosity of HC following treatment with different enzymes. Two controls were considered: one was native collagen with no treatment (non-thermal treatment; NTT), and the other was native collagen with thermal treatment (TT). Significant differences ($p \leq 0.05$) were observed between TT and NTT, with NTT having the highest viscosity. The collagen molecular structure was not modified, and the sizes of the molecular chains were retained following the NTT.

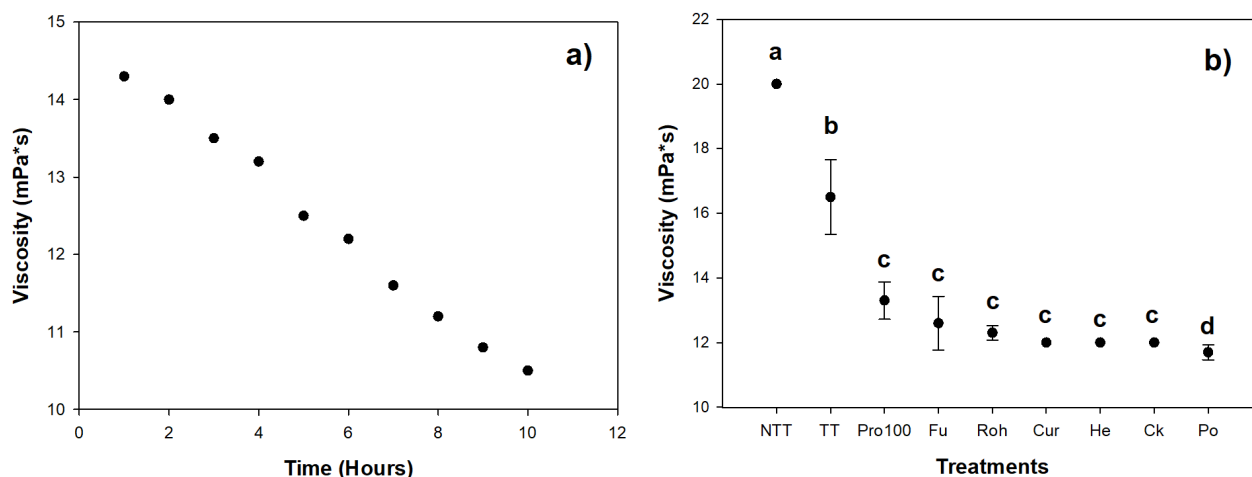The collagen monomer is a rod-like molecule

Figure 1. Viscosity of collagen hydrolysates (a) obtained with Heliozym as a function of time, and (b) obtained with commercial enzymes.

with a molecular weight of about 300 kDa (Ledward, 1986). A larger chain size leads to a higher hydrodynamic volume, and thereby a higher resistance to flow. This could be the reason why native collagen molecules without thermal treatment had a high viscosity (20 mPa*s). The TT at 50°C caused a reduction in the viscosity of the native collagen to 16.5 mPa*s due to denaturation of the native collagen, which has a denaturation temperature of around 40°C. At that temperature, thermal denaturation results in the transition from the semi-crystalline state to an amorphous random-coiled state, and the three-chain helical structure separates into three individual chains (Stainsby, 1987).

The maximum drop in viscosity was observed when the native collagen was treated under thermal conditions followed by enzymatic attack. The Po treatment showed the smallest reduction in viscosity, from 16.5 to 11.7 mPa*s. The other enzymatic treatments also reduced the viscosity to values that were significantly lower ($p \leq 0.05$) than the control values. The viscosities for Pro, Fu, Roh, Ck, Cur, and He were 13.3, 12.6, 12.29, 12.0, 12.0, and 12.0 mPa*s, respectively. These results agree with those reported by León-López et al. (2019a), who hydrolysed ovine collagen with Heliozym, and discovered a close relationship between the viscosity and molecular weight of the peptides. The viscosity changes were subsequently determined to be due to structural changes in collagen from its triple helix structure to a random coil form (Zhang et al., 2006).

Collagen can be hydrolysed by two types of proteases. The exopeptidases like pepsin act on simple compounds that contain one or more terminal polar groups such as amino, or carboxyl groups, or both. The endopeptidases are able to hydrolyse heat-denatured collagen that do not possess terminal amino or carboxyl groups (Jung et al., 2014). Most commercial proteolytic enzymes are prepared with a combination of trypsin and chymotrypsin because their combined action brings about full hydrolysis to the polypeptide stage (Wilson, 1956). These results suggest that thermally denatured collagen was attacked by these endopeptidases or proteolytic enzymes at its peptide bonds.

Yield of collagen

The yields of collagen differed significantly ($p \leq 0.05$), as illustrated in Figure 2. The NTT had the highest yield of collagen (24.68%), as calculated by the hydroxyproline assay. The TT produced a heat-denatured collagen with a reduction in collagen yield as compared to the NTT. However, treatment of the thermally denatured collagen with enzymes
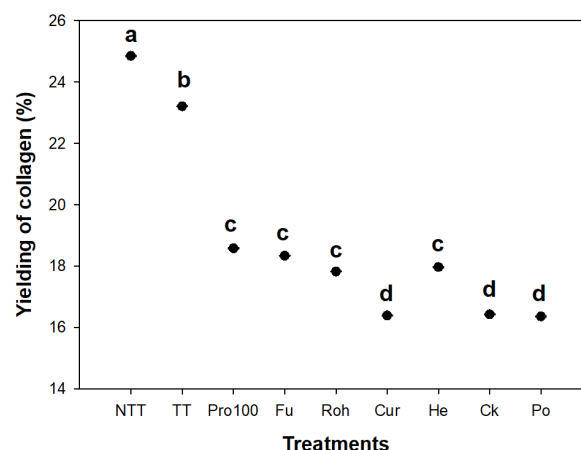

Figure 2. Yields of collagen following enzymatic treatments.

resulted in a drastic drop in yield to values of 16.3% for the Ck, Cur, and Po treatments. These results are in good agreement with previous work carried out with fish hydrolysates (Lin et al., 2015), who concluded that the lower yield of collagen also indicated a lower collagen purity. This reduction in purity could be due to a decrease in the hydroxyproline-rich fraction during the enzymatic hydrolysis process (Gustavson, 1956).

In the present work, the yield of HC was higher with Pro (papain enzymes) (around 19%) than with enzymes obtained from fish sources by Umi Hartina et al. (2017), with yields of 13.7 and 16.8%, achieved after 30 and 60 min of enzyme treatment, respectively. These differences could be due to the chemical composition of the sources of extraction (Nurul and Sarbon, 2015).

Isoelectric point of collagen

The pI represents the electrostatic properties of HC and the proportion of both acid and base amino residues. Samples of ovine collagen type I were titrated from an initial pH of 3.5 to a final pH of 9.0. The pI value was interpreted as the pH value when electrostatic repulsion was zero (Sinthusamran et al., 2013; Kaewdang et al., 2014; Chen et al., 2016). Figure 3 shows that the pI of the NTT was very similar to the value reported in the literature (Zhang et al., 2006), at around pI 8.29. The native collagen showed a shift in pI to 6.65 after thermal treatment. However, the pI value was lowest, at around pI 2.2, following any enzymatic treatment, regardless of the enzyme type. These results suggest that enzymatic hydrolysis of collagen produced low molecular weight peptides that altered the balance between acidic and basic groups. The acidic groups of amino acids were present at higher concentrations, as the pI values were consistent with low pH values. This could
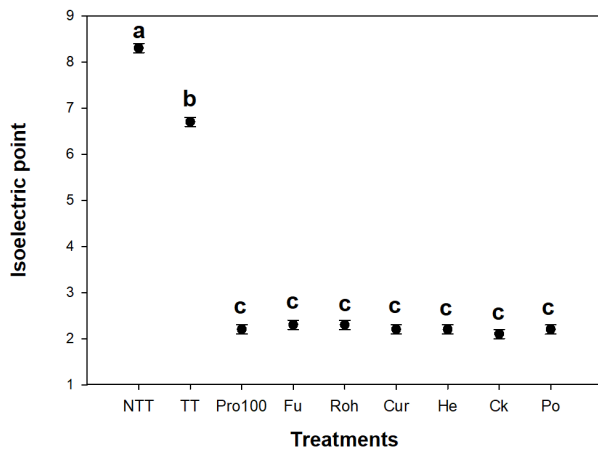
Figure 3. Isoelectric point of acidic amino acid residues in hydrolysed ovine collagen.

Figure 4. Hydroxyproline content of low molecular weight collagen hydrolysates.

reflect trypsin action during hydrolysis of collagen, as trypsin is a proteolytic enzyme that attacks only the carboxyl group of basic amino acids (Ramachandran, 1967; Jung *et al.,* 2014). Another factor that could give rise to a low pI value is the presence of alkaline products during titration. Collagen is very sensitive to deamidation of two amino acids, asparagine and glutamine, at high pH values (Gustavson, 1956). These two amino acids are converted to aspartic acid and glutamic acid, respectively, by this deamidation.

The pI values shifted from 9 to 2 after enzymatic hydrolysis, and the pI reflects the ratio of residues of acid and basic amino acids of the collagen. For the NTT, the pI remained in the alkaline range because the collagen was extracted under acidic conditions, thus retaining its lateral amide residues intact. By contrast, the pI values of hydrolysed samples were lower due to a higher concentration of carboxyl groups arising from hydrolysis of the amide groups (Zhang *et al.,* 2006).

Hydroxyproline content of collagen

Collagen differs from other proteins by its high concentration of hydroxyproline. This amino acid provides collagen with thermal stability due to hydrogen bond formation and the presence of a hydroxyl group (OH) that limit the rotation of the peptide chain (Ramachandran *et al.,* 1973; León-López *et al.,* 2019a). Figure 4 shows the hydroxyproline content of collagen after the thermal and enzymatic treatments. The highest amount of hydroxyproline was detected for NTT (13.2 mg/mL), thus indicating that the collagen molecular structure was intact, and resembled that of the native collagen (Ramachandran, 1967). However, a reduction of up to 12.8 mg/mL was observed as the native collagen was denatured under thermal conditions. The lowest
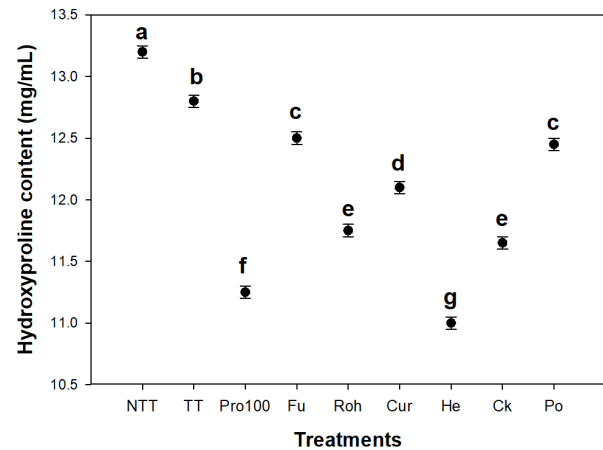
amounts of hydroxyproline were detected after the enzymatic treatments, and particularly in the He sample, which had a value of 11.0 mg/mL. These results are in agreement with literature reporting that hydrolysis of collagen drastically reduces this parameter due to hydrolysis of carboxyl bonds of arginine and lysine (Ramachandran, 1967; Jung *et al.,* 2014). The highest amount of hydroxyproline, at 12.5 mg/mL, was measured following the Fu and Po enzymatic treatments. These enzymes only weakly hydrolyse the collagen molecule when compared with the other enzymatic treatments. A strong hydrolysis is known to reduce the amount of hydroxyproline.

Molecular weight of collagen

Figure 5 shows the electrophoretic patterns of the native collagen (NTT) and collagen after TT and enzymatic treatment. Native collagen type I consists of one α-2 and two α-1 chains (Wang *et al.,* 2013). The NTT showed one dimeric β-chain at 220 kDa, and two characteristics monomeric α-chains of native collagen at 165 and 120 kDa, for α1-chain and α2-chain, respectively. Notably, no other bands were observed for this particular sample, thus indicating that the collagen type I was quite pure, and its structure was intact (Khiari *et al.,* 2014). However, the TT resulted in the appearance of a band at 100 kDa, thus indicating the denaturation of collagen and conversion of the triple helix into three single random-coiled α-chains. The subsequent application of the He enzyme to the denatured collagen resulted in the appearance of bands of low molecular weight at values below those of the α-chains, at around 20 kDa (Abdollahi *et al.,* 2018). This treatment consists of a trypsin enzyme, which is a serine protease that preferentially hydrolyses peptides at the C-terminals of lysine and arginine amino acid residues (Keil-Dlouhá *et al.,* 1971). Additional protein
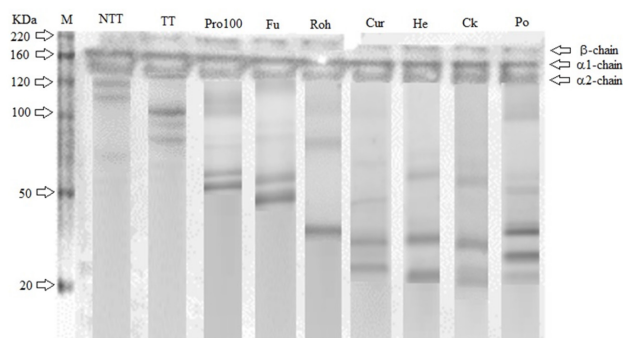
Figure 5. SDS-PAGE pattern of native collagen type I (NTT), thermally treated collagen (TT), and seven commercial enzymes. Lane 1: protein marker.

cleavage could also occur at aromatic amino acid residues, like phenylalanine and tyrosine (Perutka and Šebela, 2018).

The results reported herein are similar to those reported by Li *et al.* (2013) for skin and bone samples from Spanish mackerel. They isolated and hydrolysed collagen with trypsin at 37°C for 3 h, and produced peptides with low molecular weight of around 20 kDa. In the present work, Po treatment also resulted in the lowest molecular weight bands, of around 20 kDa. This enzyme formulation consists of a mixture of enzymes including amylase, protease, B-glucanase, B-glucosidase, and cellulase. The presence of all these different enzymes may have resulted in different substrate specificities, thus indicating a collaborative action, wherein an enzyme mixture containing various exo- and endo-activities may function in an additive manner by activating new reactive sites for the other enzymes (Khiari *et al.,* 2014). The Po treatment produced shorter collagen peptides as compared to treatments involving only a single enzyme. The Pro and Fu treatments had a smaller enzymatic effect on the cleavage of collagen. These enzyme formulations mainly consist of papain and fungus serine proteases, respectively.

Leon-López *et al.* (2019b) performed studies on the enzymatic hydrolysis of collagen type I with trypsin, and found a significant reduction in molecular weight of up to 3 - 6 kDa, as the hydrolysis time increased. This reduction in molecular weight also increased the antioxidant activity of these low molecular weight natural peptides. The absorption of low molecular weight collagen in the human bloodstream has been quantified and reported in literature which indicates that collagen molecules are absorbed and transported into blood (Ohara *et al.,* 2007; Yazaki *et al.,* 2017). Similar results were observed by Nurilmala *et al.* (2020) who obtained HC by 2% alcalase treatment of yellowfin tuna skin. A lower molecular

weight was associated with a stronger scavenging activity. A higher antioxidant activity was measured in hydrolysates with molecular weight lower than 3 kDa (Nurilmala *et al.,* 2020).

Recently, low molecular weight natural peptides like collagen hydrolysates have been widely used for their excellent biocompatibility, easy biodegradability, and weak antigenicity (Liu et al., 2015).

Conclusions

The extraction of low molecular weight hydrolysates from ovine collagen was satisfactory using commercial proteolytic enzymes. The Po enzyme showed the best ability to hydrolyse collagen, and generated a solution with low viscosity containing low molecular weights peptides. These biomaterials could be applied to a wide variety of products of the food, biomedical, cosmeceutical, and pharmaceutical industries.

Acknowledgement

References

Abdollahi, M., Rezaei, M., Jafarpour, A. and Undeland, I. 2018. Sequential extraction of gel-forming proteins, collagen and collagen hydrolysate from gutted silver carp (*Hypophthalmichthys molitrix*), a biorefinery approach. Food Chemistry 242: 568-578.

Bilek, S. E. and Bayram, S. K. 2015. Fruit juice drink production containing hydrolyzed collagen. Journal of Functional Foods 14: 562-569.

Camacho, F., González-Tello, P. and Guadix, E. M. 1998. Influence of enzymes, pH and temperature on the kinetics of whey protein hydrolysis. Food Science and Technology International 4(2): 79-84.

Chen, S., Chen, H., Xie, Q., Hong, B., Chen, J., Hua, F., … and Wu, H. 2016. Rapid isolation of high purity pepsin-soluble type I collagen from scales of red drum fish (*Sciaenops ocellatus*). Food Hydrocolloids 52: 468-477.

Cheung, I. W. Y. and Li-Chan, E. C. Y. 2017. Enzymatic production of protein hydrolysates from steelhead (*Oncorhynchus mykiss*) skin gelatin as inhibitors of dipeptidyl-peptidase IV and angiotensin-I converting enzyme. Journal of

Functional Foods 28: 254-264.

Denis, A., Brambati, N., Dessauvages, B., Guedj, S., Ridoux, C., Meffre, N. and Autier, C. 2008. Molecular weight determination of hydrolyzed collagens. Food Hydrocolloids 22(6): 989-994.

Gustavson, K. H. 1956. The chemistry and reactivity of collagen. New York: Academic Press.

Hong, H., Chaplot, S., Chalamaiah, M., Roy, B. C., Bruce, H. L. and Wu, J. 2017. Removing cross-linked telopeptides enhances the production of low-molecular-weight collagen peptides from spent hens. Journal of Agricultural and Food Chemistry 65(34): 7491-7499.

Huo, J.-X., and Zhao, Z. 2009. Study on enzymatic hydrolysis of *Gadus morrhua* skin collagen and molecular weight distribution of hydrolysates. Agricultural Sciences in China 8(6): 723-729.

Jung, K.-H., Choi, Y.-C., Chun, J.-Y., Min, S.-G. and Hong, G.-P. 2014. Effects of concentration and reaction time of trypsin, pepsin, and chymotrypsin on the hydrolysis efficiency of porcine placenta. Korean Journal for Food Science of Animal Resources 34(2): 151-157.

Kaewdang, O., Benjakul, S., Kaewmanee, T. and Kishimura, H. 2014. Characteristics of collagens from the swim bladders of yellowfin tuna (*Thunnus albacares*). Food Chemistry 155: 264-270.

Keil-Dlouhá, V., Zylber, N., Tong, N.-T. and Keil, B. 1971. Cleavage of glucagon by α- and β-trypsin. FEBS Letters 16(4): 287-290.

Ketnawa, S., Benjakul, S., Martínez-Alvarez, O. and Rawdkuen, S. 2017. Fish skin gelatin hydrolysates produced by visceral peptidase and bovine trypsin: bioactivity and stability. Food Chemistry 215: 383-390.

Khiari, Z., Ndagijimana, M. and Betti, M. 2014. Low molecular weight bioactive peptides derived from the enzymatic hydrolysis of collagen after isoelectric solubilization/precipitation process of turkey by-products. Poultry Science 93(9): 2347-2362.

Kim, S. H., Park, H. S., Lee, O. J., Chao, J. R., Park, H. J., Lee, J. M., … and Park, C. H. 2016. Fabrication of duck's feet collagen - silk hybrid biomaterial for tissue engineering. International Journal of Biological Macromolecules 85: 442-450.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Ledward, D. A. 1986. Gelation of gelatin. In Mitchel, J. R. and Ledward, D. A. (eds). Functional properties of food macromolecules, p. 171-174. United Kingdom: Elsevier Applied Sciences.

León-López, A., Fuentes-Jiménez, L., Hernández-Fuentes, A. D., Campos-Montiel, R. G. and Aguirre-Álvarez, G. 2019a. Hydrolysed collagen from sheepskins as a source of functional peptides with antioxidant activity. International Journal of Molecular Sciences 20(16): article no. 3931.

León-López, A., Morales-Peñaloza, A., Martínez-Juárez, V. M., Vargas-Torres, A., Zeugolis, D. I. and Aguirre-Álvarez, G. 2019b. Hydrolyzed collagen—sources and applications. Molecules 24(22): articles no. 4031.

Li, Z.-R., Wang, B., Chi, C.-F., Zhang, Q.-H., Gong, Y.-D., Tang, J.-J., Luo, H.-Y. and Ding, G.-F. 2013. Isolation and characterization of acid soluble collagens and pepsin soluble collagens from the skin and bone of Spanish mackerel (*Scomberomorous niphonius*). Food Hydrocolloids 31(1): 103-113.

Lin, C. C., Chiou, T. K. and Sung, W. C. 2015. Characteristics of gelatin from giant grouper (*Epinephelus lanceolatus*) skin. International Journal of Food Properties 18(11): 2339-2348.

Liu, D., Nikoo, M., Boran, G., Zhou, P. and Regenstein, J. M. 2015. Collagen and gelatin. Annual Review of Food Science Technology 6: 527-557.

Neuman, R. E. and Logan, M. A. 1950. The determination of collagen and elastin in tissues. Journal of Biological Chemistry 186(2): 549-556.

Nurilmala, M., Hizbullah, H. H., Karnia, E., Kusumaningtyas, E. and Ochiai, Y. 2020. Characterization and antioxidant activity of collagen, gelatin, and the derived peptides from yellowfin tuna (*Thunnus albacares*) skin. Marine Drugs 18(2): article no. 98.

Nurul, A. G. and Sarbon, N. M. 2015. Effects of pH on functional, rheological and structural properties of eel (*Monopterus* sp.) skin gelatin compared to bovine gelatin. International Food Research Journal 22(2): 572-583.

Ohara, H., Matsumoto, H., Ito, K., Iwai, K. and Sato, K. 2007. Comparison of quantity and structures of hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different sources. Journal of Agricultural and Food Chemistry 55(4): 1532-1535.

Oshimura, E. and Sakamoto, K. 2017. Amino acids, peptides, and proteins. In Sakamoto, K., Lochhead, R. Y., Maibach, H. I. and Yamashita, Y. (eds). Cosmetic Science and Technology, p. 285-303. United States: Elsevier.

Perutka, Z. and Šebela, M. 2018. Pseudotrypsin: a little-known trypsin proteoform. Molecules 23(10): article no. 2637.

Ramachandran, G. N. 1967. Treataise on collagen. United Kingdom: Academic Press.

Ramachandran, G. N., Bansal, M. and Bhatnagar, R. S. 1973. A hypothesis on the role of hydroxyproline in stabilizing collagen structure. Biochimica et Biophysica Acta (BBA) - Protein Structure 322(1): 166-171.

Sinthusamran, S., Benjakul, S. and Kishimura, H. 2013. Comparative study on molecular characteristics of acid soluble collagens from skin and swim bladder of seabass (*Lates calcarifer*). Food Chemistry 138(4): 2435-2441.

Stainsby, G. 1987. Gelatin gels. In Pearson, A. M., Dutson, T. R. and Bailey, A. J. (eds). Advances in Meat Research: Collagen as Food, p. 209-222. United States: AVI Publishing.

Thuanthong, M., De Gobba, C., Sirinupong, N., Youravong, W. and Otte, J. 2017. Purification and characterization of angiotensin-converting enzyme-inhibitory peptides from Nile tilapia (*Oreochromis niloticus*) skin gelatine produced by an enzymatic membrane reactor. Journal of Functional Foods 36: 243-254.

Umi Hartina, M. R., Kuan, A. Y. P., Wolyna, P., Lee, J. S. and Nor Qhairul Izzreen, M. N. 2017. Effects of different enzymatic treatment on the properties of hydrolyzed collagen from pangasius fish (*Pangasius hypophthalmus*). In International Conference on Food Science and Nutrition, p. 173-178. Sabah, Malaysia: University Malaysia Sabah.

Wang, B., Wang, Y.-M., Chi, C.-F., Luo, H.-Y., Deng, S.-G. and Ma, J.-Y. 2013. Isolation and characterization of collagen and antioxidant collagen peptides from scales of croceine croaker (*Pseudosciaena crocea*). Marine Drugs 11(11): 4641-4661.

Wilson, H. R. 1956. The practice of bating. In O´Flaherty, F. (eds). The Chemistry and Technology of Leather (volume 1), p. 323-346. New York: Reinhold Publishing.

Yazaki, M., Ito, Y., Yamada, M., Goulas, S., Teramoto, S., Nakaya, M., … and Yamaguchi, K. 2017. Oral ingestion of collagen hydrolysate leads to the transportation of highly concentrated Gly-Pro-Hyp and its hydrolyzed form of Pro-Hyp into the bloodstream and skin. Journal of Agricultural and Food Chemistry 65(11): 2315-2322.

Yu, D., Chi, C.-F., Wang, B., Ding, G.-F. and Li, Z.-R. 2014. Characterization of acid-and pepsin-soluble collagens from spines and skulls of skipjack tuna (*Katsuwonus pelamis*). Chinese Journal of Natural Medicines 12(9): 712-720.

Zhang, Z., Li, G. Y. and Shi, B. 2006. Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. Journal of the Society of Leather Technologists and Chemists 90(1): 23-28.

Zhuang, Y., Hou, H., Zhao, X., Zhang, Z. and Li, B. 2009. Effects of collagen and collagen hydrolysate from jellyfish (*Rhopilema esculentum*) on mice skin photoaging induced by UV irradiation. Journal of Food Science 74(6): H183-H188.