Journal of Chromatography B, 963 (2014) 10–15

Contents lists available at ScienceDirect

Journal of Chromatography B

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

An improved method to extract and purify cystatin from hen egg white

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ARTICLE INFO

Article history:
Received 13 January 2014
Accepted 23 May 2014
Available online 2 June 2014

Keywords:
Cystatin
Hen egg white
Affinity chromatography
Quantification
HPLC
Mass spectrometry

ABSTRACT

Hen egg white cystatin, an inhibitor of cysteine proteinase, may have wide applications for improving human health. However, its pricy cost associated with extraction and preparation has hurdled its further utilization. The objective was to develop an improved method to extract and purify cystatin from egg white. After removal of ovomucin, a fraction containing cystatin was obtained by cation exchange chromatography, and further purified by affinity chromatography using a cm-papain-Sepharose column. The prepared cystatin was then characterized by SDS-PAGE, Western-Blot, and LC–MS/MS, and its purity was determined by HPLC method instead of the conventional immunodiffusion method. The protein content of cystatin extract was 66.4 ± 2.3%. In comparison with the conventional method, the purity of cystatin was improved from 56.6 ± 1.7% to 93.3 ± 4.0%, and its yield was improved from 21.3 ± 1.2% to 33.6 ± 1.5%. Relative activities of cystatin to inhibit papain prepared by our method and the conventional method were determined to be 88 ± 7% and 91 ± 4% respectively, against a cystatin standard from Sigma. This suggested no significant loss of activity during the separation process.

1. Introduction

Chicken cystatin, belonging to the family 2 of the cystatin superfamily [1], was firstly isolated from egg white by Fossum and Whitaker [2], and then was consequently found in chicken sera, lung, gizzard, brain, heart, oviduct, and embryonic muscle cell [3,4]. Two isoelectric forms were identified as non-phosphorylated form 1 and phosphorylated form 2 (serine-80 by phosphorylation) with *pI* values of 6.5 and 5.6, respectively [3]. The cystatin form 1 was firstly reported to contain 116 amino acids with a molecular weight of 13,143 Da [5]. Afterwards, a longer chain (probably full sequence), containing 139 amino acids, was identified with a molecular weight of 15,287 Da [4]. X-ray crystal study of chicken cystatin suggested it consisted mainly of a straight five-turn α-helix, a five-stranded antiparallel β-pleated sheet, and an appending segment of partially α-helical geometry [6].

Cystatin is a well-known inhibitor of cysteine proteinases, such as ficin, papain [1,2], cathepsin B, H, L [7,8], chymopapain A, actinidin [9], and human cathepsin C (cathepsin C: chicken cystatin by a 4:1 stoichiometry) [10]. Chicken cystatin was shown to have high affinity to various papain-like proteinases [11]. The mechanism of inhibition against papain by chicken cystatin was revealed through N-terminally truncated forms and cyanogen bromide fragments of chicken cystatin [1,6]. It was also reported that chicken cystatin could inhibit osteoclast-mediated bone resorption in calvarial bone explants [12], similar to human cystatin C [13]. Cystatin C could also stimulate the differentiation of mouse osteoblastic cells and bone formation [14], present significant protection against oxidative stress-induced death in PC12 cells [15], and provide protective roles in neurodegenerative disorders (Alzheimer's disease) [16]. Cystatin could also be used as markers for clinical diagnosis or biomarker related to some human diseases, such as cardiovascular disease [17,18], chronic kidney disease [19,20], amyotrophic lateral sclerosis [21] and dysfunction of glomerular filtration rate [22].

Chicken egg white cystatin makes up 0.05% of the total egg white proteins [23], presenting a concentration of 80 μg/mL in egg white [7,24]. A three-step procedure was performed to purify cystatin from egg white, sequentially including removal of ovomucin, purification with affinity chromatography on a carboxymethylated papain–Sepharose, and chromatofocusing [7]. This method was then modified by Lindahl et al. [25] at the last step, in which chromatofocusing was replaced by DEAE chromatography. Trziszka et al. [26] developed a similar cystatin separation procedure with an additional step of Sephadex G-100 gel filtration before DEAE chromatography step. In addition, another method by Turk et al. [27] was conducted by compiling alkaline treatment

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http://dx.doi.org/10.1016/j.jchromb.2014.05.049
1570-0232/

(pH 12), heat treatment (65 °C), and separation by gel chromatography on Sephadex G-50 and ion-exchange chromatography on DEAE-Sephacel. The complicated extraction procedure and a low yield (around 20%) of cystatin extraction [7,27], as well as the expensive price (900 USD/mg, Sigma) greatly restrain its further study. Our preliminary experiment suggested some other unknown components in egg white (besides cystatin) also responded to cystatin antibody prepared from rabbit blood serum, which might complicate the conventional method of cystatin quantification by immunodiffusion [7]. The objective of the work was to develop an improved method of cystatin extraction from hen egg white.

2. Materials and methods

2.1. Materials

Fresh white-shell eggs (55–60 g/egg) were obtained from LUCERNE FOOD Company (Calgary, Alberta, Canada) for cystatin extraction. Cystatin standard was purchased from Sigma (Louis, MO, USA). Sodium dodecyl sulfate-polyacrylmide was from Bio-Rad Laboratories, Inc. (Hercules, CA), 2-mercaptoethanol was obtained from MP Biomedicals, LLC (Solon, OH, USA), and the sodium chloride was from Acros Organics (Morris Plains, NJ, USA).

2.2. Cystatin extraction

2.2.1. Removal of ovomucin

A total of 500 g (492 mL) egg white was manually separated from egg yolk and chalazae, then diluted with 3 volumes of 100 mM NaCl solution and adjusted to pH 6.0 with 6 M HCl. After homogenization and placing at 4 °C overnight the dispersion was separated by centrifugation at 15,300 × *g* (Avanti J-E refrigerated centrifuge, Beckman Coulter, Inc., Fullerton, CA, USA) for 15 min at 4 °C. The supernatant was further subjected to a cation exchange chromatography.

2.2.2. Pre-separation of cystatin by fast-performance liquid chromatography (FPLC)

The above supernatant (ovomucin free) was subjected to a cation High-Prep 16/10 column (SP Sepharose FF, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) coupled with FPLC system. The column was equilibrated with 30 mM NaCl solution at pH 4.8, which was also used as the starting buffer. Flow rate was set to 2 mL/min and the injection volume was 20 mL. After sample injection, the column was eluted isocratically using 30 mM NaCl solution for 0.5 CV, followed by a linear gradient elution (2.2 CV) till 0.95 M NaCl solution, in which buffers were made by mixing Milli-Q water and 1 M NaCl solution (pH 4.8). The elution was monitored at 280 nm.

2.2.3. Purification of cystatin by affinity chromatography

The unbound fraction above was then subjected to an affinity separation according to Anastasi et al. [7].

2.2.3.1. Activation of Sepharose. Ten grams of wet Sepharose was washed with 60–70 mL of 1 mM EDTA on a sintered glass funnel. After vacuum filtering, 40 mL Milli-Q water and 800 mg of CNBr powder were sequentially added into this solution (50 mL, 20 mg/mL). Sepharose suspension was adjusted to pH 11.0 by adding 6 M NaOH maintaining the pH at 25 °C for 6 min. The activated gel was rapidly washed with 500 mL of 0.1 M $NaHCO_3$ on a sintered glass filter under suction.

2.2.3.2. Preparation of Cm-papain-Sepharose. The commercial papain (100 mg) was activated with 50 mL of 0.1 M sodium phosphate buffer (pH 6.0) containing 2 mM cysteine and 1 mM disodium EDTA for 10 min at 20 °C, followed by addition 10 mM iodoacetic acid (final concentration), dialysis against 0.1 M $NaHCO_3$ at 4 °C for 3 h, and finally the retentate was mixed with 10 g (wet weight) of activated Sepharose 4B under overnight stirring.

2.2.3.3. Purification by affinity chromatography. After overnight stirring of activated papain and Sepharose 4B in sodium phosphate buffer, the supernatant (containing unbound papain) was carefully removed and the cm-papain-Sepharose was equilibrated with 50 mM phosphate buffer (pH 6.5) containing 0.5 M NaCl and 0.1% Brij 35 for 30 min. Then it was mixed with 100 mL egg white sample solution and stirred overnight. The adsorbent was collected by centrifugation (30 min of 340 *g* at 4 °C), and re-suspended in 50 mM phosphate buffer at pH 6.5 containing 0.5 M NaCl and 0.1% Brij 35. This step was re-performed several times until the absorbance of the washed solution was zero at 280 nm. The cm-papain-Sepharose was packed into a column (50 mm diameter × 30 cm length), and equilibrated with the same buffer but containing additional 10% (v/v) glycerol in place of the Brij 35. After passage of 2 CV, the column was eluted with 50 mM K_3PO_4 solution at pH 11.5 containing 0.5 M of NaCl and 10% glycerol. The fraction representing the single peak of protein was detected at 280 nm in the effluent. Each cystatin extraction was carried out in duplicate.

2.2.4. Extraction of cystatin by conventional method

For comparison, cystatin was also extracted from egg whites by a conventional method [7]. Briefly, a total of 100 g egg whites were separated from the yolks and blended with an equal volume of 0.25% NaCl by the use of a mixer/homogenizer. The egg white solution was adjusted to pH6.5 by adding 5 M sodium formate buffer (pH 3.0), and its precipitate was removed by centrifugation (15 min at 15,300 × *g*). The supernatant was then subjected to an affinity chromatography as described above. Each extraction was carried out in duplicate.

2.3. Identification of cystatin

2.3.1. SDS-PAGE (sodium dodecyl sulfate-polyacrylmide gel electrophoresis)

SDS-PAGE test was carried out according to Laemmli's method [28] using 10–20% ready-to-use gels at a constant voltage (200 V) mode in a Mini-PROTEAN tetra cell attached to a PowerPac Basic electrophoresis apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA). The loaded amount of proteins was 50 μg in a volume of 20 μL. Protein marker of high range molecular weight was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The gels were scanned using an Alpha Innotech gel scanner with FluorChem SP software (San Leandro, CA, USA).

2.3.2. Western-Blot

Rabbits were immunized by intramuscular injection of 100 μg of cystatin (separated from chicken egg white, Sigma, Louis, MO, USA) in Freund's complete adjuvant (Sigma, Louis, MO, USA), followed by the injection of 100 μg of cystatin in Freund's incomplete adjuvant (Sigma, Louis, MO, USA) 28 days later. After 14 days, the rabbit serum was then collected by centrifugation and kept in −70 °C until use.

Western-Blot test was performed according to the protocol online with slight modifications (http://www.westernblotting.org/). Briefly, after completing SDS-PAGE and electrophoretic transfer, the membrane was blocked by 3% Non Fat Dry Milk (NFDM) in TBS (25 mM of Tris–HCl, 0.15 M of NaCl, pH 7.4) at ambient temperature for 2 h. Then, the rabbit serum (primary antibody) was diluted 1000 times with above solution and incubated with the