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Optimum conditions for extracting collagen from the tunica albuginea of immunologically castrated pig testes and the functional properties of the isolated collagen

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ABSTRACT

This study evaluated alternative methods for extracting collagen from the tunica albuginea of pig testes and characterized the functional properties of the isolated collagen. Using the statistical tools of factorial design (2^{4-1}) and a central composite rotatable design (2^3), it was concluded that the best conditions were 0.83 mol L^{-1} acetic acid, 0.24% pepsin and 28 h of hydrolysis to isolate 82.54 g of collagen per 100 g of sample. This purified collagen had improved functional properties in relation to bovine skin collagen, including water solubility, water-holding capacity, emulsifying capacity and emulsion stability. These results suggest that isolated collagen from the tunica albuginea can be used in pharmaceutical and food products.

1. Introduction

Collagen is the dominant and ubiquitous protein in the body and accounts for approximately 30% of total vertebrate protein. The term collagen describes a family of at least 28 protein isoforms (Ricard-Blum, 2011). Overall, collagen has been extracted from mammals, particularly cattle and pig skin, for food, cosmetic, and medical products and is often treated with pepsin to increase the solubility and maximize extraction. Treatment with pepsin is necessary to digest the crosslinking sites of the protein because the triple helix structure protects collagen against this enzymatic activity. The presence of collagen crosslinking stabilizes the protein as the animal ages because the immature collagen crosslink, hydroxylysinoketonorleucine (HLKNL), is biologically replaced by the main mature crosslink derived from two reducible HLKNLs, forming pyridinoline (PYR), which stabilizes the collagen molecules and increases meat texture (Coró, Youssef, & Shimokomaki, 2002; Robins, Shimokomaki, & Bailey, 1973). PYR bridges different types of collagen and further stabilizes the extra cellular macromolecular organization (Shimokomaki, Wright, Irwin, Van Der Rest, & Mayne, 1990). Initially, pepsin digestion was used to isolate a new collagen type from pig cartilaginous tissue, known as type IX (Shimokomaki, Duance, & Bailey, 1980). Pepsin digestion was also used to identify collagen types as I, II, III and V from mechanically deboned chicken meat, and type I was found to be the major component (Tanaka & Shimokomaki, 1996). Recently, there has been growing interest in evaluating industrial byproducts; thus, there have been many studies on the extraction of collagen from different animal sources (Alves & Prudêncio-Ferreira, 2002; Benjakul et al., 2010; Nalinanon, Benjakul, Kishimura, & Osako, 2011; Sadowska, Koodziejska, & Niecikowska, 2003; Shon, Eo, Hwang, & Eun, 2011; Wang, Yang, Du, Yang, & Liu, 2008; Woo, Yua, Chob, Leea, & Kima, 2008; Yan et al., 2008).

The immunological castration of male pigs is an alternative to surgical castration and enhances animal welfare while preventing the development of the androsterone and skatole hormones, which cause an undesirable meat odor (Claus, Weiler, & Herzog, 1994; Silveira et al., 2008). Brazil produces over 10 million immunologically castrated pigs and the testis is becoming an important economical subproduct, representing around 0.3% to 0.6% of the carcass weight (Silveira et al., 2008), currently it is removed during slaughter and discarded. Pig testes are surrounded by a capsule of dense connective tissue called the tunica albuginea, which is characterized as a resistant tissue composed of collagen fibers (Copenhaver, Kelly, & Wood, 1978).

The aim of this work was to optimize the extraction and pepsin hydrolysis conditions for purifying collagen from the tunica albuginea

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of pig testes and to characterize the chemical and functional properties of the isolated collagen.

2. Material and methods

2.1. Materials and reagents

Immunologically castrated crosses of 110 to 115 kg Landrace and Large White pigs were slaughtered in a commercial slaughterhouse in the São Paulo state countryside, and the testes were removed and frozen. Only the tunica albuginea, which was manually removed from the testes, was used for collagen production. Fig. 1 shows the location of the tunica albuginea in the testes. The reagents were from different origins and of analytical grade purity.

2.2. Chemical composition and collagen content determination

The physicochemical composition of pig testes, tunica albuginea and the isolated collagen was determined on a dry matter basis according to the Association of Official Analytical Chemists (AOAC, 2005). The collagen contents of pig testes, tunica albuginea and the isolated collagen were determined by the amino acid hydroxyproline concentration according to the methodology of Woessner (1961) multiplied by a factor 8.0, (Kolar, 1990). Tukey's test was used to determine the differences the chemical composition means of the testes and the tunica albuginea, and $p < 0.05$ was adopted as the threshold of statistical significance.

2.3. Removal and preparation of the tunica albuginea

The tunica albuginea was manually removed from the testes, thoroughly washed in distilled water and carefully cut into cubes. The collagen was prepared by immersing 10 g of tunica albuginea in 100 mL of distilled water and continuously stirring the immersion in a refrigerated incubator (Shaker, Marconi, São Paulo, Brazil) at 4 °C for 48 h, with several water changes daily. To remove other proteins and contaminating components, the distilled water was replaced with 100 mL of 0.05 mol L^{-1} Tris − base and 1.0 mol L^{-1} NaCl (pH 7.5) under continuous stirring in at 4 °C for 48 h, and the solution was changed daily. At the end of treatment, the solution was removed and the tunica albuginea hydrolyzed with pepsin.

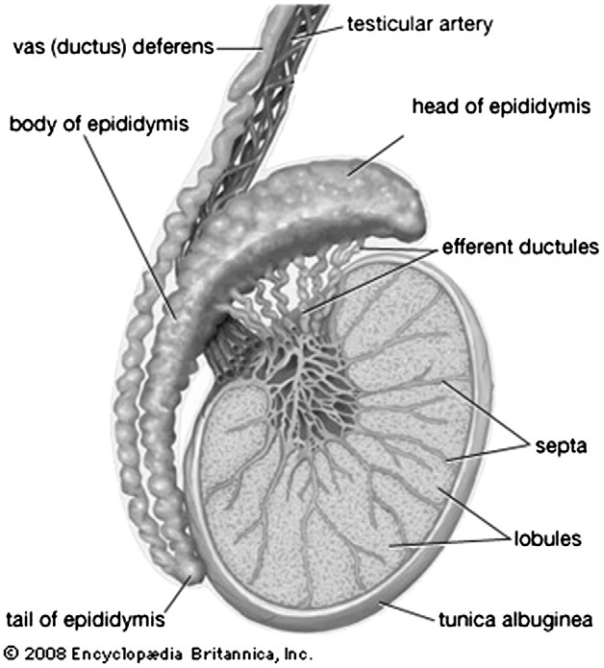

Fig. 1. Location of tunica albuginea in the testes. Source: Encyclopedia Britannica Online, 2013.

2.4. Pepsin hydrolysis conditions

To obtain isolated collagen (IC), the effect of the pepsin hydrolysis extraction conditions in an acetic acid solution was examined using the factorial design (2^{4-1}) with four independent variables (X_1 = acetic acid concentration mol L^{-1}, X_2 = time of acetic acid treatment, X_3 = pepsin percentage and X_4 = time of pepsin hydrolysis) at three levels, with three replicates at the central point for a total of 11 assays (Table 2), which were performed randomly.

Collagen extraction was performed as described in Shimokomaki et al. (1980). In each assay, a 1:10 ratio of the tunica albuginea mass per mL of acetic acid solution, expressed as mol L^{-1} (X_1), was used, and the acetic acid treatment was performed for different times, expressed in hours (X_2), the pH values of this solution varied from 2.0 to 2.5. At the end of the defined time period, the mixture was homogenized for 10 min in a turrax homogenizer (Marconi São Paulo, Brazil). Then, a percentage of pepsin (X_3) was added (Sigma E.C. 3.4.23.1 pepsin), and the hydrolysis was performed for different times, expressed in hours (X_4). The extraction and hydrolysis were carried out in refrigerated incubator (Shaker, Marconi, São Paulo, Brazil) at 4 °C with continuous stirring. After hydrolysis, the pH was adjusted to 7.5 using 1.0 mol L^{-1} NaOH to inactivate the pepsin activity. The material was centrifuged (Eppendorf centrifuge, Hamburg, Germany) for 30 min at 10,000 × *g* and 4 °C, and the precipitate (P_1) discarded. The supernatant (S_1) was subjected to saline precipitation with 3.0 mol L^{-1} NaCl and was centrifuged for 30 min at 10,000 × *g* and 4 °C. The supernatant (S_2) was discarded, and the precipitate (P_2) was dialyzed in 0.5 mol L^{-1} acetic acid solution for 72 h at 4 °C; the solution was replaced daily. The gelatinous precipitate was lyophilized (Christ Alpha lyophilizer, Osterode am Harz, Germany), resulting in the isolated collagen (IC), shown in Fig. 2.

The response function (Y_1) was expressed as g of collagen in 100 g of IC. Based on the evaluation of the response function (Y_1), the main effects of the independent variables (X_1, X_2, X_3 and X_4) and the interactions of the variables were calculated and the contour curves were performed using STATISTICA 7.0 (Statsoft Inc. Corporate Tulsa, OK, EUA) (Statsoft, 2004).

2.5. Optimal conditions for the extraction and hydrolysis to obtain the isolated collagen (IC)

The optimization of the extraction and hydrolysis conditions to obtain the IC was performed after the analysis of the independent variable effects X_1, X_2, X_3 and X_4 and interactions. To implement this optimization, a new experiment was performed by applying a central composite rotatable design (CCRD) (2^3) with five variation levels and three replicates at the central point for a total of 17 assays (Table 4), which were conducted randomly. The following independent variables were evaluated: X_5 (mol L^{-1} acetic acid), X_6 (pepsin percentage) and X_7 (h of pepsin hydrolysis).

Collagen extraction with acetic acid and pepsin hydrolysis was performed as described by Shimokomaki et al. (1980). For each assay, a ratio of 1:10 tunica mass per mL of mol L^{-1} acetic acid solution (X_5) was used, and the acetic acid treatment time was 12 h. At the end of this time, the mixture was homogenized for 10 min in a turrax homogenizer (Marconi São Paulo, Brazil). Then, a percentage of pepsin (X_6) was added and the hydrolysis was performed for different periods, measured in hours (X_7). The extraction and hydrolysis were performed in a refrigerated incubator (Shaker, Marconi, São Paulo, Brazil) at 4 °C with

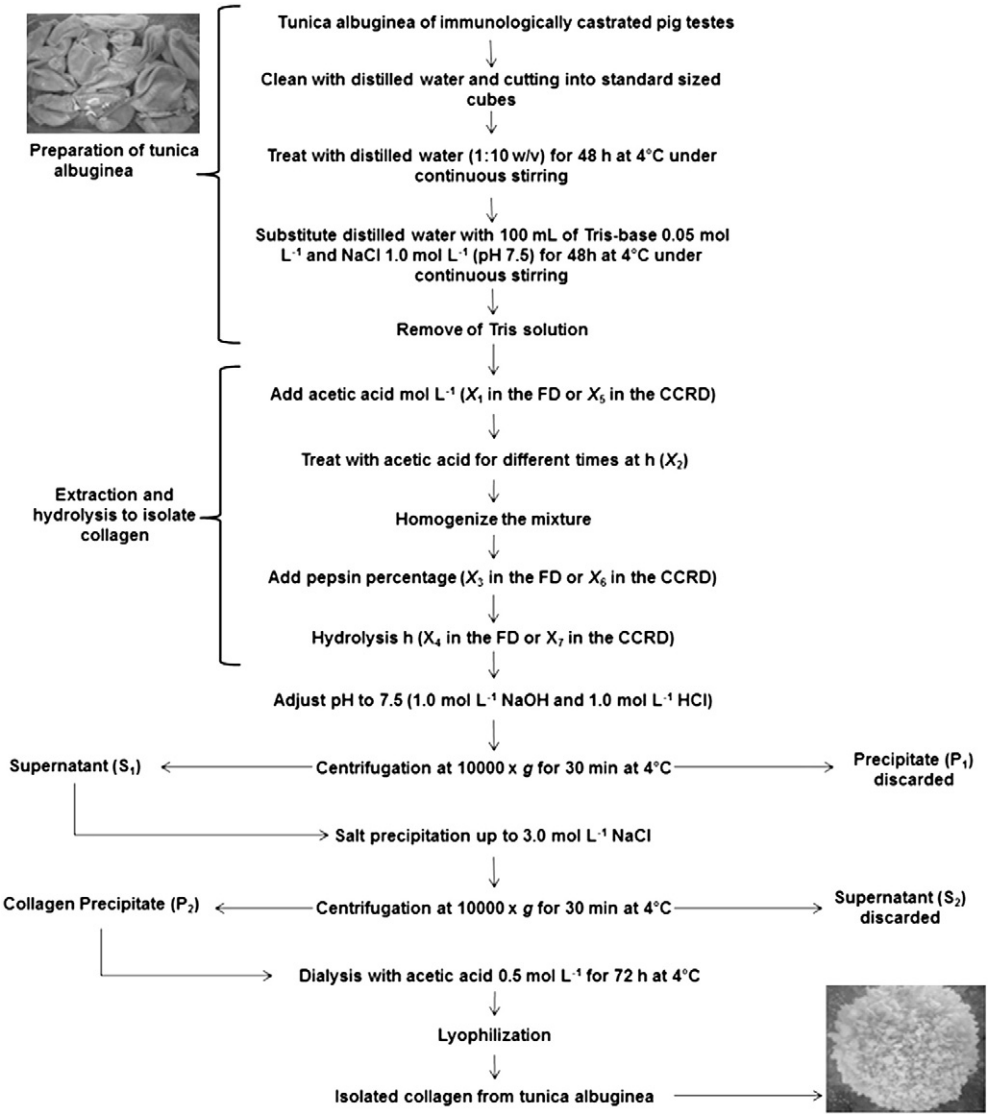

Fig. 2. Fluxogram showing the step-by-step extraction of collagen from the tunica albuginea of pig testes. FD: factorial design; CCRD: central composite rotatable design.

continuous stirring. The other procedures for IC isolation were performed as described in Section 2.4 and shown in Fig. 2.

The response function (Y_2) was expressed as g of collagen in 100 g of IC. The model fit to the experimental data was verified by analysis of variance (ANOVA) and the coefficient of determination (R^2). The contour curves were plotted, and the desirability parameters were estimated using STATISTICA 7.0 (Statsoft Inc. Corporate Tulsa, OK, EUA) (Statsoft, 2004). The responses obtained through the CCRD (2^3) (Table 4) were fitted to the model according to Eq. (1):

$$Y_2 = \beta_o + \beta_5 x_5 + \beta_{55} x_5^2 + \beta_6 x_6 + \beta_{66} x_6^2 + \beta_7 x_7 + \beta_{77} x_7^2 + \beta_{56} x_5 . x_6 + \beta_{57} x_5 . x_7 + \beta_{67} x_6 . x_7 + e \quad (1)$$

where Y_2 = response function; x_5, x_6 and x_7 = coded variables; β = estimated coefficient, e = residual (experimental error).

2.6. Model validation

The proposed model for the optimization of the extraction and hydrolysis conditions for IC isolation was validated in triplicate on the completion of a new experiment with the optimized conditions. The experimental results were compared with those estimated by the model using Student's *t* test ($p < 0.05$).

2.7. Yield and degree of purification of the isolated collagen

The yield of the IC and the degree of purification were determined after applying the optimal extraction method with pepsin. The yield was calculated as the ratio between the weight (g) of the IC and the initial weight (g) of the tunica albuginea multiplied by 100. The degree of purification was calculated as the ratio between collagen content of the

Table 1
Proximate chemical composition and collagen content of testis and tunica albuginea (g per 100 g).

Sample	Moisture*	Protein*	Lipids*	Ashes*	Collagen content*
Testis	82.47[a] (±0.62)	14.40[b] (±0.07)	5.78[a] (±0.57)	0.93[a] (±0.02)	10.90[b] (±0.91)
Tunica albuginea	76.15[b] (±0.52)	23.92[a] (±0.64)	6.19[a] (±0.53)	0.99[a] (±0.35)	23.24[a] (±0.11)

[a,b] Means followed by different letters in the same column differ in Tukey's test at the 5% significance level ($p < 0.05$); *g per 100 g of sample, dry matter basis.

Table 2
Factorial design (2^{4-1}) with the independent variables and response function (Y_1) of the conditions of pepsin hydrolysis.

	Independent variables and variation levels				Response function
Assays	X_1 (level) mol L^{-1} acetic acid	X_2 (level) h of acetic acid treatment	X_3 (level) pepsin percentage	X_4 (level) h of pepsin hydrolysis	Y_1* g of collagen per 100 g
1	0.20 (−1)	12 (−1)	0.05 (−1)	12 (−1)	48.31 ± 0.005
2	0.80 (+1)	12 (−1)	0.05 (−1)	36 (+1)	55.62 ± 0.010
3	0.20 (−1)	36 (+1)	0.05 (−1)	36 (+1)	49.01 ± 0.004
4	0.80 (+1)	36 (+1)	0.05 (−1)	12 (−1)	54.04 ± 0.090
5	0.20 (−1)	12 (−1)	0.15 (+1)	36 (+1)	54.92 ± 0.005
6	0.80 (+1)	12 (−1)	0.15 (+1)	12 (−1)	69.94 ± 0.006
7	0.20 (−1)	36 (+1)	0.15 (+1)	12 (−1)	54.55 ± 0.002
8	0.80 (+1)	36 (+1)	0.15 (+1)	36 (+1)	75.84 ± 0.012
9 (C)	0.50 (0)	24 (0)	0.10 (0)	24 (0)	61.91 ± 0.007
10 (C)	0.50 (0)	24 (0)	0.10 (0)	24 (0)	60.93 ± 0.002
11 (C)	0.50 (0)	24 (0)	0.10 (0)	24 (0)	62.10 ± 0.004

* Means and standard deviation (n = 3). (C) = Central Point.

IC (g per 100 g) and collagen content (g per 100 g) of the tunica albuginea.

2.8. Amino acid profile of isolated collagen

The IC amino acid profile was determined in duplicate at the Protein Chemistry Center, Faculty of Medicine of Ribeirao Preto (FMRP-USP). The sample was previously defatted and pyrolyzed at 400 °C for 8 h and hydrolyzed with HCl, 6.0 mol L^{-1} at 110 °C for 22 h. The amino acid derivatives were separated and quantified according to Bidlingmeyer, Cohen, and Tarvin (1984) and Atherton (1989).

2.9. Determination of the functional properties of the isolated collagen in comparison to bovine skin collagen

The functional properties analyzed in the IC and in the bovine skin collagen (NovaProm Food Ingredients Ltda, São Paulo, Brazil) were: water solubility at 70 °C, water-holding capacity at 25 °C and 60 °C, emulsifying capacity and emulsion stability. All analyses were performed in triplicate and the difference between means determined by Tukey's test ($p < 0.05$).

The water solubility at 70 °C (Timpl et al., 1975) and the soluble collagen content (Woessner, 1961) were determined and the results expressed as a percentage of soluble collagen compared to the total sample collagen content. The water-holding capacity (WHC) at 25 °C and 60 °C was evaluated following the method of Montero, Alvarez, Martí, and Borderías (1995), and the results expressed as g of water held per g of dry sample. The emulsifying capacity was assessed following the method of Montero and Borderias (1991), substituting acetic acid for distilled water to solubilize the sample, and the ratio of sample to water was 1:40 (w/v). The emulsifying capacity was expressed as mL of emulsified oil per mg of soluble collagen. The stability of the emulsion was evaluated following the method of Ribeiro, Prudencio, Myagui, and Ribeiro (2009).

The pH values were determined in triplicate using a pH meter (Mettler Toledo, Switzerland) after homogenization of 1 g of sample in 10 mL of distilled water.

3. Results and discussion

3.1. Proximate chemical composition

Table 1 shows the proximate chemical composition of the testes and tunica albuginea. The tunica albuginea had approximately twice as much collagen because of its structural and protective role surrounding the testes (Copenhaver et al., 1978). Conversely, the testes showed a higher amount of moisture, but the lipid and ash contents were not significantly different ($p > 0.05$). The collagen content of the skin, tendons, bones and stomachs of mammals varies from 15% to 95% (Bailey & Light, 1989; Balian & Bowes, 1977). Comparison of the chemical composition of the tunica albuginea with calf skin and pig skin showed that the moisture contents of these tissues were similar, while the protein content was lower in the tunica, as reported in several other studies.

3.2. Conditions of pepsin hydrolysis

A factorial design (2^{4-1}) was used to evaluate the effects of the variables X_1 (mol L^{-1} acetic acid), X_2 (h of acetic acid treatment), X_3 (pepsin percentage) and X_4 (h of pepsin hydrolysis) in the extraction and hydrolysis to obtain IC (Table 2).

The variables X_1, X_3 and X_4 showed a positive and significant effect ($p < 0.05$) on the Y_1 response function (Table 3). These results indicate that the optimal conditions for extraction and hydrolysis occurred when using positive levels of variables X_1 (mol L^{-1} acetic acid), X_3 (pepsin percentage) and X_4 (h of pepsin hydrolysis). Assay 8 (Table 2) showed that the isolated collagen had a value of $Y_1 = 75.84 \pm 0.012$ g of collagen per 100 g of IC. This result was obtained by applying the highest levels of the four variables, i.e., $X_1 = 0.8$ mol L^{-1} acetic acid, $X_2 = 36$ h of acetic acid treatment, $X_3 = 0.15\%$ pepsin and $X_4 = 36$ h of hydrolysis. However, the effect of variable X_2 on the Y_1 response

Table 3
Effect estimates for the response function* from the results in the factorial design (2^{4-1}) of the conditions of pepsin hydrolysis.

Independent variable	Regression coefficient	Standard error	t(2)	p-value
Mean	58.834	0.189	310.774	0.000**
X_1 (mol L^{-1} acetic acid)	6.081	0.444	27.394	0.001**
X_2 (h of acetic acid treatment)	0.581	0.444	2.618	0.120
X_3 (pepsin percentage)	6.034	0.444	27.180	0.001**
X_4 (h of pepsin hydrolysis)	1.069	0.444	4.814	0.040**
$X_1.X_2$	0.499	0.444	2.246	0.153
$X_1.X_3$	2.996	0.444	13.497	0.005**
$X_1.X_4$	0.801	0.444	3.609	0.068

*Y_1 = g of collagen per 100 g; **significant factors ($p < 0.05$).

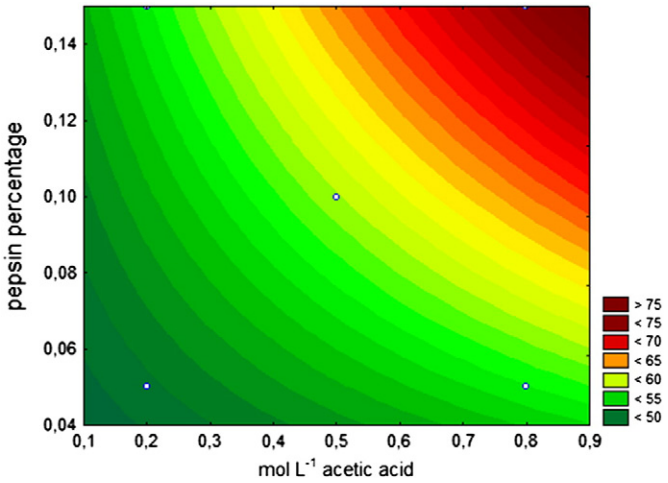

Fig. 3. Contour curves for conditions of pepsin hydrolysis to obtain IC as a function of the X_1 (mol L^{-1} acetic acid) and X_3 (pepsin percentage), setting the variable X_4 (h of pepsin hydrolysis) to 24 h.

function was not significant; therefore, the effect of X_2 was removed from the model.

The effect of the interaction between the variables X_1 (mol L^{-1} acetic acid) and X_3 (pepsin percentage) on the Y_1 response function (Table 3) was significant and positive ($p < 0.05$). This result was observed in assay 6 (Table 2), in which the highest levels of the variables X_1 and X_3, i.e., $X_1 = 0.8$ mol L^{-1} acetic acid and $X_3 = 0.15\%$ pepsin, were used, and an isolate with 69.94 ± 0.006 g of collagen per 100 g was produced.

The contour curves were plotted based on the Y_1 response function (g of collagen per 100 g) and Fig. 3 shows that using the variables X_1 (mol L^{-1} acetic acid), X_3 (pepsin percentage) and X_4 (h of pepsin hydrolysis) at higher levels results in a product with a higher collagen concentration ($Y_1 > 70$). Thus, these results confirmed that these three variables have a significant effect on acetic acid extraction and pepsin hydrolysis.

3.3. Optimal conditions for pepsin hydrolysis

In the first design, the acetic acid treatment time had no significant effect on IC production. Thus, to optimize the process, the CCRD (2^3) was used for the variables X_5 (mol L^{-1} acetic acid), X_6 (pepsin percentage) and X_7 (h of pepsin hydrolysis) (Table 4), and the acetic acid treatment time was fixed at 12 h. The effects of the variables on the Y_2 response function and multiple regression coefficients are shown in Table 5.

The linear effects of the variables X_5 (mol L^{-1} acetic acid), X_6 (pepsin percentage) and X_7 (h of hydrolysis) were positive and significant (Table 5). These results indicate the +1 level of these variables produced an IC with high collagen concentration. This result could be observed in assay 8 (Table 4), in which the variables $X_5 = 0.9$ mol L^{-1} acetic acid, $X_6 = 0.20\%$ pepsin and $X_7 = 30$ h of pepsin hydrolysis were used and an IC with 77.75 ± 0.006 g of collagen per 100 g was produced.

The quadratic effects of the variables X_5, X_6 and X_7 were significant and negative (Table 5). In assays 9 and 10 (Table 4), in which the lowest level of X_5 (0.3 mol L^{-1} acetic acid) and the highest level of X_5 (1.1 mol L^{-1} acetic acid), respectively, were used with X_6 (0.15% pepsin) and X_7 (24 h of hydrolysis) for the central points, the response function Y_2 was reduced. These results indicate that the optimum point of the variable X_5 (mol L^{-1} acetic acid) is among these values. Because of the high extraction capacity, acetic acid is the most widely used organic solvent for collagen extraction, but the acetic acid concentration can affect the collagen extraction (Cheng, Hsu, Chang, Lin, & Sakata, 2009; Wang et al., 2008). In the present study, the optimal concentration of acetic acid predicted by the model was 0.8 mol L^{-1}.

Assay 12 (Table 4) showed that applying the variable X_6 at the highest level (0.25% pepsin) with X_5 (0.7 mol L^{-1} acetic acid) and X_7 (24 h of hydrolysis) at the central point increased the response function Y_2 (81.68 ± 0.056 g of collagen per 100 g of IC). However, in assay 11 (Table 4), in which X_6 was used at the lowest level (0.05% pepsin) with X_5 (0.7 mol L^{-1} acetic acid) and X_7 (24 h of hydrolysis) at the central points, the response function Y_2 was reduced (48.04 ± 0.001 g of collagen per 100 g of IC). These results show the real positive effect of the variable X_6 (pepsin percentage). The pepsin digestion in an acidic medium increased tissue collagen extraction yield because this enzyme removes the non-helical ends of the collagen molecule and the intermolecular crosslinks (Skierka & Sadowska, 2007).

With respect to the variable X_7 (h of pepsin hydrolysis), assays 13 and 14 (Table 4), in which higher (36 h) or lower levels (12 h) and X_5 (mol L^{-1} acetic acid) and X_6 (pepsin percentage) at the central points were used, showed similar response functions Y_2 (72.70 ± 0.026 and 74.63 ± 0.005 g of collagen per 100 g of IC). An extended hydrolysis time increases the yield of collagen, as observed by Wang et al. (2008) and Shon et al. (2011), but in this study, a low effect of pepsin hydrolysis time on the response function Y_2 was observed.

Table 4
Central composite rotatable design (2^3) with the independent variables, levels and experimental responses (Y_2 = g of collagen per 100 g) of the extraction and hydrolysis to obtain the IC.

Assay	Independent variables			Response function
	X_5 (level) mol L^{-1} acetic acid	X_6 (level) pepsin percentage	X_7 (level) h of pepsin hydrolysis	Y_2^* g of collagen per 100 g
1	0.5 (−1)	0.10 (−1)	18 (−1)	57.26 ± 0.003
2	0.5 (−1)	0.10 (−1)	30 (+1)	58.07 ± 0.001
3	0.5 (−1)	0.20 (+1)	18 (−1)	62.89 ± 0.010
4	0.5 (−1)	0.20 (+1)	30 (+1)	63.61 ± 0.005
5	0.9 (+1)	0.10 (−1)	18 (−1)	58.32 ± 0.021
6	0.9 (+1)	0.10 (−1)	30 (+1)	58.39 ± 0.002
7	0.9 (+1)	0.20 (+1)	18 (−1)	76.96 ± 0.020
8	0.9 (+1)	0.20 (+1)	30 (+1)	77.75 ± 0.006
9	0.3 (−1.68)	0.15 (0)	24 (0)	41.12 ± 0.013
10	1.1 (+1.68)	0.15 (0)	24 (0)	50.76 ± 0.007
11	0.7 (0)	0.05 (−1.68)	24 (0)	48.04 ± 0.001
12	0.7 (0)	0.25 (+1.68)	24 (0)	81.68 ± 0.056
13	0.7 (0)	0.15 (0)	12 (−1.68)	72.70 ± 0.026
14	0.7 (0)	0.15 (0)	36 (+1.68)	74.63 ± 0.005
15	0.7 (0)	0.15 (0)	24 (0)	75.17 ± 0.037
16	0.7 (0)	0.15 (0)	24 (0)	74.80 ± 0.011
17	0.7 (0)	0.15 (0)	24 (0)	75.00 ± 0.016

*Means and standard deviation (n = 3).

Table 5
Effect estimates for the response function* from the results in the central composite rotatable design (2^3) of the extraction and hydrolysis to obtain the IC.

Independent variable	Regression coefficient	Standard error	t(2)	p-value
Mean	74.874	0.102	732.826	0.000**
(X_5) mol · L^{-1} acetic acid (L)	3.242	0.093	70.018	0.000**
(X_5) mol · L^{-1} acetic acid (Q)	−7.277	0.084	−172.922	0.000**
(X_6) pepsin percentage (L)	7.466	0.093	161.242	0.000**
(X_6) pepsin percentage (Q)	−2.547	0.084	−60.523	0.000**
(X_7) h of pepsin hydrolysis (L)	0.203	0.093	4.387	0.048**
(X_7) h of pepsin hydrolysis (Q)	−0.346	0.084	−8.215	0.014**
$X_5.X_6$	3.729	0.131	56.946	0.000**
$X_5.X_7$	−0.459	0.131	−7.006	0.019**
$X_6.X_7$	−0.296	0.131	−4.524	0.045**

* Response function Y_2 = g of collagen per 100 g;
** significant factors ($p < 0.05$).

The effect of the $X_5.X_6$ interaction (mol L^{-1} acetic acid and pepsin percentage) was significant and positive, whereas the interactions $X_5.X_7$ (mol L^{-1} acetic acid and h of hydrolysis with pepsin) and $X_6.X_7$ (pepsin percentage and h of pepsin hydrolysis) were significant and negative (Table 5).

Table 6
ANOVA of the quadratic model for the extraction and hydrolysis to obtain the IC.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	$F_{calculated}$
Regression	2327.795	9	258.644	65.314*
Residual	27.720	7	3.96	
Total	2355.515	16		

$F_{tabulated\ (0.05;\ 9;\ 2)} = 19.38$; *$F_{calculated} > F_{tabulated}$ = significant regression.
Coefficient of determination (R^2) = 0,98.

Analysis of variance indicated that the proposed model was significant (Table 6). The high coefficient of determination ($R^2 = 0.98$) showed that the response function adequately fits the experimental data and that the model can be used to predict the extraction and hydrolysis of collagen of the tunica albuginea to obtain IC. Thus, considering the significant variables and interactions, the proposed model (Eq. (2)) can be expressed as:

$$Y_2 = 74,874 + 3,242x_5 - 7,277{x_5}^2 + 7,466x_6 - 2,547{x_6}^2 + 0,203x_7 - 0,346{x_7}^2 + 3,729x_5.x_6 - 0,459x_5.x_7 - 0,296x_6.x_7 \quad (2)$$

where Y_2 = g of collagen per 100 g of IC; X_5 = mol L^{-1} acetic acid; X_6 = pepsin percentage and X_7 = h of pepsin hydrolysis.

The surface response was constructed based on the Y_2 response function and the desirability parameters were estimated (Figs. 4 and 5). The surface response (Fig. 4) shows a favorable region to obtain IC with collagen content above 80% ($Y_2 > 80$ g of collagen per 100 g of IC). This area was observed where X_5 is between 0.7 and 0.9 mol L^{-1} acetic acid and X_6 is greater than 0.20% pepsin. The estimated desirability parameters (Fig. 5) indicated that the optimal response function was 81.71 g of collagen per 100 g of isolated collagen and that this response was obtained with $X_5 = 0.86$ mol L^{-1} acetic acid, $X_6 = 0.25\%$ pepsin and $X_7 = 36$ h of pepsin hydrolysis. However, there was a smaller effect of variable X_7 (h of hydrolysis). According to the estimated desirability parameter (Fig. 5), 24 h of hydrolysis may be sufficient to obtain an isolate with 81.71 g of collagen per 100 g.

The desirability parameters (Fig. 5) indicate that increasing the pepsin percentage (variable X_6) results in an IC with higher collagen content. However, when increasing the pepsin percentage from 0.30% to 0.40% and 0.50% while maintaining the other variables at 0.86 mol L^{-1} acetic acid and 24 h of pepsin hydrolysis, there was no significant change ($p > 0.05$) in the collagen content of the IC. Therefore, a level of 0.25% pepsin was considered to be sufficient to obtain IC with high collagen content.

Collagen extraction from different animal sources has been evaluated, but these experiments used different methods of extraction and obtained different yields compared to the present study. Wang et al. (2008) found that the best conditions for extracting acid-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*) applying the Box–Behnken design, were using 0.54 mol L^{-1} acetic acid, at 24.7 °C for 32.1 h, but it only produced a concentrate containing 19.3 g of collagen per 100 g. Muralidharan, Shakila, Sukumar, and Jeyasekaran (2011) found that pepsin extraction in an acidic medium resulted in a higher

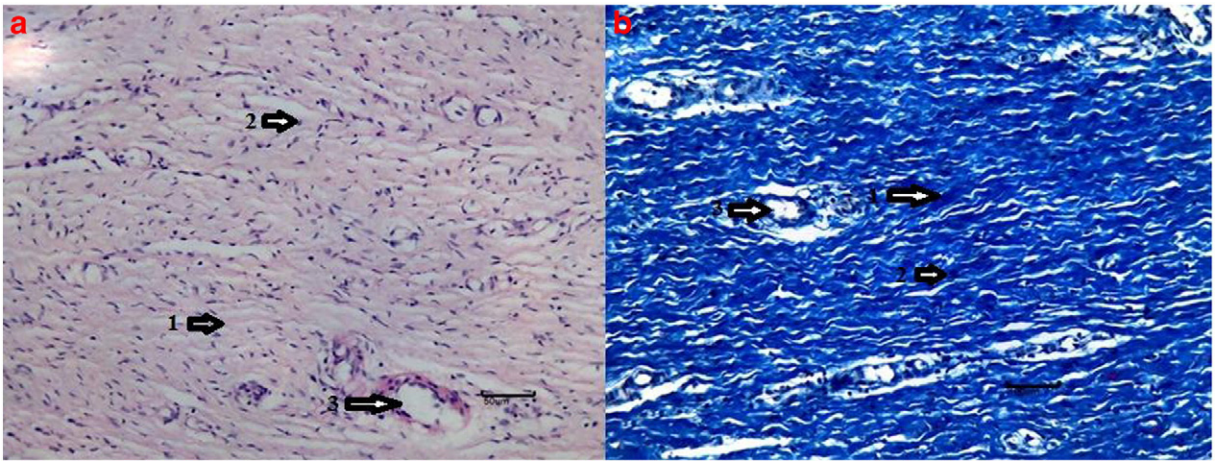

Fig. 4. Surface response model for extraction and hydrolysis to obtain IC as a function of the X_5 (mol L^{-1} acetic acid) and X_6 (pepsin percentage), setting the variable X_7 (h of pepsin hydrolysis) to 24 h.

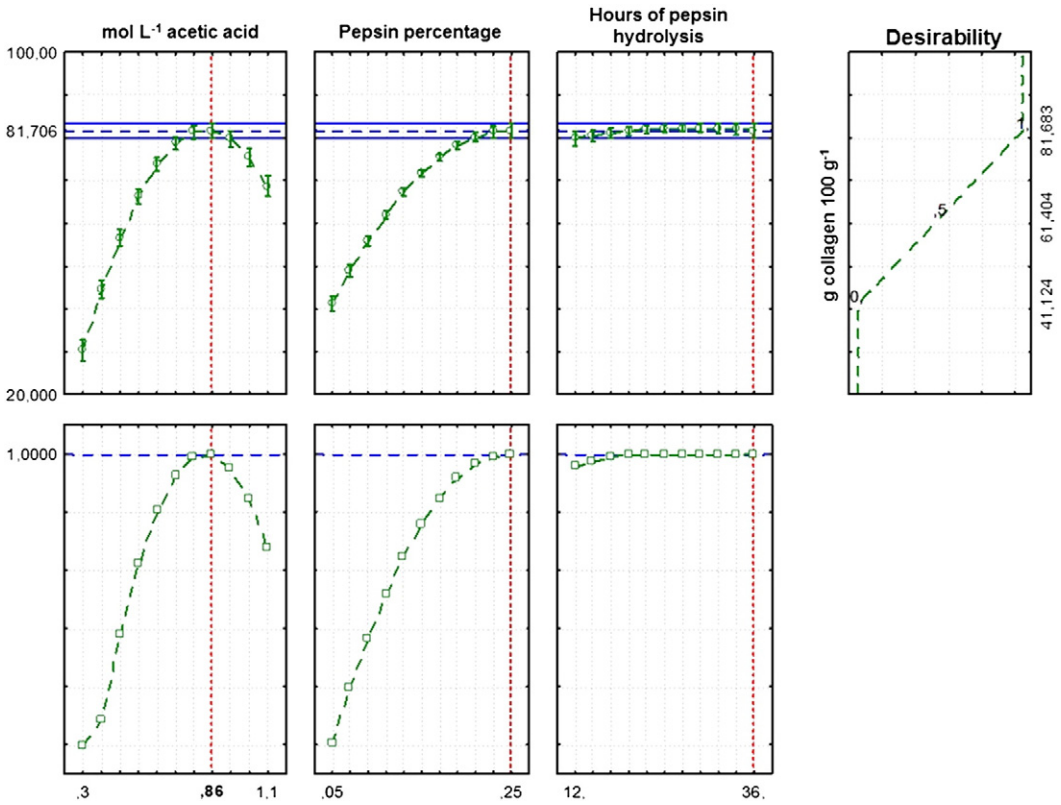

Fig. 5. Parameters of estimated desirability for the variables X_5 (mol L^{-1} acetic acid), X_6 (pepsin percentage) and X_7 (h of pepsin hydrolysis) for extraction and hydrolysis to obtain the IC.

yield than extraction with only acetic acid in the red-toothed triggerfish (*Odonus niger*), and obtained a concentrate of 70.94 g of collagen per 100 g when using pepsin. The optimum conditions for the extraction of a concentrate with 26.7 g of collagen per 100 g from the dorsal skin of yellowfin tuna (*Thunnus albacores*) were observed by Woo et al. (2008) using the central composite rotatable design and 0.92 mol L^{-1} NaOH, 24 h of NaOH treatment, 0.98% pepsin and 23.5 h of pepsin hydrolysis.

Table 7
Amino acid profile of the isolated collagen from the tunica albuginea (results are expressed as residues per 1000 total amino acid residues).

Amino acids	Residues 1000 total residues^{-1}
Aspartic acid *	60
Glutamic acid *	118
Alanine **	72
Arginine *	87
Cysteine ***	0
Phenylalanine **	26
Glycine **	223
Hydroxyproline **	101
Histidine *	11
Isoleucine **	13
Leucine **	34
Lysine *	29
Methionine **	12
Proline **	112
Serine **	43
Tyrosine **	13
Threonine **	25
Valine **	22
Total	1000

* Hydrophilic amino acids.
** Hydrophobic amino acids.
*** nd = not detected.

3.4. Model validation

Based on the response function (Y_2) and the model proposed in the optimization process it was estimated that the values of $X_5 = 0.83$ mol L^{-1} acetic acid, $X_6 = 0.24\%$ pepsin and $X_7 = 28$ h of pepsin hydrolysis should be used to obtain an IC with 82.54 g of collagen per 100 g.

Thus, the optimal points were experimentally reproduced in triplicate, and the response obtained was 81.9 ± 0.40 g of collagen per 100 g of IC. The means of the experimental results and the results estimated by the model were not significantly different ($p > 0.05$), thereby confirming the validity of the proposed model.

3.5. Yield and degree of purification of the collagen isolate

Applying the optimal conditions for the acetic acid extraction and pepsin hydrolysis, 3 g of IC from 10 g of tunica albuginea was obtained. Thus, the yield of IC was 30.00 ± 1.33%, as the tunica albuginea had 23.24 ± 0.11 g of collagen per 100 g and the IC had 81.9 ± 0.40 g of collagen per 100 g; the degree of purification of IC was 3.5. The yield of collagen depends on the raw material, the extraction method and the hydrolysis. The extraction yield of collagen from different raw materials varied from 30.0% to 70.0% (Liu, Lin, & Chen, 2001; Montero, Borderias, Turnay, & Leyzarbet, 1990; Muralidharan et al., 2011; Yamaguchi, Lavéty, & Love, 1976).

3.6. Amino acid profile of isolated collagen

The amino acid profile of the tunica IC (Table 7) indicates the presence of 69.60% hydrophobic amino acids and 30.40% hydrophilic amino acids. Bailey and Light (1989) reported that collagen molecules consist of approximately 60% hydrophobic amino acids and 40% hydrophilic amino acids. The high content of hydrophobic amino acids

Table 8
Mean and standard deviation of the water solubility at 70 °C, the hot and cold water absorption capacity, the emulsifying capacity and the emulsion stability of the isolated collagen from the tunica albuginea and bovine skin collagen.

	Isolated collagen from the tunica albuginea	Bovine skin collagen
Water solubility at 70 °C*	70.02 ± 0.96[a]	60.00 ± 0.96[b]
Cold water-holding capacity**	5.98 ± 0.06[a]	3.82 ± 0.10[b]
Water-holding capacity at 60 °C**	35.63 ± 0.42[a]	24.00 ± 1.80[b]
Emulsifying capacity ***	31.56 ± 1.40[a]	23.63 ± 0.80[b]
Emulsion stability****	84.50 ± 0.55[a]	65.2 ± 0.76[b]

[a,b] Means followed by different letters in the same row differ in Tukey's test at the 5% significance level ($p < 0.05$).
* Percentage soluble collagen relative to the total collagen content of the sample.
** g water·g^{-1} dry residue^{-1}.
*** mL emulsified oil.mg^{-1} soluble collagen.
**** Percentage remaining in the emulsion relative to the initial emulsion quantity.

provides suitable properties for an emulsifier in emulsified products (Bailey & Light, 1989; Montero & Borderias, 1991).

Glycine was the most abundant amino acid, followed by proline, hydroxyproline and glutamic acid (Table 7). These results were similar to those observed in collagen extracted from various raw materials, such as pig skin (Lin & Liu, 2006), fish skin (Benjakul et al., 2010; Yan et al., 2008), yak bones (*Bos grunniens*) (Li, Jia, & Yao, 2009) and skin streak (Shon et al., 2011).

It was observed that 21.3% of the total amino acids were the imino acids proline and hydroxyproline (Table 7). These imino acids are crucial in the formation of the triple helix of the molecule and are related to thermal stability (Matsumura, 1972). Lin and Liu (2006) found that the proportion of hydroxyproline and proline in collagen from pig skin ranged from 17.00 to 18.50%, which is lower than the content in collagen isolated from the tunica albuginea; however, Li et al. (2009) observed that these imino acids constituted 20.19% of yak bone collagen (*B. grunniens*), which is similar to the content in collagen isolated from the tunica albuginea. The content of imino acids in collagen influences its functional properties; collagen with 15% imino acids showed reduced emulsifying capacity and gel formation (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). Therefore, the amino acid profile indicates that the tunica albuginea IC could have properties suitable for gelling and emulsification. The amino acids present in lower amounts were methionine, tyrosine, isoleucine, and histidine. The low content of these amino acids was also observed in collagen extracted from materials, such as pig skin (Lin & Liu, 2006) fish skin (Benjakul et al., 2010; Yan et al., 2008), yak bones (*B. grunniens*) (Li et al., 2009) and skin streak (Shon et al., 2011).

3.7. Chemical characterization and functional properties

The IC produced under optimal conditions (0.83 mol L^{-1} acetic acid, 0.24% pepsin and 28 h of pepsin hydrolysis) presented 11.16 ± 1.10 g of moisture, 82.17 ± 2.08 g of protein, 3.24 ± 1.20 g of lipids and 3.43 ± 0.07 g of ash (values expressed in g per 100 g of IC). Olivo and Shimokomaki (2002) obtained an isolated collagen from bovine tendons with chemical characteristics similar to those of tunica albuginea IC, however, the IC obtained from chicken feet by Alves and Prudêncio-Ferreira (2002) contained high lipid content.

Because of the recent increase in the economic value of collagen for industrial byproducts, several studies have investigated alternative sources for various applications (Gómez-Guillén et al., 2011). Collagen may confer stability to the fat globules in emulsions and improve the texture of meat products (Gordon & Barbut, 1992; Olivo & Shimokomaki, 2002). Thus, the functional properties of tunica albuginea IC were determined and compared with those of bovine skin collagen (Table 8).

The IC had higher ($p < 0.05$) water solubility at 70 °C than bovine skin collagen (Table 8), collagen solubility is influenced by its molecular structure, the presence of ionizable polar groups formed during extraction by the hydrolysis conditions (Li et al., 2013; Shon et al., 2011). Montero et al. (1990) observed that the solubility of fish skin collagen (*Merluccius merluccius* L.) was 90.60 ± 1.9%, while the solubility of fish muscle collagen (*M. merluccius* L.) was 69.6 ± 2.1%. Alves and Prudêncio-Ferreira (2002) extracted collagen from chicken feet with a solubility of 66.09%. The IC showed a higher ($p < 0.05$) WHC at 25 °C and 60 °C than bovine skin collagen (Table 8). Ranganayaki, Asghar, and Henrigkson (1982) observed that the WHC of bovine hide collagen ranged from 5.0 to 6.0 g of water per gram of collagen, and heating to 60 °C increased the WHC. The effect of heating at 60 °C was observed in the IC and bovine skin collagen (Table 8). Olivo and Shimokomaki (2002) reported that an isolated collagen with a WHC at 25 °C of 5.91 ± 0.31 improved the performance of emulsified meat products of low fat content. Finally, the IC presented a higher ($p < 0.05$) emulsifying capacity and emulsion stability than bovine skin collagen (Table 8). In emulsified meat products, the main function of collagen is to improve the stability of the product during processing (Bailey & Light, 1989). Olivo and Shimokomaki (2002) observed that bovine tendon IC with an emulsifying capacity of 24.47 ± 0.90 mL of oil emulsified per g of collagen improved the texture of emulsified meat products.

In the present study, the extraction conditions with acetic acid and pepsin hydrolysis were optimized, and produced an IC with functional properties suitable for use in processed meat products. The tunica albuginea of pigs testes is an excellent source of collagen.

4. Conclusions

Optimum collagen isolation from immunologically castrated pigs testes was observed under the following conditions: 0.83 mol L^{-1} acetic acid, 0.24% pepsin and 28 h of pepsin hydrolysis at 4 °C. These conditions resulted in the isolation of 82.54 g of collagen per 100 g with a yield of 30.00 ± 1.33%. The isolated collagen presented an amino acid profile and chemical and functional properties suitable for use in emulsified meat products.

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