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Molecular structure and physicochemical properties of pepsin-solubilized type II collagen from the chick sternal cartilage

H. CAO, F.X. SHI, F. XU, J.S. YU

Medical Equipment and Food Institute, University of Shanghai for Science and Technology, Shanghai, China

Abstract. – BACKGROUND AND OBJECTIVES: Recently, type II collagen (CII) was found to be effective clinically for treatment of rheumatoid arthritis (RA). However, the molecular properties of CII could be changed during the preparation process. In the present study, we isolated CII from chick sternal cartilage and studied the structural characteristics of purified CII.

MATERIALS AND METHODS: Pepsin-solubilized CII was purified from sternal cartilage of the chick using a combination of pepsin digestion, NaCl precipitation and DEAE-Sepharose CL 6B ion exchange chromatography. Then, the molecular structure and physicochemical properties of pepsin-solubilized CII were investigated.

RESULTS: According to the electrophoretic patterns, the purified preparation consisted of a single band (α chain) and dimmers (β chains) with a subunit Mr of 110 kDa, were characterized to type II, and contained imino acid of 232 residues/1000 residues. The maximum transition temperature (Tmax) of the pepsin-solubilized CII measured by DSC was 45.60°C. Circular dichroism (CD) spectra analysis revealed that pepsin-solubilized CII retained more intermolecular crosslinks during the preparation process. Investigation results of atomic force microscope (AFM) indicated that the collagen fibrils from chick cartilage were about 146 nm in width and highly periodic with a banding pattern of –68.3 nm spacing. Analysis of physical properties indicated that pepsin-solubilized CII were highly solubilized in the pH range of 1-3.5 and the optimal NaCl concentration was 0.6 mol/L.

CONCLUSIONS: Chick sternal cartilage can be used as an alternative CII source.

Key Words:
Pepsin-solubilized type II collagen, Sternal cartilage, Physicochemical properties, Purification.

Introduction

Type II collagen (CII) is the main structural component of cartilage and, together with other tissue-specific collagens and proteoglycans (GAG), supports the tissue with its shock-absorbing properties and resiliency to stress. CII with specific molecular structures has been widely used in the food, pharmaceutical, and cosmetic industries because of its excellent biocompatibility and biodegradability[1]. Recently, there is an increasing demand for CII as research found that CII was a clinically effective treatment for rheumatoid arthritis (RA), and the mechanism of action of CII in suppressing RA could be related to the shift in cytokine secretion patterns and the suppression of inflammatory cytokine release by Th1-type cells[2,3].

Most commercial CII are obtained from articular cartilage of mammals. Nevertheless, these collagens have the limitation for applications because of their low yield and intricate sample pretreatment techniques. Consequently, increasing interest has been paid to the alternative CII sources, especially sternal cartilage from poultry processing by-products due to their abundance and low cost. With the rapid development of poultry processing industries, huge quantities of by-products have been discarded which may cause pollution and emit offensive odors[4]. Hence, the comprehensive utilization of these by-products, especially the production of value-added products, is a promising means to increase revenue for the products and accelerate the development of the poultry processing industry.

Native CII from cartilage, known as procollagen, is composed of three identical α-chains with non-collagenous telopeptide extensions. It has been reported that the telopeptides of procollagen are insoluble, and are considered to be responsible for causing an immunogenic response when introduced into xenogenic hosts[5]. To minimize this problem, telopeptide extensions must be removed by specific procollagen peptidases (such

Corresponding Author: Fei Xu, MD; e-mail: caohuian@sina.com

as pepsin, trypsin, pancreatin, ficin, brome lain or papain), converting the procollagen into soluble CII[6]. Pepsin has been widely used to cleave peptides in the telopeptide region, since the extraction of collagen partially cleaved by pepsin resulted in a higher yield[7]. In addition to the hydrolysis of telopeptides, the enzymatic treatment of connective tissue can change the molecular properties of collagen[8]. However, no biochemical information on pepsin-solubilized CII from chick sternal cartilage has been reported to date.

Isolation of CII from dumped cartilage will not only greatly promote the value of the poultry byproducts, but also partly resolve the CII resource problems. Therefore, the present study describes the method to prepare pepsin-solubilized CII from the chick sternal cartilage. Subsequently, the physicochemical properties and structure including the protein pattern, amino acid composition, thermal stability, solubility and secondary structure of the pepsin-solubilized CII were characterized.

Materials and Methods

Materials and Chemicals

Chick sternal cartilage was provided by Nanjing YuRun Co., Ltd. (2-3 months old, Nanjing, China), and stored in refrigerator at –20°C until use. Resins of DEAE-Sepharose CL 6B were purchased from Pharmacia Biotech (Uppsala, Sweden). Standard protein (e.g., myosin heavy chain 200 kDa, Calmodulin-binding protein, 130 kDa, Rabbit Phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; rabbit actin, 43 kDa) for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Shanghai Huamei Biotech (Shanghai, China). Standard CII, O-phthalaldehyde and 9-fluorenylmethoxycarbonyl, pepsin (EC 3.4.23.1, enzyme activity, 3,200-4,500 units/mg) and 1, 9-dimethyl methylene blue were purchased from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals were in reagent grade or higher.

Proximate Composition and Ultra-structure of Chick Sternal Cartilage

Sternal cartilages were removed from chickens and the composition was determined. Crude fat and ash content of cartilage were determined according to the Association Of Analytical Communities (AOAC) methods. The percentage (%) of CII was determined using the method of Woessner (1961)[9]. The glucosaminoglycan (GAG) content was determined by 1,9-dimethyl methylene blue method[10]. Briefly, 1 ml effluent was assayed for GAG after the addition of 1.2 ml 1,9-dimethyl methylene blue. The absorbance was measured at 525 nm. Spark chondroitin sulphate was used as a standard.

The cartilage sheets were fixed in 2.5% gluteraldehyde in 0.05% phosphate buffer (pH 7.2) for 24 h at 4°C and refixed by 10% Osmium tetroxide in the same buffer for 2h. Samples were dehydrated in a series of graded alcohol, infiltrated and then embedded in Spurr's resin. Samples were subsequently subjected to ultra thin sectioning with a glass knife fitted on an ultra Microtome. Ultra thin sections of 2 μm thickness were mounted on grids and the sections were stained with saturated aqueous uranyl acetate and counter stained with 4% lead citrate. The mounted grids were scanned for cartilages by Transmission Electron Microscope (JEM 2000EX) using a 10 kV accelerating voltage.

Preparation of CII from Chicken Sternal Cartilage

The method used for the isolation of pepsin-solubilized CII from sternal cartilage has been previously described[11]. Briefly, 100 g of cartilage pieces were digested in 1 L of pepsin solution for 32 h at 20°C, with an enzyme to cartilage ratio of 1:100. The resulting viscous solution was aggregated by centrifugation at 8.000 g at 4°C for 30 min, and allowed to precipitate for 16 h in 0.9 M NaCl solution. The precipitated collagen was then dissolved in 0.5 M acetic acid (pH 2.5). Three milliliters of extract was then loaded onto a 1 × 20 cm column of diethylaminoethanol (DEAE)-sepharose CL 6B equilibrated with 0.2 M NaCl (0.05 M Tris-HCl, pH 7.5). The column effluent was monitored and recorded at 280 nm. After the sample was applied to the column, elution with 0.2 M NaCl (0.05 M Tris-HCl, pH 7.5) was continued until no further ultraviolet-absorbing material was detected in the effluent. At this time, the eluting solvent was changed to 1.0 M NaCl (0.05 M Tris-HCl, pH 7.5) and elution with the latter buffer was continued until additional peaks were eluted from the column.

The effluent from all peaks was analyzed for CII and GAG content. The effluent containing CII was pooled and concentrated by precipitation using 3 M NaCl. Finally, the precipitate was redissolved in 0.5 M acetic acid, dialyzed against a large volume of the same solvent, and then lyophilized.

Characterization of Pepsin-solubilized CII from Sternal Cartilage

Protein Pattern

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the purity and molecular mass determination of pepsin-solubilised CII as described by Laemmli, using a 5% (w/v) stacking gel and a 7.5% (w/v) separating gel[12]. The samples were prepared by mixed the purified collagen at 1:1 (v/v) ratio with distilled water containing 10 mM Tris-HCl pH 6.8, 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue. Gels were stained with protein Coomassie brilliant blue R250. The molecular weight of the collagen was estimated using a high molecular weight calibration kit as markers.

Amino Acid Composition Analysis

CII samples were hydrolyzed with 6 M hydrochloric acid for 24 h at 120°C. The resulting mixture was analyzed by an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) following online derivatisation with O-phthalaldehyde and 9-fluorenylmethoxycarbonyl for proline. Calibration kit was served as marker.

Denaturation Temperature (Td)

The Td was determined by means of a differential scanning calorimetry (Perkin-Elmer DSC-7, Lauderdale, FL, USA). CII samples were prepared by dissolving 100 mg dry material in 5 ml acetic acid (0.1 M) and stirring for 3 h at 4°C. Subsequently, the wet samples were wiped with filter paper to remove excess water and hermetically sealed in aluminum pans. Heating rate of 5°C/min was applied from 20 to 90°C and the endothermic peak of the thermogram was monitored.

Circular Dichroism (CD) Spectra

CII samples were prepared by dissolving 5 mg dry material in 5 ml acetic acid (0.1 M) and stirring for 3 h at 4°C. Subsequently, the CD was used to assess the secondary structure of CII. Briefly, the CII solution was placed into a quartz cell with a path length of 1 mm. CD spectra measurements were performed in triplicate. Data was collected in the range of 250-190 nm with a scan speed of 100 nm/min at an interval of 1.0 nm. A reference spectrum containing acetic acid was also recorded. The CD spectra of the samples were obtained after subtracting the reference spectrum.

Atomic Force Microscopy (AFM)

Pepsin-solubilized CII samples (200 µg/ml) were allowed to adsorb to mica discs for approximately 30 min. The samples were then rinsed extensively with deionized water and allowed to dry before being placed in the instrument. The morphological characteristics of CII were imaged using a Nanowizard AFM (Veeco, Santa Barbara, CA, USA). The AFM was operated in the contact mode in air using Si_3N_4 tips. The typical force constant was 20 N/m.

Solubility of Pepsin-solubilized CII

The solubility of CII in 0.5 M acetic acid at different NaCl concentrations and pH levels was determined. Briefly, 2 mL of the CII solution (3 mg/mL) in 0.5 M acetic acid was mixed with 2 mL of cold NaCl solution to reach the final NaCl concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2 mol/L, respectively. After vortexing, each mixture was stored at 4°C for 30 min, followed by centrifuging at 10,000 g at 4°C for 30 min. Protein content in the supernatant was measured by the method of Lowry et al[13], using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the NaCl concentration rendering highest solubility.

Similar analysis was conducted to determine the correlation between the solubility of the CII and pH values of the solution. 8 mL of the CII solution (3 mg/mL) was transferred to a series of centrifuge tubes, adjusted to pH values ranging from 1.5 to 9.5 by addition of proper amount of 1 M NaOH or 1 M HCl, respectively, and vigorously stirred. Each mixture then was centrifuged at 10.000 g at 4°C for 30 min. Protein content in the supernatant was determined by the method of Lowry et al[13] using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH rendering highest solubility.

Statistical Analysis

Data were expressed as the mean ± SD. Statistical significance was determined by one-way analysis of variance with Dunnett's post-hoc test using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). *p*-values less than 0.05 were considered to be significant.

Results

Proximate Composition and Ultra-structure

As shown in Figure 1, chick sternal cartilage was a specialized tissue with a large amount of

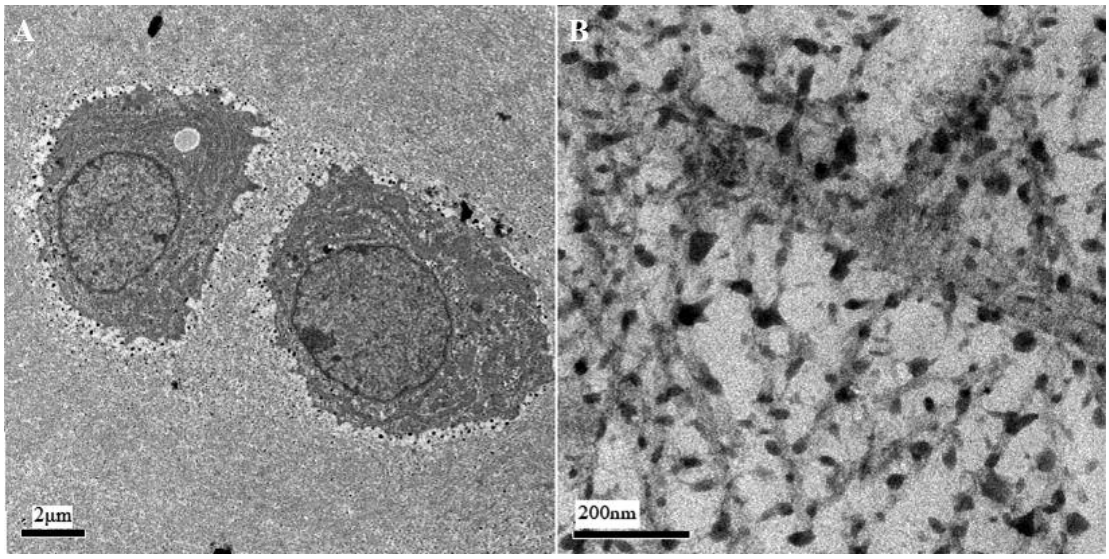

Figure 1. TEM images of chick sternal cartilage with ***(A)*** chondrocytes and ***(B)*** collagen fibers.

extracellular matrix. This matrix was composed of a dense network of collagen fibers and the chondrocytes appear flattened. Previous papers indicated that the collagen fiber network provides the shape and serves as confinement to the entrapped proteoglycans, which cause the tissue to swell because of the large osmotic pressure[4,7]. Distributed with the matrix was a sparse population of cells, the chondrocytes, which were responsible for the synthesis and the maintenance of the matrix components.

The proximate composition of chick sternal cartilage was as shown in Table I. The collagen content was 50.85%, which was slightly smaller than the results obtained for the chick articular cartilage. The crude lipid, ash and GAG content of the chick sternal cartilage on a dry basis were 1.01%, 0.30% and 6.97%, respectively.

Purification and Yield of CII

The isolation and purification procedures of CII consisted of pepsin digestion, chromatography techniques and NaCl precipitation. As shown in Figure 2, DEAE-sepharose CL 6B chromatography of pepsin-solubilized CII resulted in the fractionation of the total extract into three major components. The effluent from all peaks were dialyzed against water and lyophilized, then analyzed for hydroxyproline and GAG content. The results of the analyses showed that the collagen in the extracts, which had been eluted with 0.2 M NaCl in the first unretained fraction from the DEAE column, contained abundant hydroxyproline. On the other hand, the fractions eluted with 1.0 M NaCl did not contain any hydroxyproline, but have abundant GAG. This result indicated that DEAE-sepharose CL 6B chromatography under the conditions outlined above provided a rapid and efficient method for separating collagen from proteoglycans in cartilage extracts. This was accordant with the results of Miller[14,15].

Pepsin-solubilized CII was extracted from the chick sternal cartilage with the yield 27.91% based on the weight of cartilage. Sternal cartilage contained the CII at the level of 50.85%/100 g. Therefore, the yield based on the initial collagen presented in the cartilage or % recovery was 54.89%.

Table I. Proximate composition of sternal and articular cartilage (based on dry weight, %).

	Hyp	collagen	Crude fat	GAG	Ash
Sternal cartilage	7.32	50.85	1.01	0.30	6.97
Articular cartilage	9.08	63.08	0.81	0.26	6.21

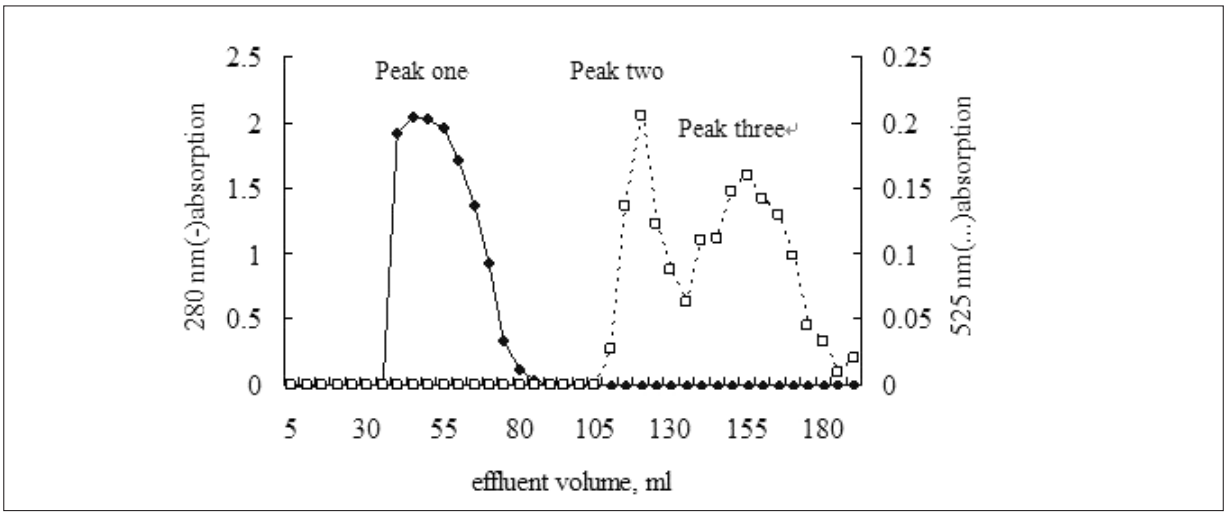

Figure 2. Elution curve of DEAE-sephalose CL 6B chromatography for pepsin- solubilized CII (peak1, CII eluted by 0.2 mol/L NaCl; peak2 and peak3, GAG eluted by 1 mol/L NaCl).

Protein Pattern of CII from Chick Sternal Cartilage

Figure 3 shows a protein pattern of CII from sternal and articular cartilage under reducing conditions. It was found that the purified collagen samples had similar migration bands to the reference CII, which consisted of α- and β-chains. The α-chain was found to be the dominant constituent with a subunit Mr of 110 kDa. However, only small amounts of β-components were showed in the electrophoretogram with Mr of about 220 kDa (dimers of the α-chain). The results suggested that pepsin was able to hydrolyze pocollagen, as indicated by the decreased band intensity of β-components.

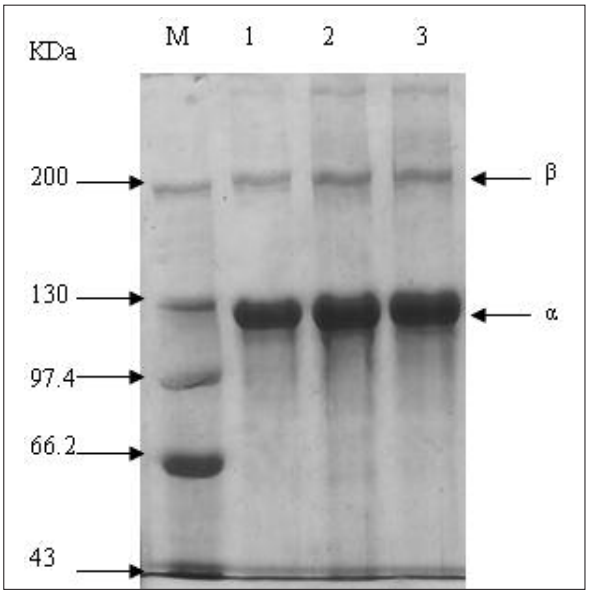

Figure 3. SDS-PAGE profile for the Sigma-Aldrich standard CII (Lane 1), CII purified from chick articular cartilage (lane 2), CII purified from sternal cartilage (lane 3) and MW standards (Lane M).

Amino Acid Composition

Table II shows the amino acid composition of CII samples. It was found that the purified CII samples from sternal cartilage had similar amino acid composition to the reference CII and articular cartilage CII, which had high content of glycine, hydroxyproline (Hyp) and proline (Pro) residues, with 310, 117 and 115 residues per 1000 amino acids residues respectively, and small amounts of tyrosine, cysteine, histidine and methionine residues with 5, 18, 4 and 10 residues per 1000 amino acids residues respectively. From these results it can be concluded that glycine, which constitutes about 1/3 of all residues in pepsin-solubilized CII, would be present as every third residue in the sequence. High amounts of Pro and Hyp could be accommodated while maintaining planar peptide bonds[16]. These assumptions led to the construction of the correct model of pepsin-solubilized CII as a (Gly-X-Y) n pattern, where X and Y are frequently Pro or Hyp, respectively. Indeed, it is the most commonly found triplet in collagen chains space[17,18].

Table II. Amino acid composition of type II collagen from reference Sigma-Aldrich, sternal and articular cartilage.

	Sigma residues/1000 Residues	Articular collegan residues/1000 Residues	Sternal collagen residues/1000 Residues
Aspartic acid/asparagine	47	45	46
Glutamic acid/glutamine	94	88	85
Serine	25	24	22
Histidine	4	4	4
Glycine	313	309	310
Threonine	30	31	26
Alanine	102	99	104
Arginine	53	50	52
Tyrosine	5	4	5
Cysteine	17	18	18
Valine	22	19	19
Methionine	2	8	10
Phenylalanine	15	14	15
Isoleucine	13	10	11
Leucine	31	28	27
Lysine	15	12	14
Proline	94	116	115
Hydroxyproline	118	121	117

The Thermal Stability of CII

The heat transformation of collagen is interpreted as disintegration of the collagen triple helical structure into random coils. DSC is used to investigate the thermal stability of CII in 0.05 M acetic acid, their maximum transition temperature (T_{max}) and total denaturation enthalpy (DH). As shown in Table III, CII from articular and sternal cartilage showed transition curves with maximum temperatures (T_{max}) of 47.83°C and 45.60°C, respectively. T_{max} of purified CII was similar to that of reference CII, suggesting no difference in the denaturation temperature between the two fractions, and the triple helix structure was still predominant in the CII fraction when the sternal cartilage was limited to digestion by pepsin. In addition, a slightly lower enthalpy was also observed for CII from sternal cartilage (H = 14.05 J/g) compared with that of articular CII (H = 15.13 J/g), dispersed in 0.05 M acetic acid.

Circular Dichroism (CD) Studies

To gain information on the triple helical secondary structure presented in the pepsin-solubilized CII, CD measurements were performed (Figure 4). The ellipticity and secondary structure information deduced from CD spectra are given in Table IV. The results show purified CII and reference CII samples have the characteristic triple helical conformation of collagen, specifically a rotatory maximum at 221 nm (positive band), a rotatory minimum at 198 nm (negative band), and a consistent cross over point (zero rotation) at ~212 nm[19].

Atomic Force Microscope (AFM)

Due to recent developments in single molecule techniques, AFM has been successfully used to unravel the biomechanical properties of a number of biomolecules including titin, tenascin, fibronectin, as well as fibrillar biological structures[20]. Here, we used AFM as a tool to investigate the biomechanical properties of pepsin-solubilized CII fibrils. As shown in Figure 5A, pepsin-solubilized CII presented a rigid rod-like structure and the length of the fibers was approximately 300 nm. The holes in the network were

Table III. Thermal stability of type II collagen from reference Sigma-Aldrich, sternal and articular cartilage[a].

	Reference CII	Sternal cartilage CII	Articular cartilage CII
Denaturation temperature (°C)	48.25 ± 2.65	45.60 ± 2.02	47.83 ± 1.87
Denaturation enthalpy (J/g)	19.99 ± 1.03	14.05 ± 1.52	15.13 ± 2.02

[a]Data show the means of three determinations for the identical collagen samples.

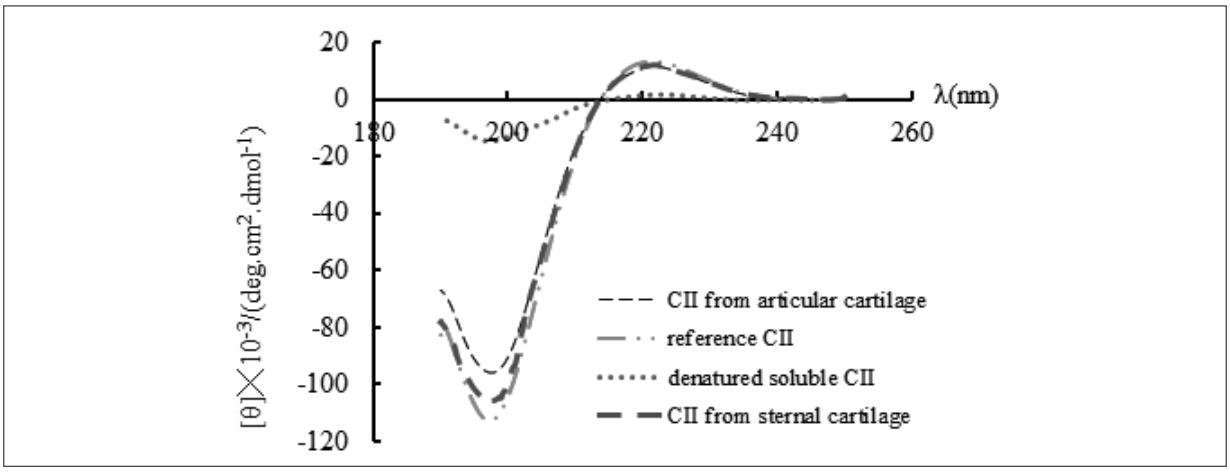

Figure 4. Circular dichroism (CD) spectra of CII in 0.1 M acetic acid at 25°C. All the spectra were obtained after subtraction of baseline spectrum for the acetic acid.

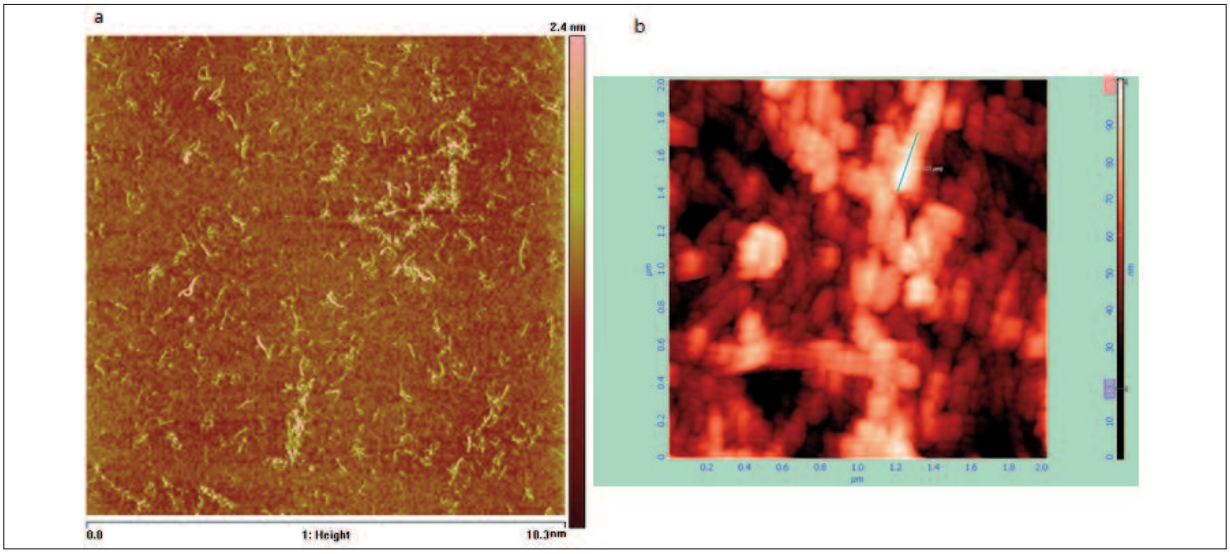

Figure 5. Atomic force microscopy of pepsin-solubilized CII from chick sternal cartilage: ***(A)*** Fibrils were adsorbed to the surface from a dilute solution (200 µg/mL) of fibrils. ***(B)*** Three-dimensional reconstruction of the area of collagen ***(C)*** Representative line scans of a collagen fibril showing periodicity of 68.3 nm (5 D-repeats).

Table IV. Secondary structure of type II collagen with different sources[a].

Samples	Ellipticity [θ]× 10^{-3}/(deg.cm^2.$dmol^{-1}$)		Secondary structure	
	221 nm	198 nm	β-sheet	Random coil
CII from sternal cartilage	11.91 ± 1.03	-95.75 ± 5.77	43.10 ± 2.12	56.90 ± 2.88
CII from articular cartilage	12.08 ± 1.22	-103.25 ± 7.21	44.10 ± 2.67	55.90 ± 2.62
Sigma-Aldrich standard CII	13.19 ± 1.08	-112.15 ± 7.08	43.30 ± 2.48	56.70 ± 2.09
Denatured CII	1.52 ± 0.23	-14.54 ± 1.36	33.20 ± 2.01	66.80 ± 2.31

[a]Results are presented as the means (n = 3).

determined to be mica, so there was only a single layer of collagen covered the entire surface. The results are similar to those described by Goh et al[21]. Figure 5B showed a pseudo-three-dimensional reconstruction of CII fibrils from sternal cartilage with a higher magnification 2×2 μm view. The CII preparation possessed the evidently structure of collagen. The collagen fibrils were about 146 nm in width and highly periodic, with a banding pattern of ~68.3 nm spacing (5 D-repeats). The results resemble type I collagen fibrils in their native form, which typically display a banding pattern with 60-70 nm spacing[22].

Solubility of Pepsin-solubilized CII

The effect of pH on the solubility of pepsin-solubilized CII in 0.5 M acetic acid was depicted. As shown in Figure 6A, pepsin-solubilized CII was soluble to a greater extent in the pH ranging from 1 to 3.5 and the highest solubility was observed at pH 2.5 ($p < 0.05$). When the pH goes above 4, a sharp decrease in solubility was showed and the lowest solubility was observed at pH 5.5 ($p < 0.05$). Previous research has shown that different collagens have varying solubilities at pH ranging from 2 to 10. The variation in solubility of collagens might be associated with a different isoelectric point (pI), which is altered by pepsin treatment[23]. When the pH is close to the pI, the molecular charges of collagen become diminished and a decrease in solubility occurred[24]. From this result, it appears that pIs of pepsin-solubilised CII are more likely to be above pH 5.5 and alkaline ranges, as evidenced by the lowered solubility in these ranges.

AS shown in Figure 6B, the solubility of pepsin-solubilized CII remained a constant high level in the presence of NaCl up to 0.6 mol/L ($p > 0.05$), and sharply decreased when the NaCl concentration was 0.9 ($p < 0.05$), after which solubility remained at a constant low level ($p > 0.05$). Greater solubility of pepsin-solubilized CII at lower salt concentration might be due to the proteolytic action of pepsin in altering collagen structure and reducing the chain length of resultant collagens[25]. The decrease in solubility of CII with the increase of NaCl concentration might be attributed to the salting-out effect[25,26]. These solubility behaviours of collagens with changes in pH and NaCl concentrations may play a crucial role in their extraction and application.

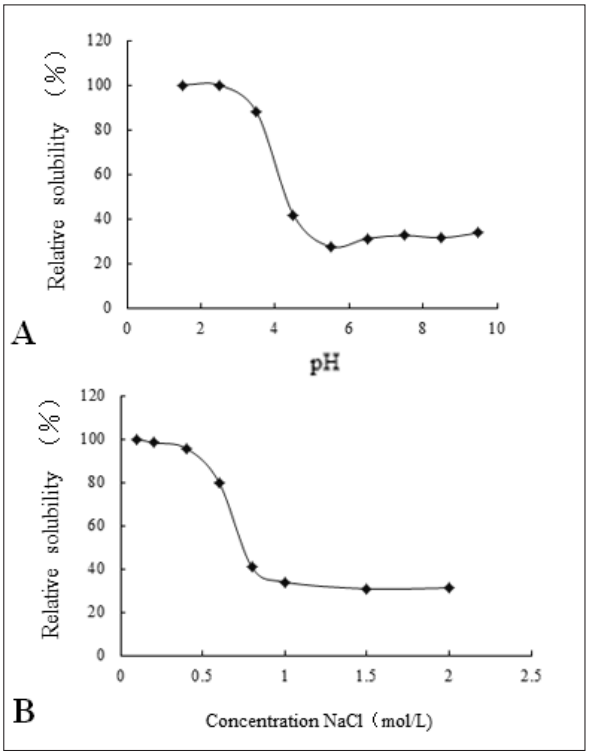

Figure 6 Relative solubility (%) of pepsin-solubilized CII at different pHs ***(A)*** and NaCl concentration ***(B)***.

Discussion

RA is a complicated refractory autoimmune disease characterized by a number of the inflammatory and destructive events, such as joint pain and swelling, synovial hyperplasia, cartilage and bone erosions, joint malformation. Accumulative findings demonstrate that oral administration of intact antigen protein could be a simple, safe and effective new method for autoimmune diseases. CII, as auto-antigen in RA, is recognized as a potential therapeutic approach for RA. However, it has been found that functional properties of CII are highly related to its structure integrity and the molecular structure of CII could be changed during preparation process. Therefore, at present study, we investigated the structure character of pepsin-solubilized CII.

We found collagen constitutes more than half of the dry weigh of chick sternal cartilage, in which CII accounts for almost 90% of the total collagen. Based on the subunit composition and electrophoretic mobility, it was suggested that the collagen from chick sternal cartilage was typical CII, which comprised of three identical α chains with a subunit Mr of 110 kDa like articular cartilage, and collagen molecule is depicted as $[\alpha_1 (II)]_3$[28].

The amounts of imino acid (Pro and Hyp) are important for the structural integrity of collagen. It is known that the pyrrolidine rings of Pro and Hyp impose restrictions on the conformation of a polypeptide chain and help to strengthen the triple helix. Furthermore, the imino acid content contributes to the thermal stability of the helix structure of collagen, due to the fact that the Pro + Hyp rich zones of the molecules are most likely to be the involved in the formation of junction zones stabilized by hydrogen binding[29]. In our study, the imino acid content of CII from sternal and articular cartilage was 232 and 237 residues/1000 residues, respectively, which was similar to other land animals collagen (190-240 residues per 1000 residues), but higher than that of edible jellyfish, dusky spine foot, cod, ocellate puffer and bullfrog, which contained imino acids ranging from 122 to 167 residues per 10,000 residues[26,30]. This result supported that of Love et al[31] who indicate that the difference in the imino acid content of animals was associated with the differences in their habitates.

DSC is used to investigate the thermal stability of CII in 0.05 M acetic acid. The difference in H might be governed by hydrogen bonding in collagen structure. The T_{max} values of CII from the chick sternal cartilage were similar to that of land animals collagen (40.8°C)[32], but higher than those of collagens from the skins of fish species (28.7°C)[26]. The T_{max} values of collagen from different sources have been correlated with the contents of imino acids, temperature of normal habitat, seasons and age[33-35]. Collagen from sternal cartilage had a higher imino acid content (about 232 residues per 1000 total residues) than did collagen from fish species (122-167 residues per 1000 total residues), and hence showed a higher T_{max} value[32]. Generally, collagens from land animal species with a high habitat temperature had higher amounts of imino acids and they also had higher thermal stability than those from fish with a low habitat temperature[33,34]. The comparatively high T_{max} values of sternal cartilage CII indicated their high heat resistance and great structural stability, which might be beneficial when using them as potential substitutes for articular cartilage collagen. The results also suggested that the molecular structure of collagen could be maintained mainly by restrictions on changes in the secondary structure of the polypeptide chain, imposed by the pyrrolidine rings of Pro and Hyp, and also maintained partially by the hydrogen bond ability through the hydroxyl group of Hyp. The higher the imino acid content, the more stable are the helices[10,36].

The main objective of the preparation of CII is to use it as a potential therapeutic approach for RA. As structure integrity of CII is highly related to its functional properties, CD spectrum was applied to investigate the secondary structure of CII from the chick sternal cartilage. It has been reported that a rotatory maximum at 221 nm in the CD spectrum of native collagen is characteristic of a triple-folded helix. On complete denaturation, the positive peak at 221 nm disappears completely and the negative band is red-shifted[19]. In this investigation, there was neither significant change in the red shift of the negative band nor any disappearance of the positive band at 221 nm for purified CII samples compared with the reference and denatured CII. Therefore, CD spectral analysis indicated that purified CII retained most of its intra- and inter-molecular cross-links following the extraction process. The general shape and peaks of the spectrum change when the collagen is denatured and are in agreement with literature data[37].

The telopeptides of procollagen are insoluble and pepsin has been widely used to cleave peptides in the telopeptide region. From our results, pepsin-solubilized CII showed greater solubility, especially at high salt concentration and very acidic pH. Greater solubility of pepsinsolubilized collagen might be due to the proteolytic action of pepsin in altering collagen structure and reducing the chain length of resultant collagens.

Conclusions

DEAE-sepharose CL 6B chromatography under the conditions outlined above provided a rapid and efficient method for separating collagen from proteoglycans in cartilage extracts. Collagen from chick sternal cartilage was typical CII which comprised of three identical α chains, and the collagen molecule was depicted as $[\alpha_1 (II)]_3$. Pepsin-solubilized CII retained more intermolecular crosslinks during the purification process. The collagen fibrils from sternal cartilage were about 146 nm in width and highly periodic with a banding pattern of ~68.3 nm spacing. Generally, pepsin-solubilized CII were highly soluble in the pH range of 1-3.5 and the optimal NaCl concentration was 0.6 mol/L.

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Competing Interest

The Authors declare that they have no competing interests.

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