

ISOLATION AND PARTIAL CHARACTERIZATION OF PEPSIN-SOLUBLE COLLAGEN FROM PIGS TESTICLES TUNICA ALBUGINEA

Simões, G. S.¹; Bracarense, A. P. F. R. L.²; Silveira, E. T. F.³; Ida, E. I.¹; Shimokomaki, M.^{1,2,4*}

¹ Food Science Graduate Program, Dept. of Food Science and Technology, Londrina State University, Londrina, PR, Brazil

² Animal Sciences Graduate Program, Dept. of Preventive Veterinary Medicine, Londrina State University, Londrina, PR, Brazil

³ Meat Technology Centre, CTC - Institute of Food Technology, Campinas, SP, Brazil

⁴ Food Technology Graduate Program, Federal Technological University of Paraná, Londrina, PR, Brazil.

*email: mshimo@uel.br

Abstract – Pig testicles are surrounded by a capsule of dense connective tissue called tunica albuginea, and the objective of this work was to extract and characterize the obtained collagen fractions from immunocastrated pig. Results showed the tissue contained a high total collagen content of 17.82% and a solid presence within the tissue as shown by optical microscopy with Masson staining. In order to extract the collagen fraction, tunica albuginea was digested with pepsin and the solubilized protein were subjected to salt fractionation firstly by acid and subsequently the 0.7M pellet was also sequentially precipitate at neutral pH. The precipitates obtained were evaluated by SDS-PAGE and the electrophoretic patterns showed the omnipresence of type I and also types III and V. Various reducible collagenous fragments were also observed.

Key Words – extraction of pepsin solubilized collagen, salt fractionation, histological assessment.

I. INTRODUCTION

Collagen is the dominant and ubiquitous protein in the body which accounts for about thirty per cent of the total vertebrate protein. It has been extracted from mammals in particular cattle and pig skin for food, cosmetics, and medical products. In order to make it soluble for its maximum extraction routinely is treated with pepsin under specific conditions. There is a need to have this enzymatic treatment by digesting the crosslinking sites of the protein as its triple helix structure protects it against this enzyme activity. The presence of this collagen crosslinking renders stability to the protein as the animal become older due to firstly the presence of immature collagen crosslink, hydroxylysino-ketone-leucine (HLKNL) lately is biologically replaced by the main mature crosslink derived from two reducible HLKNL, pyridinoline stabilizing the collagen molecules

(1,2). In the early days, pepsin digestion methodology has been applied in various conditions as the isolation of new collagen type from pig cartilaginous tissue and today known as type IX (3) and also to identify different collagen types as I, II, III and V from mechanically deboned chicken meat of chicken (4). Furthermore, in recent decades, there has been growing interest in the evaluation of industrial by-products, and thus there have been many studies on the collagen extraction from different animal sources. Brazil produces over 10 mi of immunocastrated pigs thus the testicles is quantitatively becoming a rather important economical sub product representing around 0.3 to 0.6% of carcass weight. The immunological castration of male pigs is used as a modern alternative to surgical castration in order to provide animal welfare and further is an efficient odour control and appropriate animal performance (4). Pig testis are surrounded by a capsule of dense connective tissue called tunica albuginea, which is characterised as a resistant tissue composed of collagen fibres (6). Experiments on the characterisation of the tunica albuginea collagen are scarce, thus the objective of this work was to extract and to characterize the obtained collagen fractions by pepsin digestion and by sequential salt precipitation fractionation from immunocastrated pig testicles tunica albuginea.

II. MATERIALS AND METHODS

Raw material

The immunocastrated crosses Landrace and Large White pigs of 110 to 115 kg were slaughtered in a commercial pig slaughterhouse and testicles were removed and frozen. The testis tunica albuginea was manually removed

and used for collagen extraction. The Fig. 1 shows the location of the tunica albuginea in the pig testicles.

Chemical composition and total collagen content determination

The proximate chemical composition of tunica albuginea was determined as in (7) and the total collagen content was determined by hydroxyproline content (8) multiplied by 8.0 (9).

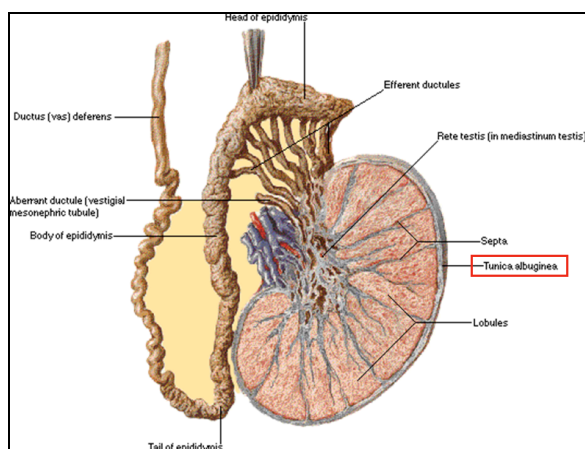


Figure 1- Location of tunica albuginea in the pig testicles <http://www.becomehealthynow.com/popups/male testes.htm>

Histological assessment

Samples of tunica albuginea were collected immediately after slaughter, fixed in 10% buffered formalin solution, and subsequently dehydrated through graded alcohols and embedded in paraffin wax. Classic staining of 3- μ m sections by hematoxylin/eosin and Masson stainings were performed to characterize collagen fibers and visualize under optical microscopy (Leica).

Preparation of the tunica albuginea

The tunica albuginea was manually removed from the testi, thoroughly washed in distilled water and cut in small size. For cleaning, 10 g of the tunica albuginea was immersed in 100 mL of distilled water under several changes daily and subjected to continuous stirring at 4°C for 48 h. In order to remove the non collagenous components distilled water was replaced with 100 mL of a solution containing 0.05 mol L⁻¹

Tris-base and 1.0 mol L⁻¹ NaCl (pH 7.5) with continuous stirring at 4°C for 48 h with several daily solution changes. Finally the solution was poured out and collagen was extracted.

Extraction of pepsin solubilized collagen

The extraction of collagen followed the method as described in (10). A ratio of 1:10 (w/v) of cut tunica albuginea in 0.5 mol L⁻¹ acetic acid (pH 2.5) and the mixture was treated in Turrax homogenizer and pepsin was added in ratio of 1:10 (w/w). Hydrolysis was performed for 24 h at 4°C under continuous stirring and interrupted by raising the solution to neutral pH and the solubilized collagens were separated by sequential salt fractionation.

Salt fractionation

The solubilized collagens were separated by sequential salt fractionation as described in (3). This technique is summarized in Fig. 2. All procedures were carried out 4°C. All pellets obtained by centrifugation were exhaustively dialyzed with 0.5 mol L⁻¹ acetic acid and lyophilized.

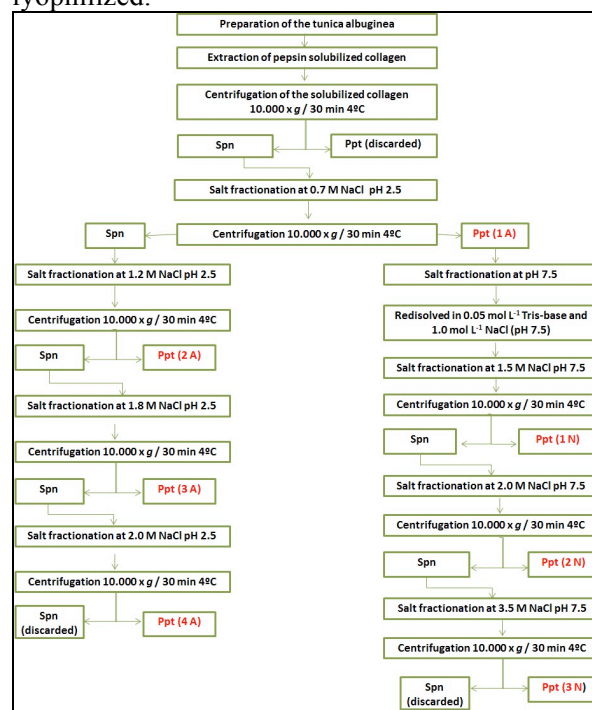


Figure 2 – Fractionation of pepsin-solubilized collagen from tunica albuginea by sequential salt precipitation. Ppt: Precipitate; Spn: Supernate.

Sodium Dodecyl Sulfate-Polyacrilamide Gel Electrophoresis SDS-PSGE

SDS-PAGE was performed as described in (3,4). Essentially, lyophilized samples (5mg) were dissolved in 1.0 mL of SDS 2% solution and 1.0 mL of the sample buffer (Tris-HCl pH 6.8, glycerol, bromophenol blue, SDS 5% and β -mercaptoethanol (+) only for the reducing conditions) and heated at 60°C for 30 min. Then 10 μ l were loaded with molecular weight markers and standard of type I collagen from rat tail tendon. Electrophoresis was carried out at 25 mA and 100 V. Bands were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 dissolved in water, methanol and acetic acid (9:9:2, v/v/v) for 12 h, then destained using a solution containing water, methanol and acetic acid (8:1:1, v/v/v).

III. RESULTS AND DISCUSSION

Chemical composition, total collagen content of the tunica albuginea and histological assessment

The Table 1 shows the proximate chemical composition and a high total collagen content was observed in tunica albuginea. The tissue presented somehow quantitatively moisture, protein, lipids and ashes similar to calf and pig skins (10). The Fig 3 evidenced the high content of collagen with application of the Masson staining in the tunica albuginea, where collagen fibers were stained in blue.

Table 1 – Pig testis tunica albuginea proximate chemical composition and total collagen content (%)

Components	g 100 g ⁻¹ sample*
Moisture	76.15 (\pm 0.52)
Protein	18.2 (\pm 0.64)
Lipid	4.71 (\pm 0.53)
Ash	0.75 (\pm 0.35)
Collagen content	17.82 (\pm 0.11)

*in triplicate.

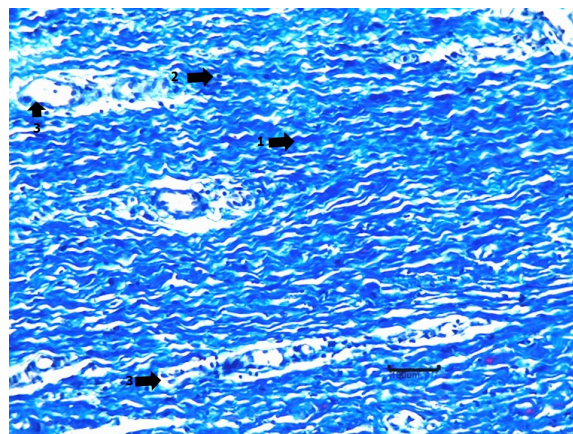


Figure 3- Optical microscopy with Masson trichrome staining for testis tissue albuginea. 1- collagen fibers were wavy distributed; 2- fibroblast cell nucleus; 3- blood vessel. Scale bars: 100 μ m.

Collagen Types Evaluation by SDS-PAGE

The electrophoretic pattern of samples obtained in the salt fractionation of pepsin solubilized collagens is show in the Figures 4 and 5.

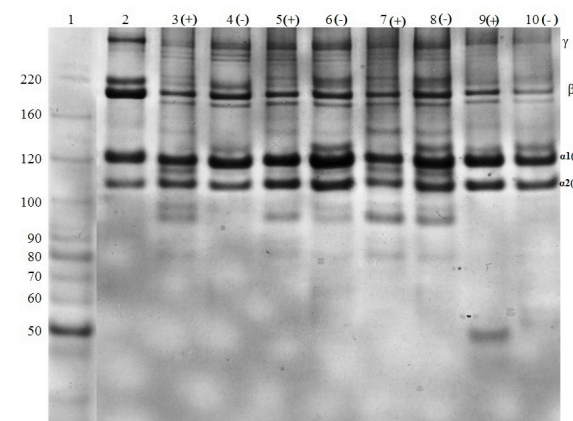


Figure 4 – SDS-PAGE pattern of samples obtained in the salt fractionation of pepsin solubilized collagen. Lines: 1- MW markers; 2- Type I collagen from rat tail (control); 3,4 – Ppt 1A 0.7M NaCl, pH 2.5; 5,6 – Ppt 2A 1.2M NaCl, pH 2.5; 7,8 - Ppt 3A 1.8M, NaCl, pH 2.5; 9,10 - Ppt 4A 2.0M NaCl, pH 2.5. (+) with and (-) without β -mercaptoethanol (ME).

Fig 4 shows the omnipresence of type I composed by $\alpha_1(I)$ and $\alpha_2(I)$ throughout the salt precipitates at acidic pH. Other unreducible bands were observable as on the lines 9 and 10 above the $\alpha_1(I)$ probably one of type V $\alpha_s(V)$ type. Lines 7 and 8 also show a band of MW of app 95K. Under ME treatment, other reduced

bands were observable and the major one appeared at line 9 of MW app. 50K and it was not possible to see its unreducible component at line 10. A reducible band was also possible to see at lines 5 and 7 of MW above $\alpha_1(I)$ chain and the reduced fragments were not detected under this SDS-PAGE conditions.

Fig 5 shows a further salt fractionation obtained originally at acidic pH in 0.7M salt. Again Type I seems to be omnipresent appearing in every pellet irrespective of ME treatment. A major reducible component was again detected at lines 5 and 7 and its reduced peptic fragments were not seen probably because they were with a lower molecular weight not detected under this 5% gel acrilamide. Other bands of higher MW above β size were not seen after ME treatment suggesting their reducible structure nature.

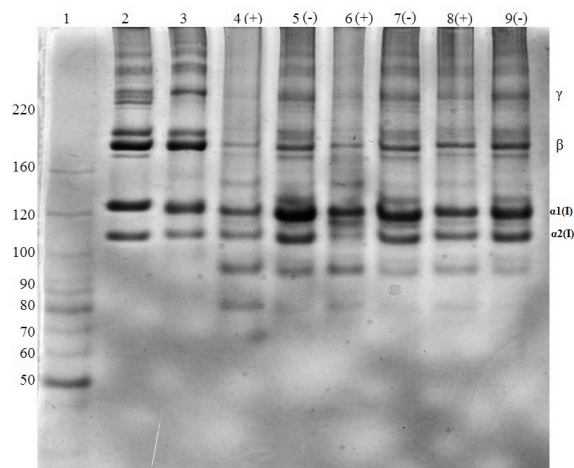


Figure 5— SDS-PAGE pattern of samples obtained in the salt fractionation of pepsin solubilised collagen originally precipitated at 0.7M NaCl, pH 2.5. Lines: 1 - MW markers; 2,3 - Type I collagen from rat tail (control); 4,5 - Ppt 1N, 1.5M NaCl pH 7.5; 6,7 - Ppt 2N, 2.0M NaCl pH 7.5; 8,9 - Ppt 3N, 3.5M NaCl pH 7.5; (+) with and (-) without β -mercaptoethanol (ME).

IV. CONCLUSION

Testis tunica albuginea is comprised solely by collagen fractions as determined by chemical and histological means. Types I, III and V are present and also other reducible components not chemically identified may constitute the collagen fibers in order to perform their structural role within the tissue. Therefore its high content makes the tunica albuginea a relevant source of collagen.

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