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Selective Extraction of Collagen Peptides with High Purity from Cod Skins by Deep Eutectic Solvents

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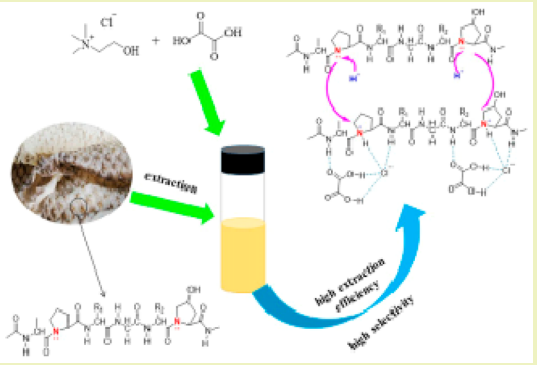
Supporting Information

ABSTRACT: This paper reports an efficient and eco-friendly extraction and separation method of collagen peptides from cod skins based on a nascent class of green and sustainable deep eutectic solvents (DESs). Six kinds of DESs were designed and synthesized. On the basis of the principle of high extraction efficiency and high purity, choline chloride−oxalic acid was selected as the optimal extraction solvent. In the process of collagen peptides purification, ethanol was the optimal precipitation reagent due to the low toxicity. Afterward, single factor experiments proved that the extraction efficiency and purity of collagen peptides were influenced by the molar ratios of choline chloride−oxalic acid, extraction temperature, reaction time and solvent-to-solid ratio. Under the optimal conditions, higher and lower molecular weight collagen peptides were obtained. The values of their respective extraction efficiencies were up to 91.57% and 96.01% and the corresponding purity was up to 93.14% and 100%, respectively. Finally, UV−vis and Fourier transform infrared spectrometry were utilized to study the extraction mechanism. The results from this study demonstrate that choline chloride−oxalic acid is a green, efficient and promising solvent for extracting collagen peptides from cod skins.



KEYWORDS: *Deep eutectic solvents, Extraction, Purification, Cod skins, Collagen peptides*

INTRODUCTION

Collagen, the most abundant and ubiquitous protein in the living body,[1−5] is a key component of the extracellular matrix.[6] Type I collagen, the most common type of collagen, is widely occurring in connective tissues, including tendons, dentin, cornea, bones and skins.[3,7−11] The basic composition of type I collagen is composed of three polypeptide α chains,[5,12] each of them being found to contain the repeating units of Gly−X−Y, where X and Y can be any amino acid, most frequently proline and 4-hydroxyproline.[2,8,13−15]

Collagen is a natural material with excellent biocompatibility,[1] low immunogenicity,[16] biodegradability,[17] nontoxicity and weak antigenicity.[9] It has a wide range of applications in food, biomedical, cosmetic, pharmaceutical, leather and film industries.[2,18,19] Collagen can be further hydrolyzed by many enzymes to produce collagen peptides that have received increasing attention due to their reparative ability to skin.[20,21] Also, collagen peptides possess antihypertensive[22,23] and antioxidant activities[24,25] that are associated with the presence of low molecular weight peptides. It is known that traditional sources of collagen are limited to those from skins of cattle and pig, as well as bones.[3,9] However, the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and the foot-and-mouth disease (FMD) crisis has resulted in anxiety and suspicions for the collagen from bovine, whereas those obtained from porcine is unacceptable for some religions.[19,26,27] As a consequence, cod skins, containing a large quantity of collagen, no risk of disease transmission and no religious barriers, as a replacement for mammalian source have received increasing attention.[28]

In recent years, it is reported that two separate operations have been required to extract the collagen peptides from aquatic byproducts for industrial production purposes. In the first step, gelatin is obtained from raw materials by a chemical pretreatment with dilute acid or alkali, which destroys hydrogen bonding and cleaves a number of intra- and intermolecular covalent cross-links that are present in collagen, thus destabilizing the triple-helix and producing collagen solubilization.[20,29] The step of acid or alkali pretreatment would result in the leaching of noncollagenous proteins because the collagen can not be specifically identified by acid or alkali solution, which makes it difficult to separate and purify the product. In addition, It has been reported that the extraction efficiency of gelatin is relatively low, which could be due to the loss of extracted collagen, through leaching, during the series of washing steps.[30] In the second step, collagen peptides are produced by enzymatic hydrolysis of the gelatin based on the principle that the peptide bonds would be cleaved by enzymes

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in the characteristic site.[29] However, the enzymes are high price and could not be reused.[31] In summary, the traditional methods used for extraction and separation of collagen peptides are complicated, time-consuming and sometimes harmful to the environment. Therefore, a new, green and efficient method for the extraction of collagen peptides is critically needed.

Since being first reported by Abbott et al. in 2003,[32] deep eutectic solvents as potential alternatives to conventional ionic liquids (ILs) and organic solvents[33−35] with many advantages over traditional solvents, such as biocompatibility,[36] easiness of preparation,[37] environmentally friendly properties[38] and low cost,[39] have attracted great attention. Deep eutectic solvents (DESs) can be obtained by simply mixing two or more compounds acting as either hydrogen bond donors (HBD) or hydrogen bond acceptors (HBA),[40,41] the mechanism of which is that the complexing agent (typically a H-bond donor) interacts with the halide anion and increases its effective size, which in turn reduces the anion interaction with the cation and thus makes the mixture melting points far below that of the individual components.[42−45] One of the most widely used as cationic salts for DESs is choline chloride (ChCl), which is not only cheap and easy to make but also biodegradable and nontoxic.[46] It is reported that DESs have been employed in different areas of chemistry, such as electrochemistry,[47] metal dissolution,[48] organic synthesis,[49] materials chemistry[50] and enzyme reaction.[51] The recent discovery and application of the deep eutectic solvent (DESs) provided new insight toward the extraction and separation bioactive compounds including DNA extraction,[52] lignin extraction,[53] flavonoid extraction[54] and phenolic acids separation[55] based on the principles of HBD or HBA interaction with the target compounds, such as hydrogen bonding, acid−base catalysis, or $\pi-\pi$, etc.[51,53] The successful application using deep eutectic solvents for separation and extraction of natural compounds has attracted our attention to the extraction of collagen peptides.

To the best of our knowledge, there has not been a detailed investigation applying DESs for extraction of collagen peptides from cod skins. This study mainly aims to propose a green, highly selective, simple and efficient method for the extraction and purification of collagen peptides based on the knowledge of DESs. In the current work, six kinds of DESs were used for extraction of collagen peptides. The influences of experimental conditions on the extraction efficiency and purity of collagen peptide were investigated. The structure and purity of the targets were analyzed by UV−vis, high performance liquid chromatography (HPLC) and SDS-polyacylamide gel electrophoresis (SDS-PAGE). Finally, the potential mechanism of DESs extraction of collagen peptide from cod skins was studied.

EXPERIMENTAL SECTION

Materials. Cod skins were provided by Dalian Hongyun fish skin processing Co. Ltd. (Liaoning, China). Choline chloride (AR, 98.0−101.0%), urea (AR, ≥99.0%), ethylene glycol (AR, ≥99.0%), glycerol (AR, ≥99.0%), lactic acid (AR, ≥99.0%), acetic acid (AR, ≥99.5%) and oxalic acid (AR, ≥99.5%) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Coomassie Blue R-250 and Tris(hydroxymethyl) aminomethan were analytical grade and purchased from Sangon Biotech (Shanghai) Co., Ltd. Acrylamide (AR, 99.0%), prestained protein ladder marker (11−245 kDa) and Type I collagen peptides were procured from Beijing Solarbio Science & Technology Co., Ltd. All other chemicals and reagents if not declared were obtained from Sinopharm Chemical Reagent Co., Ltd.

Proximate Analyses. The determinations of moisture, fat, ash and protein content of skin were carried out according to the AOAC methods. The hydroxyproline content was analyzed by using a colorimetric method recommended by ISO (the standard curve of hydroxyproline shown in Figure S1). The conversion factor for calculating the content of cod skins collagen from hydroxyproline was 14.7.[56]

Viscosity Measurement. The viscosity of DESs was measured using a NDJ-8S rotational viscometer with a spindle attachment at room temperature. Reading was taken after 10 min. The results are shown in Table S1.

Extraction of Collagen Peptides from Cod Skins with DESs. Accurately weighed 0.2 g of powdered cod skins was added to 20 mL of six kinds of DESs in a 40 mL sealed vial, and then the mixture was magnetically stirred for 4 h at 45 °C in a water bath. The extract was centrifuged at 8000 r/min for 20 min. Part of the supernatant was used for the determination of hydroxyproline contents and the remainder was precipitated by adding methanol, ethanol, acetone and acetonitrile, respectively. The resultant precipitate was collected by centrifuging at 5000 r/min for 15 min and dried in the oven at 40 °C for one night to remove the remaining ethanol.

UV−vis Spectra. The collagen peptide samples obtained from Beijing Solarbio Science & Technology Co., Ltd. and the product treated with DESs were dissolved in 0.1 M acetic acid to be measured using a TU-1810 UV−visible (UV−vis) spectrophotometer.

HPLC Analysis. HPLC analysis was performed on an Agilent 1200 series HPLC system. The chromatographic separation was carried out on a ZORBA × 300SB-C18 column (4.6 mm × 250 mm, 5 μm). The separation was conducted on a 5 μL sample at 30 °C at 0.4 mL/min for 20 min using a binary elution as a mobile phase between solvent A (60%): 5% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid and solvent B (40%): 90% (v/v) acetonitrile. The detection wavelength was 210 nm.

SDS-Polyacylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed using the discontinuous Tris−HCl/glycine buffer system with 15% and 7.5% resolving gel, respectively. The prestained protein ladder marker (11−245 kDa) and samples were loaded per well and subjected to electrophoresis at discontinuous voltages of 80 and 120 V using a mini double vertical electrophoresis instrument (DYCZ-24DN, Beijing Liuyi Biotechnology Co., Ltd. Beijing, China).

Fourier Transform Infrared Spectroscopy (FT-IR). Fourier transform infrared spectroscopy (FT-IR) of the samples was obtained using FTIR (Nicolet 380) spectroscopy from 4000 to 400 cm^{-1} at a data acquisition rate of 2 cm^{-1} per point and was compared to a background spectrum recorded from not placing on a sample stage at 25 °C.

RESULTS AND DISCUSSION

Leaching of Collagen or Collagen Peptides with Different DESs. The cod skins possessed the chemical composition of 18.21 ± 0.05% moisture, 1.57 ± 0.10% fat, 5.81 ± 0.07% ash, 73.23% protein, and 61.90% collagen, which indicated that cod skins are good sources for extraction of collagen peptides. In this study, six DES mixtures were applied in the collagen peptides extraction process, and the extraction efficiencies (%) are shown in Figure S2. It is clear that the extraction abilities of the DESs are established as follows: ChCl-OA > ChCl-HAC > ChCl-La > ChCl-EG > ChCl-G > ChCl-U. These DESs have the same hydrogen-bonding acceptor (HBA) but different hydrogen-bond donor (HBD). It is worth noting that the acidity of HBD followed the order: OA > La > HAC > G > EG > U, which indicates that the acidity of the HBD is the main factor that determines the extraction efficiency of the product. In addition, the viscosity of DESs can also affect the extraction abilities in the case of little difference in acidity of DESs, which would be explained by the phenomenon of the ChCl-HAC > ChCl-La and ChCl-EG > ChCl-G in extraction abilities. It is obvious that ChCl-U has a weak ability to extract

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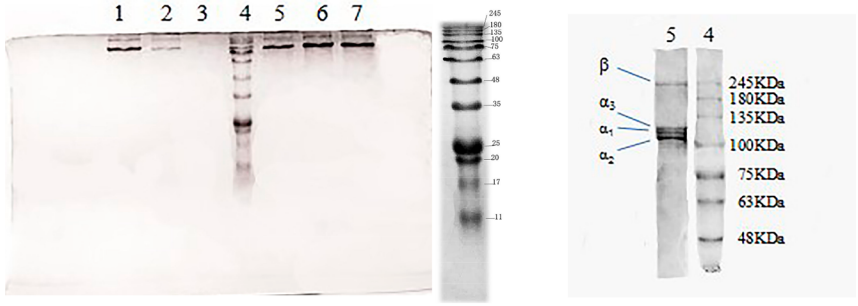

Figure 1. SDS-PAGE of products obtained from different DESs. Lane 1: products precipitated by ethanol in ChCl-La. Lane 2: products precipitated by methanol in ChCl-La. Lane 3: products precipitated by ethanol in ChCl-OA. Lane 4: prestained protein ladder marker (11−245 kDa). Lane 5: products precipitated by ethanol in ChCl-HAC. Lane 6: products precipitated by methanol in ChCl-HAC. Lane 7: products precipitated by acetonitrile in ChCl-HAC.

collagen or collagen peptides from cod skins due to the lack of free protons in the solution. Therefore, the method of using ChCl-U as a leaching solvent for the product was excluded.

Purification of Products with Different Precipitation Mediums. In this study, methanol, ethanol, acetone, and acetonitrile were used as preliminary screening of precipitation mediums. The results are shown in Table S2, which demonstrated that methanol, ethanol and acetonitrile could be miscible with ChCl-EG and accompanied by the formation of white flocculent precipitations that were used for UV−vis analysis (Figure S3). It can be observed that the white flocculent precipitations may be the mixture of collagen (230 nm), noncollagenous proteins and nucleic acid,[57] the result of which was similar to ChCl-G (Figure S4). They were not suitable for the extraction of collagen or collagen peptides due to low purity and yield of the products.

The results obtained from ChCl-La, ChCl-HAC and ChCl-OA are shown in Figure S5−S7. It is clear that the acidic deep eutectic solvents can get the collagen or collagen peptides with high purity. In order to identify the molecular weight of the product treated with ChCl-La, ChCl-HAC and ChCl-OA, HPLC was performed, and the results are displayed in Figures S8−S10. From the results, ChCl-La and ChCl-HAC can be used to obtain the high molecular weight collagen (4.228−4.233 min). It can be seen from Figure S10 that the products obtained from ChCl-OA were mainly collagen peptides (6.323−6.330 min). It is worth noting that the strength of hydrogen bonds increases with increasing the acidity of DESs,[58] so the hydrogen bonds formed by ChCl-OA is the strongest compared with ChCl-La and ChCl-HAC. The strong hydrogen bonding would contribute to the acquisition of low molecular weight collagen peptides. According to the retention time of HPLC, the properties of the products precipitated by methanol, ethanol and acetonitrile were similar. Therefore, the ethanol was used as a precipitation medium to separate and purify the products depending on the principle of low toxicity.

To determine further the molecular weight of products, the white flocculent precipitations from acidic deep eutectic solvents were analyzed by SDS-PAGE (Figure 1). It is shown that the collagen from ChCl-La and ChCl-HAC is a heterotrimer with α chain composition of α1, α2, α3 and their cross-linke β chains. The existence of three different α subunits shows that the major collagen from cod skins extracted by ChCl-La and ChCl-HAC is type I collagen.[59] Moreover, the products obtained from ChCl-OA were mainly collagen peptides that possessed a molecular weight of approximately 11 KDa below. Apparently, compared with other DESs, ChCl-OA possesses the greatest extraction efficiency of collagen peptides and it was employed in the subsequent single factor experiments.

Optimization of the Extraction Process. *Effect of the Molar Ratios of ChCl-OA.* In this study, choline chloride was combined with oxalic acid at various molar ratios from 1:0.6 to 1:1.4. The results are shown in Figure S11. It is clear that the solid particles were formed at room temperature when the molar ratio was 1:1.4. Then further selections were made using the other molar ratios of ChCl-OA to identify the effect on the extraction efficiency at 45 °C. As shown in Figure 2a, when the

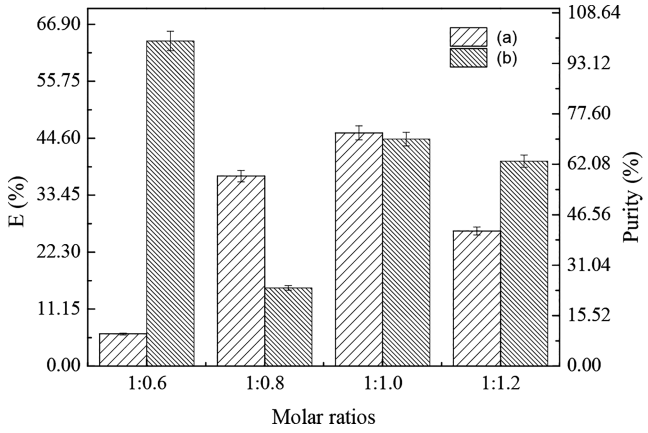

Figure 2. Effect of the molar ratios on the extraction efficiency (a) and the purity of collagen peptides (6.321−6.325 min) (b).

molar ratios of ChCl-OA were decreased from 1:0.6 to 1:1.2, the extraction efficiency increased at first and then decreased, which might be affected by the free hydrogen proton and viscosity of DESs. Generally, the number of free hydrogen protons increased with adding oxalic acid under the condition of the content of choline chloride remaining constant, resulting in a lot of binding sites formed between hydrogen protons and the imino of proline or hydroxyproline in collagen. In addition, the low viscosity would contribute to an increase in the mass transfer effect between solute and solvent. That is why the extraction efficiency of the target compounds obviously increased from 1:0.6 to 1:1.0 mol ratios. When the mole ratio was 1:1.2, the extraction efficiency slightly decreased. The

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reason is that, with a larger amount of oxalic acid, viscosity of ChCl-OA increased, which leaded to a low extraction efficiency of collagen peptides obtained.

HPLC was carried out to identify the effect of molar ratios of ChCl-OA on the purity. The results are presented in Figure S12. The changes in the percentage of collagen peptides (6.321−6.325 min) are shown in Figure 2b. From the results, when the mole ratio was 1:0.6, the collagen extracted from cod skins all became collagen peptides (6.321−6.325 min) under the action of hydrogen bonds in ChCl-OA. On the contrary, when the mole ratio was 1:0.8, the purity of collagen peptides (6.321 min) was lowest due to the depolymerization rate of collagen less than the rate of leaching, resulting in the presence of higher molecular weight collagen peptides (5.239 min). The purity of collagen peptides (6.325 min) dramatically increased when the mole ratio was 1:1.0. Then, the purity decreased a little when the mole ratio reached 1:1.2 due to the increase in viscosity. According to the above results, the mole ratio of 1:1.0 was suitable for the extraction of the target compounds in further experiments.

Effect of Extraction Temperature. The effect of extraction temperature was investigated systematically as shown in Figure 3a. It is indicated that the extraction efficiency increased with

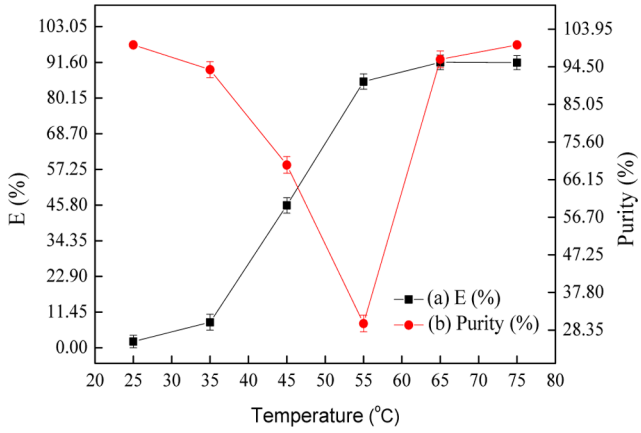

Figure 3. Effect of the extraction temperature on the extraction efficiency (a) and the purity of collagen peptides (6.308−6.325 min) (b).

temperature changed from 25 to 65 °C. A possible reason for this phenomenon was that at higher temperature, the viscosity of ChCl-OA (1:1.0) decreased and the diffusivity increased, improving the mass transfer effect between solute and solvent. In addition, there would be more hydrogen ions ionized with increasing temperature, resulting in more binding sites between hydrogen protons and the imino of proline or hydroxyproline in collagen. When the temperature was increased from 65 to 75 °C, the extraction efficiency remained essentially unchanged.

Considering that the extraction temperature may influence the purity of collagen peptides, HPLC was carried out (Figure S13). For the convenience of analysis, the percentage of collagen peptides (6.308−6.325 min) was calculated. As shown in Figure 3b, the purity decreased at first and then increased with the temperature changed from 25 to 75 °C. When the temperature was 25 °C, the purity was high. The probable reason for this was that a small amount of collagen leached from cod skins was all depolymerized into low molecular weight collagen peptides (6.308−6.325 min). When the temperature was increased from 25 to 55 °C, a large number of target compounds were extracted, resulting in the depolymerization rate of collagen less than the rate of leaching, which made the purity decrease dramatically. Further increasing the temperature might seriously impair the hydrogen bonds in collagen, resulting in more collagen peptides (6.308−6.325 min) obtained. Taking into account the extraction efficiency, purity and energy saving, 65 °C was selected for further study.

Effect of Extraction Time. As an example, the extraction efficiency of target compounds was studied over a time range of 1−7 h at 65 °C in ChCl-OA (1:1.0), and the results are presented in Figure 4a. It was shown that within 2 h, the

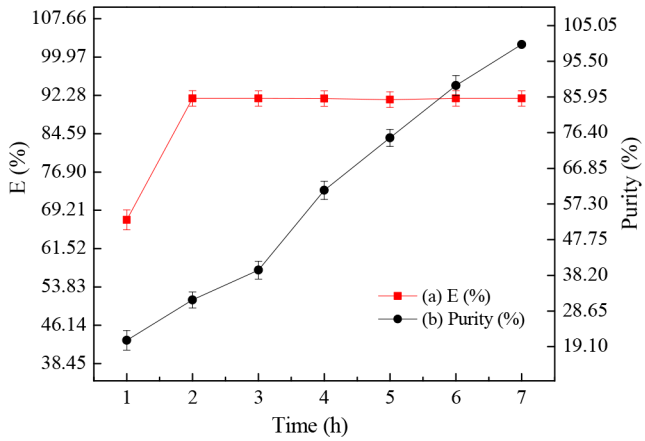

Figure 4. Effect of the extraction time on the extraction efficiency (a) and the purity of collagen peptides (6.281−6.296 min) (b).

extraction efficiency increased with an increase in extraction time, and almost 91.69% of collagen peptides were leached into the solvent at about 2 h. The extraction efficiency no longer increased when the time was further increased.

To ascertain that the extraction time clearly had an effect on the purity of collagen peptides (6.281−6.296 min), the target compounds were further studied by HPLC (Figure S14). The results of purity versus time are shown in Figure 4b. It can be seen that with the extension of the extraction time, higher purity of collagen peptides (6.281−6.296 min) was achieved. The probable reason for this was that when the extraction time was increased from 1 to 2 h, the extraction efficiency increased quickly, resulting in the depolymerization rate of collagen less than the rate of leaching, which made the purity of collagen peptides (6.281−6.296 min) at a low level and increase slowly. When the extraction time was more than 2 h, almost all of the target compounds have been leached and the main reaction in the solution was the depolymerization of collagen, resulting in more and more collagen peptides (6.281−6.296 min) obtained.

From the above results, we can conclude that when the reaction time was 2 h, most of the collagen peptides (5.255 min) were obtained and when the reaction time was 6 h, most of collagen peptides (6.294 min) were obtained, which indicated that the extraction time played a significant role in getting what kinds of collagen peptides. To obtain different kinds of collagen peptides, it is necessary for further research.

Effect of Solvent-to-Solid Ratio (2 h). The solvent-to-solid ratio was studied when the extraction time was 2 h. As shown in Figure 5a, the extraction efficiency of the target compounds increased obviously with the increase of the solvent volume before solvent-to-solid ratio reached 80:1, and then the extraction efficiency remained unchanged with a further

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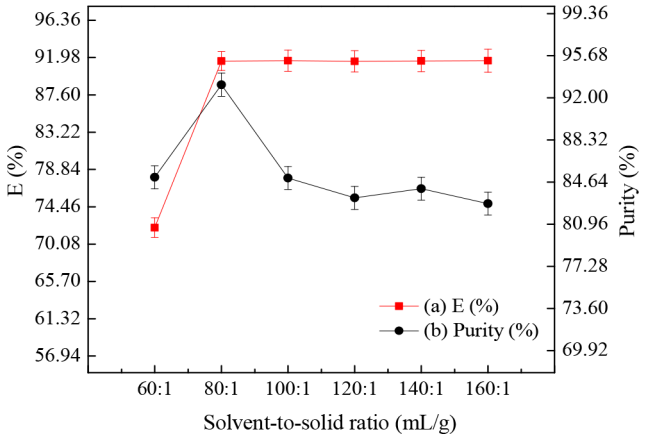

Figure 5. Effect of the solvent-to-solid ratio (2 h) on the extraction efficiency (a) and the purity of collagen peptides (5.133−5.300 min) (b).

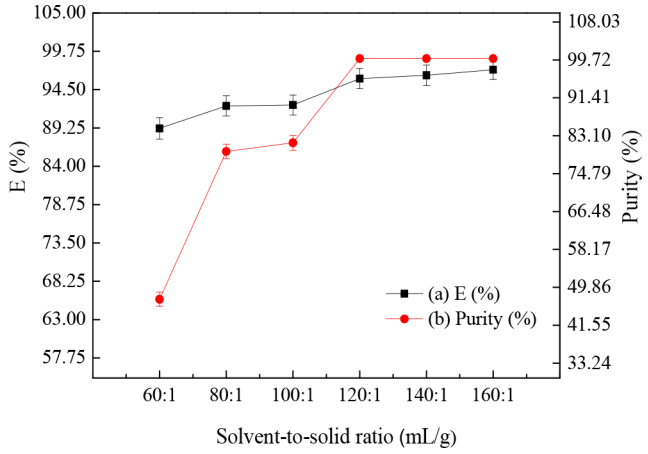

Figure 6. Effect of the solvent-to-solid ratio (6 h) on the extraction efficiency (a) and the purity of collagen peptides (6.293−6.354 min) (b).

increase of the solvent-to-solid ratio. Generally speaking, solvent-to-solid ratio can influence the contact area between solvent and solute, which in turn affects the binding probability of free hydrogen ions and imino groups in the solution, resulting in the change of the extraction efficiency.

HPLC was further used to analyze the purity of different kinds of collagen peptides, as shown in Figure S15. On the basis of the calculation of peak area, the purity of collagen peptides (5.133−5.300) is shown in Figure 5b. It was clear that the purity of target compounds maintained a slow growth with an increase of solvent-to-solid ratio below 80:1, but it slightly decreased first and then remained unchanged when the ratio was more than 80:1. The reason for this was that the effect of solvent-to-solid ratio on the depolymerization rate of collagen peptides (5.133−5.300) was not significant under this reaction condition. In addition, it can be concluded that when the solvent-to-solid ratio was 80:1 at 2 h, the depolymerization rate of target compounds was far less than the rate of leaching, resulting in a large number of collagen peptides (5.133−5.300) obtained. Considering the extraction efficiency and the purity, a solvent-to-solid ratio of 80:1 was used for the extraction of collagen peptides (5.133−5.300).

Effect of Solvent-to-Solid Ratio (6 h). As an example, the effect of solvent-to-solid ratio on the extraction efficiency was studied at 65 °C in ChCl-OA (1:1.0) for 6 h. As shown in Figure 6a, the extraction efficiency remained a slow growth before solvent-to-solid ratio reached 120:1, and then extraction efficiency was not improved with a further increase of the solvent amount, which indicated that almost all of the collagen peptides had been leached when the time was 6 h.

The purity detection of different kinds of collagen peptides is presented in Figure S16. It is shown in Figure 6b that the purity of collagen peptides (6.293−6.354 min) varied with the solvent-to-solid ratio. From the results, the collagen peptides (6.293−6.354 min) had a lower purity when the solvent-to-solid ratio was increased from 60:1 to 100:1. It might be because when the reaction time was 6 h, almost all of the target compounds were extracted, which made the collagen peptides (5.592−5.623 min) keep a high concentration in the low volume of solvent, resulting in the reaction of the depolymerization obstructed. The purity of collagen peptides (6.293−6.354 min) reached 100%, when the solvent-to-solid ratio was 120:1, which indicated that the collagen peptides (5.592−5.623 min) obtained from cod skins were all depolymerized as lower molecular weight collagen peptides (6.293−6.354 min). Hence, the optimum solvent-to-solid ratio was established as 120:1 in the process of extracting collagen peptides (6.293−6.354 min).

After the single factor experiments, the optimum extraction conditions of the higher molecular weight collagen peptides were ChCl-OA (1:1.0)/cod skins (80:1, mL/g) at 65 °C for 2 h. The optimum extraction conditions of the lower molecular weight collagen peptides were ChCl-OA (1:1.0)/cod skins (120:1, mL/g) at 65 °C for 6 h.

Extraction Mechanism. *UV−vis Spectroscopy.* For preliminary exploring the extraction mechanisms, 2 g/L collagen peptides in ChCl-OA, 2 g/L collagen peptides in ChCl-La, 2 g/L collagen peptides in ChCl-HAC and 0.4 g/L collagen peptides in ChCl-EG were analyzed by UV−vis spectra. As shown in Figure S17, collagen peptides dissolved in ChCl-EG had a strong absorbance at 218 nm corresponding to the n→π^* transitions of C═O in the peptide bonds,[9] which indicated neither the structure of peptide bonds was destroyed by ChCl-EG nor the new chemical bonds were formed between ChCl-EG and collagen peptides. The explanation for this phenomenon was that ChCl-EG might contribute to the formation of intermolecular hydrogen bonds between Cl^- and the amino or carboxyl groups at the end of the collagen peptides, resulting in the driving force for the dissolution of collagen peptides. However, the mixtures based on acidic deep eutectic solvents as solvents were no absorption peaks detected in UV region. This is probably due to the fact that the hydrogen ions in acidic deep eutectic solvents bind to the imino with the lone pair electrons, resulting in the formation of ammonium ions in collagen peptides.

To verify the above inference, further research was carried out. L-Alanine, L-glutamic acid, L-lysine, glycine and L-hydroxyproline were respectively added to the solvent of ChCl-EG and ChCl-OA. It was found that all the amino acids used for the study can be dissolved in ChCl-EG, whereas in ChCl-OA only L-hydroxyproline was completely dissolved. The mixtures of dissolving amino acids were used for UV−vis analysis. The results are shown in Figure 7. It is clear that the maximum absorption peak of amino acids dissolved in ChCl-EG existed at 215 nm, which is similar to the studies of Kuipers et al.,[60] indicating that there were no new chemical bonds formed among ChCl-EG and amino acids. It was interesting to

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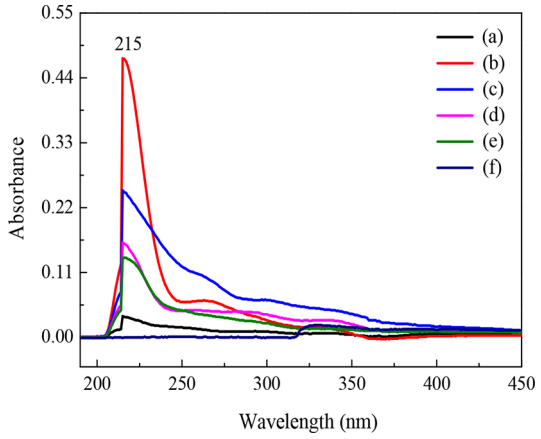


Figure 7. UV−vis spectra of different amino acids in ChCl-EG and in ChCl-OA. (a) 2 g/L L-alanine in ChCl-EG, (b) 2 g/L L-glutamic acid in ChCl-EG, (c) 2 g/L glycine in ChCl-EG, (d) 2 g/L L-hydroxyproline in ChCl-EG, (e) 0.5 g/L L-lysine in ChCl-EG, (f) 2 g/L L-hydroxyproline in ChCl-OA.

find that the characteristic peaks of L-hydroxyproline disappeared in ChCl-OA, which denoted that the structure of L-hydroxyproline was affected by ChCl-OA and a new chemical bond might be generated. Compared with the phenomenon that only L-hydroxyproline was completely dissolved in ChCl-OA, it can be concluded that Cl^- in ChCl-OA was difficult to form hydrogen bonds with amino and carboxyl groups due to the strong hydrogen bond in DES itself. It was worth noting that a unique imino structure is only presence in L-hydroxyproline among the amino acids used in the experiment. As a result, the possible reaction between ChCl-OA and L-hydroxyproline was that the free hydrogen ions attacked the imino groups with lone pair electrons to form ammonium salts, which confirmed the extraction mechanisms of collagen peptides obtained from cod skins in ChCl-OA and also explained why the products being extracted were of high purity when ChCl-OA was used as the solvent.

FT-IR Spectra. FT-IR spectra of pure ChCl-OA, pure collagen peptides, and collagen peptides dissolved in ChCl-OA were performed, and the results are displayed in Figure 8. It can be seen that collagen peptides had characteristic absorption bands at 1630 cm^{-1} for amide I (C═O stretching), 1530 cm^{-1} for amide II (N—H bending and C—H stretching), and 1240 cm^{-1} for amide III (carboxyl OH). However, when the collagen peptides were dissolved in ChCl-OA, the characteristic absorption bands of the collagen peptides disappeared, which indicated that the functional group of peptide bonds might be broken under the influence of ChCl-OA. Moreover, it is surprising to find that the FT-IR spectra of collagen peptides dissolved in ChCl-OA was consistent with the pure ChCl-OA. The reason for this might be that the ammonium salt formed by the reaction of hydrogen ions with the imino of collagen peptides would attract a lot of Cl^- by electrostatic interaction, causing most of the oxalic acid also to be attracted due to the strong hydrogen bonding between ChCl and oxalic acid, which made the intermolecular and intramolecular hydrogen bond in collagen peptides damaged. The formation of new hydrogen bond between collagen peptides and ChCl-OA would contribute to the cleavage of amide bonds at a certain temperature and time, resulting in a new DES formed, which also explained the reason for a large number of collagen peptides obtained by using ChCl-OA as solvent. The reaction process is shown in Scheme 1.

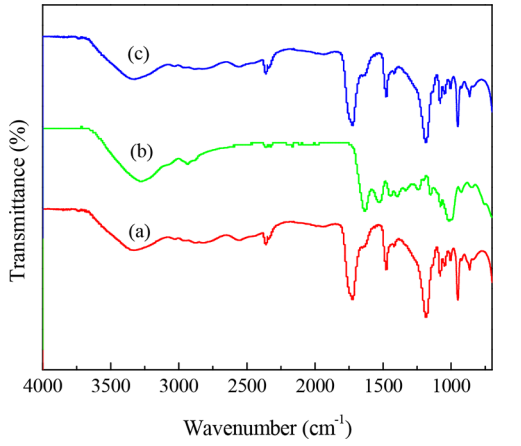
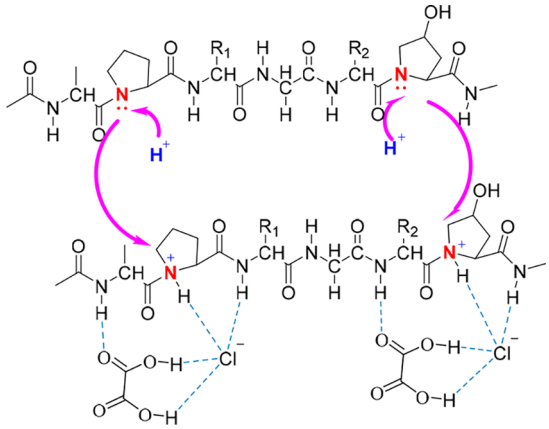


Figure 8. FT-IR analysis of ChCl-OA (a), collagen peptides (b) and 2 g/L collagen peptides in ChCl-OA (c).

Scheme 1. Reaction Process between Collagen Peptides and ChCl-OA



CONCLUSION

In summary, a very simple, efficient and green extraction method was proposed that applying deep eutectic solvents (DESs) to extract high purity collagen peptides from cod skins in high yield. In this study, ChCl-OA was proved to be the best solvent for the extraction of collagen peptides and ethanol was selected as the optimal precipitation reagent to separate and purify the products after a systematic screening, and then the single factor experiments were carried out to obtain the optimal extraction conditions of two types of collagen peptides. Finally, the extraction mechanism was proved by UV−vis and FT-IR spectra. The ammonium salt formed by the hydrogen ions and imino in collagen was the driving force for extracting target compounds from cod skins.

ASSOCIATED CONTENT

Supporting Information

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General procedure for DESs preparation; precipitation effect of organic solvent in different DESs; standard curve of hydroxyproline; extraction results of different kinds of DESs; UV−vis spectra of product obtained from ChCl-EG, G, La, HAC, OA; HPLC of the products in ChCl-La, ChCl-HAC and ChCl-OA; HPLC of the products in different molar ratios, extraction temperature, extraction time, solvent-to-solid ratios (2, 6 h) in ChCl-OA; UV−vis spectra of collagen peptides in acidic DESs and ChCl-E (PDF)

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Notes

The authors declare no competing financial interest.

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