Process Biochemistry 46 (2011) 2021–2029

Contents lists available at ScienceDirect

Process Biochemistry

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

Impact of legume seed extracts on degradation and functional properties of gelatin from unicorn leatherjacket skin

Mehraj Ahmad, Soottawat Benjakul*

Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

ARTICLE INFO

Article history:
Received 9 March 2011
Received in revised form 25 June 2011
Accepted 23 July 2011

Keywords:
Unicorn leatherjacket skin
Degradation
Gelatin
Trypsin inhibitor
Legume seed
Functional properties

ABSTRACT

Trypsin inhibitor was extracted from the seed flour of soybean (SB; *Glycine max*), mung bean (MB; *Vigna radiata*), cowpea bean (CP; *Vigna unguiculata*) and adzuki bean (AB; *Vigna angularis*) using 0.15 M NaCl, followed by heat precipitation at 70 °C. The extract from SB showed the highest specific trypsin inhibitory activity, followed by those from MB, CP and AB, respectively. Based on inhibitory activity staining, molecular weights (MWs) of trypsin inhibitor from SB, MB, CP and AB were 20.1, 14, 10 and 13 kDa, respectively. The SB extract powder (SBEP) containing trypsin inhibitor in the range of 10–100 TIU/g effectively prevented the degradation of γ-, β- and α-chains of collagenolytic proteins of leatherjacket skin subjected to incubation at 50 °C for 30 min. The impact of SBEP on the extraction yield, physical and functional properties of gelatin from leatherjacket skin was investigated. The gelatin extracted in the presence of SBEP contained α_1 and α_2 chains as the predominant components with some degradation peptides. FTIR spectra indicated the significant loss of molecular order of triple helix and higher degradation was found in gelatin extracted in the absence of SBEP. Gelatin extracted in the presence of SBEP had the higher gel strength (232.8–268.5 g) than that extracted in the absence of SBEP (90.4 g). Higher foam stability (FS) but lower emulsion stability index (ESI) was observed in the former. Therefore, the addition of SBEP effectively prevented the degradation of gelatin from the skin of unicorn leatherjacket, thereby yielding the gelatin with improved gel strength and foam stability.

1. Introduction

Indigenous proteases associated with skin matrix degrade, destabilize and disintegrate the major components of native structure of collagen by disrupting the intra- and intermolecular cross-links of high molecular weight components like γ-, β-, and α-chains during gelatin extraction at higher temperature, resulting in the substantial decrease in gel strength, foam ability, foam stability and viscosity of gelatins [1]. Gelatins which retained high molecular weight components including γ-, β-, and α-chains have been known to possess the maximal functional properties, which can be applicable for food, medicine and pharmaceutical industries [2]. Collagenases are proteases capable of cleaving the peptide bonds in the native triple helical collagen molecules under physiological conditions [3]. These collagenases are classified into two major groups, metallo- and serine-collagenases. Collagenases are endopeptidases that have the unique ability to hydrolyse the major structural extracellular matrix proteins, especially the triple helical strand of type I and type II collagens at Gly^{775}-Leu (Ile)^{776}, giving rise to ¾- and ¼-cleavage peptide products [4]. Furthermore, non-collagenase proteases can cleave the collagen molecule in the telopeptide region and contribute to hydrolysis of the collagen molecule by disrupting the regions, in which intermolecular cross-links are formed [5]. Heat-activated serine proteases in unicorn leatherjacket skin (*Aluterus monoceros*) were involved in the drastic degradation of the γ-, β- and α-chains of the gelatin extracted at 50 °C [1].

To produce the high-quality gelatin with negligible hydrolysis of peptides, the use of an appropriate protease inhibitor from natural sources could be a new approach and an effective means to suppress the indigenous protease-induced degradation of gelatin molecules due to its safety and lower price, in comparison with commercial protease inhibitors. The major protease inhibitors from seeds belonging to Gramineae, Leguminosae and Solanaceae families are the Kunitz and Bowman–Birk inhibitors [6]. Kunitz inhibitors are usually 8–22 kDa proteins, with two disulphide linkages and a single reactive site of trypsin, whereas Bowman–Birk inhibitors are usually 8–10 kDa proteins, with seven disulphide linkages and two reactive sites of trypsin and chymotrypsin [7]. The extracts from legume seeds were reported to prevent the autolysis of surimi, thereby improving the surimi gel properties [8].

Unicorn leatherjacket (*A. monoceros*) is one of the marine tropical fish, which is harvested in large quantities throughout the world. This species has been used for fillet production in Thailand, in

* Corresponding author. Tel.: +66 7428 6334; fax: +66 7455 8866.
E-mail address: soottawat.b@psu.ac.th (S. Benjakul).

1359-5113/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2011.07.018

which a large quantity of skin has been produced as by-product. Due to its thick skin, it can be a potential source of value-added products like collagen and gelatin. As a consequence, the increased revenue for processors can be achieved. Furthermore, the comprehensive utilization of leatherjacket skin is a promising means to bring about the biomaterial with environmental protective aspect. Nevertheless, there is no information regarding the suppression of indigenous protease-induced degradation in the skin of unicorn leatherjacket by the extracts of legume seed. Therefore, the objective of this investigation was to suppress the unwanted proteolysis of collagenolytic proteins in the skin of unicorn leatherjacket by legume seed extract and to elucidate the impact of extract on the functional properties of gelatin extracted by different processes.

2. Materials and methods

2.1. Materials

Soybean (*Glycine max*), mung bean (*Vigna radiata*), cowpea bean (*Vigna unguiculata*) and adzuki bean (*Vigna angularis*) were purchased from the local market, Hat Yai, Thailand. *N*-α-Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), trypsin from bovine pancreas, casein from bovine milk, type I collagen from calf skin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphoric acid was obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) and Coomassie blue R-250 were procured from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

2.2. Fractionation of trypsin inhibitor from legume seeds

2.2.1. Preparation of crude extracts

Legume seeds were finely ground using a blender (Model MX-T2GN, National, Taipei, Taiwan). Seed flour was defatted by mixing with hexane at a ratio of 1:5 (w/v). The mixture was shaken for 10 min and filtered through Whatman No. 1 filter paper (Whatman International, Ltd., Maidstone, England). The retentate was rinsed twice with hexane to remove the residual oil in the ground sample. Defatted sample was then air-dried at room temperature (28–30 °C) until dry and free of hexane odour. The dried defatted samples were extracted with 0.15 M NaCl at room temperature (26–28 °C) at the sample/medium ratio of 1:5 (w/v), with continuous stirring for 3 h, using IKA® Model Colour Squid [white] magnetic stirrer (BEC THAI, BKK, Thailand) [9]. The mixture was then subjected to centrifugation at 8000 × *g* at 25 °C for 30 min using a Beckman Model Avanti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was referred to as 'crude extract'.

2.2.2. Heat treatment of crude extract

Crude extracts were subjected to heat treatment at 70 °C for 15 min, followed by cooling with iced water. To remove coagulated debris, the extracts were centrifuged at 8000 × *g* for 5 min at room temperature. The supernatants were then dialysed extensively at 4 °C for 24 h against 20 volumes of distilled water with a change of solution every 6 h. The dialysates obtained were freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). The powder obtained was referred to as 'seed extract powder'.

2.3. Characterisation of trypsin inhibitor in seed extracts

2.3.1. Trypsin inhibitory activity assay

Trypsin inhibitory activity of all seed extract powders was measured by the method of Welham and Domoney [10] with a slight modification using BAPNA as substrate. A solution containing 200 μl of inhibitor solution (2 mg/ml), 200 μl of bovine pancreas trypsin (1 mg/ml) and 1000 μl of 50 mM Tris–HCl, pH 7 containing 10 mM $CaCl_2$ was pre-incubated at 37 °C for 15 min. To initiate the reaction, 200 μl of BAPNA (0.4 mg/ml in DMSO) (pre-warmed to 37 °C) were added and vortexed immediately to start the reaction. After incubating for 10 min, 200 μl of 30% acetic acid (v/v) was added to terminate the reaction. The reaction mixture was centrifuged at 8000 × *g* for 5 min (Eppendorf Micro Centrifuge, MIK-RO20, Hettich Zentrifugan, Germany). Residual activity of trypsin was determined by measuring the absorbance at 410 nm due to *p*-nitroaniline released. One unit of proteolytic activity was defined as an increase of 0.01 absorbance unit ml^{-1} min^{-1} under the assay condition. One unit of trypsin inhibitory activity (TIU) was defined as the amount of inhibitor, which reduced trypsin activity by one unit.

2.3.2. Protein determination

Protein concentration was determined by the Biuret method using BSA as a standard [11].

2.3.3. Protein pattern and inhibitory activity staining

2.3.3.1. Protein pattern. SDS-PAGE was performed under non-reducing conditions, using 12% separating and 4% stacking gels according to the method of Laemmli [12]. The samples were mixed with the sample buffer (0.5 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 10% (w/v) SDS and 0.1% (w/v) bromophenol blue) at a ratio of 1:1 without heating. Samples (20 μg protein) were loaded onto the gel and then subjected to electrophoresis at 15 mA/gel using a Mini Protean Tetra Cell units (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, one gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with the mixture of 30% (v/v) methanol and 10% (v/v) acetic acid.

2.3.3.2. Inhibitory activity staining. After electrophoresis, another identical gel was washed in 2.5% (v/v) Triton X-100 for 15 min to remove SDS and renature the proteins. The gel was washed extensively with cold distilled water before soaking in 30 ml of a trypsin solution (1 mg/ml in 50 mM Tris–HCl, pH 7 containing 10 mM $CaCl_2$) for 30 min at 4 °C to allow the trypsin to diffuse into the gel. The gel–trypsin solution was then incubated at 37 °C for 40 min and then rinsed with cold distilled water, fixed and stained with 0.05% (w/v) Coomassie brilliant blue R-250 to develop inhibitory zones. The apparent molecular weight (MW) of the trypsin inhibitor was estimated by comparing its Rf with those of protein standards.

2.4. Effect of soybean extract powder (SBEP) towards autolysis of pretreated skin

2.4.1. Collection and preparation of fish skin

The skin of unicorn leatherjacket (*A. monoceros*) was obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand. Upon arrival to the Department of Food Technology, Prince of Songkla University, Hat Yai, the skin was cleaned and washed with iced tap water (0–2 °C). Prepared skin was then cut into small pieces (0.5 cm × 0.5 cm), placed in polyethylene bags and stored at −20 °C until use. The storage time was less than 2 months.

2.4.2. Pretreatment of unicorn leatherjacket skin

Prepared skin was pretreated following the method of Ahmad and Benjakul [13] with a slight modification. To remove non-collagenous proteins, the prepared skin was mixed with 0.1 M NaOH at a skin/alkali solution ratio of 1:20 (w/v). The mixture was stirred for 6 h at 4 °C using an overhead mechanical stirrer (W20.n, IKA-Werke GmbH & CO.KG, Stanfen, Germany) at a speed of 300 rpm. The alkali solution was changed every 2 h. The samples were then washed with iced tap water until neutral or faintly basic pH was obtained. Thereafter, the prepared skin was swollen by mixing the skins with 0.2 M phosphoric acid at a ratio of 1:10 (w/v). The mixture was stirred for 24 h at 4 °C. Finally, the swollen skin was washed thoroughly with iced tap water until neutral or faintly acidic pH of wash water was obtained. The pretreated skin was stored at −20 °C until use, but not longer than 2 months. Prior to study, the frozen pretreated skin was powderised in liquid nitrogen using a blender. The powder obtained was used for autolysis study.

2.4.3. Autolysis inhibition study

Powderised pretreated skin (1 g) was homogenised with 3 ml of McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate, pH 7) at a speed of 11,000 rpm at room temperature for 2 min using a homogeniser (Model T25 basic, IKA, Labortechnik, Selangor, Malaysia). The homogenate was mixed with SBEP at various levels (5–100 TIU/g pretreated skin). The mixtures were allowed to stand at 4 °C for 2 h to ensure the interaction between inhibitor and target serine proteases. To initiate the autolysis, the mixtures were incubated at 50 °C for 30 min in a temperature controlled water bath (Memmert, Schwabach, Germany). The reaction was then terminated by addition of 5 ml of 10% (w/v) SDS (85 °C). The reaction mixture was further incubated at 85 °C in a water bath for 1 h, followed by centrifugation at 8000 × *g* for 10 min. After complete solubilisation, the autolytic pattern was determined by SDS-PAGE using 7.5% separating gel and 4% stacking gel. The control was performed in the same manner, except de-ionised water was added instead of SBEP.

2.5. Effects of SBEP on extraction and properties of gelatin from unicorn leatherjacket skin

To inhibit the indigenous serine proteases associated with pretreated skin, the mixtures of pretreated skin and distilled water at the ratio of 1:10 (w/v) were subjected to extraction using different processes as follows (Fig. 1):

(1) *Process I*. Addition of SBEP at a level of 100 TIU/g pretreated skin, followed by pre-incubation at 4 °C for 12 h and extraction using distilled water at 50 °C for 12 h.
(2) *Process II*. Same manner with process No. 1 except that SBEP was also added in distilled water at the level of 100 TIU/g used as the extracting medium.
(3) *Process III*. Extraction using distilled water at 50 °C for 12 h in the presence of 100 TIU/g pretreated skin without pre-incubation with SBEP.

After extraction at 50 °C for 12 h, the mixtures from three processes were then filtered using two layers of cheesecloth. The filtrates were further filtered using a Whatman No. 4 filter paper with the aid of an electrical aspirator (Model VE-11, JEIO TECH, Seoul, Korea). The resultant filtrates were freeze-dried. Gelatins obtained from processes I, II and III were referred to as G1, G2 and G3, respectively. Gelatin extracted at 50 °C for 12 h without the addition of SBEP was used as the control and