Meat Science 96 (2014) 1460–1468

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

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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ARTICLE INFO

Article history:
Received 26 August 2013
Received in revised form 25 October 2013
Accepted 29 October 2013

Keywords:
Factorial design
Central composite rotatable design
Hydroxyproline
Collagen emulsified products

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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0309-1740/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.meatsci.2013.10.038

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.

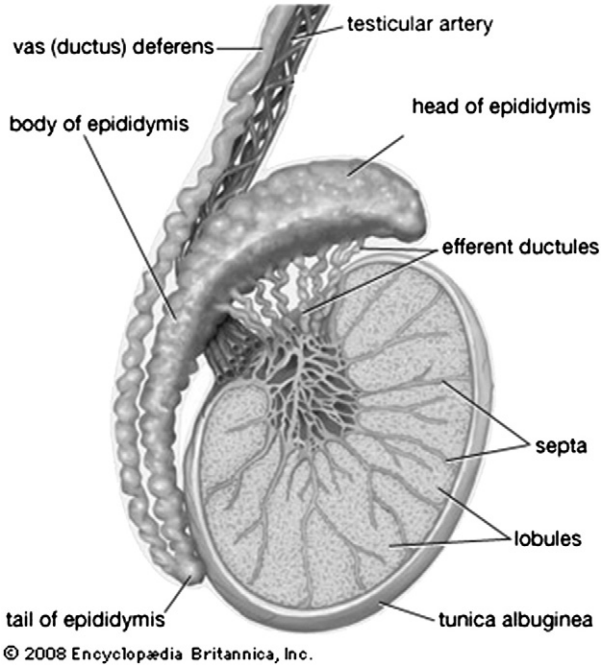

Fig. 1. Location of tunica albuginea in the testes. Source: Encyclopedia Britannica Online, 2013.

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