

Mass spectrometric detection of marker peptides in tryptic digests of gelatin: A new method to differentiate between bovine and porcine gelatin

Guifeng Zhang^a, Tao Liu^a, Qian Wang^b, Li Chen^c, Jiandu Lei^a, Jian Luo^a, Guanghui Ma^a, Zhiguo Su^{a,*}

^aState Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100080, China

^bCollege of Life Science and Technology, Beijing University of Chemical Technology, Beijing 1000029, China

^cBioinformatics Institute, Singapore 138671

ARTICLE INFO

Article history:

Received 7 April 2008

Accepted 24 March 2009

Keywords:

Bovine gelatin

Porcine gelatin

Tryptic digestion

Marker peptide

HPLC/MS

ABSTRACT

Gelatin is a mixture of polypeptides obtained by hydrolysis of collagen primarily from bovine and porcine skin and bones. The similarity between different gelatins makes it difficult to trace their species origin. In this work, a new method for differentiation between bovine and porcine gelatin was developed based on detection and identification of marker peptides in digested gelatins. Sequence alignment analysis indicates that bovine and porcine Type I collagen contain differential sequences. The gelatins were digested by trypsin, and the resulting peptides were analyzed by high performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS). The marker peptides specific for bovine and porcine were successfully detected in the digested bovine and porcine gelatin, respectively. Comparative analysis indicated that more marker peptides could be detected in gelatin with a higher mean molecular weight. It was found that proline hydroxylation was a key factor affecting the peptide identification. For peptides such as GPPGSAGSPGK and GPPGSAGAPGK detected in digested bovine and porcine gelatin, respectively, the sequence should be verified manually since the mass shift caused by proline hydroxylation can be confused with the mass difference between Ser and Ala residues. The results indicate that detection of marker peptides in the digested gelatin sample using HPLC–MS/MS is an effective method to differentiate between bovine and porcine gelatin.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Gelatin is a mixture of polypeptides obtained by partial hydrolysis of collagens extracted from connective tissues of animals. Because of its unique gel forming properties, gelatin is widely used in food, cosmetic and pharmaceutical industries. The rheological and functional properties of gelatin, such as the gel strength, viscosity, gelling and melting point, are related to its chemical characteristics (Johnston-Banks, 1990). Both the source of raw material and the manufacturing process affect the properties of the gelatin (Cho, Jahncke, Chin, & Eun, 2006; Gomez-Guillen et al., 2002). Traditionally, most commercial gelatins are manufactured from porcine or bovine skin and/or bones. However, in some cases, such as outbreaks of bovine spongiform encephalopathy, it may be required to differentiate between bovine and porcine gelatin for safety consideration. Another aspect is that some religions ban consumption or use of porcine derivatives. Therefore, it is necessary to develop a method to trace the species origin of gelatin.

Bovine and porcine gelatin have similar structures and physicochemical properties. It is difficult to distinguish them by conventional spectroscopic methods. For gelatins derived from mixed sources, the situation is more complex. Thus, physicochemical methods based on principal component analysis of amino acid residues obtained after acid hydrolysis or calcium phosphate precipitation tests have not been proved able to detect a mixture of porcine gelatin in bovine gelatin (Hidaka & Liu, 2003; Nemati, Oveisi, Abdollahi, & Sabzevari, 2004). It was reported that bovine gelatin could be discriminated by an immunochemical analysis method (Venien & Levieux, 2005). Research by Arbogast, Gunson, and Kefalides (1976) indicated that the hydroxylation of proline plays an important role in determining the antigenicity of collagens. So, immunochemical method might be influenced by the extent of proline hydroxylation in gelatin peptides. Ocana et al. (2004) reported that some species specific ions could be detected using mass spectrometry after bovine gelatin was hydrolyzed with 3 mol/l HCl, which could be used for detection of bovine gelatin. However, the content of target ions might be affected by the hydrolysis time and temperature.

Type I collagen contains two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain, and is the most abundant collagen in connective tissues, especially

* Corresponding author. Tel.: +8610 62561817; fax: +8610 62561813.

E-mail address: zgsu@home.ipe.ac.cn (Z. Su).

in skin, tendon and bone (Cleary, 1996). Theoretically, gelatins should contain peptides from hydrolyzed Type I collagen. The sequences of bovine and porcine Type I collagens can be obtained from the Swiss-prot database and from patent texts (Bell, Neff, Polarek, & Seeley, 2001). Alignment-based sequence analysis indicates that the amino acid sequences of bovine and porcine collagen type I are not identical. Amino acid variants were found both in the $\alpha 1(I)$ chain and in the $\alpha 2(I)$ chain of bovine and porcine collagens. The variant-containing peptides generated by theoretical tryptic digestion of bovine and porcine collagen type I are listed in Table 1. These peptides might be used as potential marker peptides for differential analysis of bovine and porcine gelatins. Thus, detection of marker peptides in the digested gelatin might be a possible method to trace the species origin of gelatin. High performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) is a powerful and important technique for protein identification. MS/MS data can be used to identify a certain peptide in complex peptide mixture based on a peptide sequence tag, which has been widely used in proteomics (Domon & Aebersold, 2006; Kinter & Sherman, 2000; Yates, 2004). HPLC–MS/MS has been successfully used in analysis of digest mixtures of collagens for identification of some peptides or analysis of post-translational modifications on

some amino acid residues of peptides (Li, Chen, Wang, Ji, & Wu, 2007; Mikulikova, Eckhardt, Pataridis, & Miksik, 2007).

The purpose of this work is to develop a method to differentiate bovine and porcine gelatin. Gelatins were digested by trypsin and the digest mixtures were analyzed by HPLC/MS. The differential peptides identified in the digested bovine and porcine gelatins can be used as potential marker peptides.

2. Experimental

2.1. Materials

Bovine gelatin (G9382, type B), porcine gelatin (G2500, type A) standards, sinapinic acid, and α -cyano-4-hydroxycinnamic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bovine gelatins with different molecular weight ranges were purchased from Sinopharm chemical reagent Beijing Co. Ltd. (Beijing, China). Trypsin (sequencing grade) was obtained from Promega (Madison, WI, USA). Syringe filter (0.22 μ m) was purchased from Millipore (Billerica, MA, USA). Trifluoroacetic acid (TFA) and acetonitrile (ACN) was purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Table 1
Marker peptides used for the differentiation of bovine and porcine gelatin.

No.	Position ^a	Bovine Type I collagen ^{b,e,f}	m/z^d	Porcine Type I collagen ^{c,e,f}	m/z^d
1.	$\alpha 1$ 315–324	PGAPGPAGAR	850.5	PGPPGPAGAR	876.5
2.	$\alpha 1$ 451–470	GEPGPTGIQPPGPAGEEGK	1831.9	GEPGPTGVQGGPPGPAGEEGK	1817.9
3.	$\alpha 1$ 508–522	GPAGERGAPGPAGPK ^g	1318.7	GPAGERGSPGPAGPK ^g	1334.7
4.	$\alpha 1$ 784–798	GEAGPSGPAGPTGAR	1281.6	GETGPSGPAGPTGAR	1311.6
5.	$\alpha 1$ 921–936	PGEVGPVGGPPGPAGEK	1442.7	PGEAGPPGPPGPAGEK	1414.7
6.	$\alpha 1$ 937–960	GAPGADGPAGAPGTPGPQGIAGQR ^g	2057.0	GSPGADGPAGAPGTPGPQGIAGQR ^g	2073.0
7.	$\alpha 1$ 987–996	QGSPGASGER	945.5	QGSPGSPGER	971.5
8.	$\alpha 1$ 1029–1035	DGSPGAK	631.1	DGAPGPK	641.3
9.	$\alpha 1$ 1039–1064	GETGPAGPPGAPGAPGAPGVPVGPAGK	2121.1	GESGPAGPPGAPGAPGAPGVPVGPAGK	2107.1
10.	$\alpha 1$ 1069–1086	GETGPAGPAGPIGVPVGPAGK	1560.7	GETGPAGPAGVPVGPVGPAGK	1546.8
11.	$\alpha 1$ 1144–1154	GPPGSAGSPGK ^g	911.4	GPPGSAGAPGK ^g	895.5
12.	$\alpha 2$ 235–264	GSDGSVGPVGPAGPIGSAGPPGFPGAPGPK	2541.3	GNDGSVGPVGPAGPIGSAGPPGFPGAPGPK	2568.3
13.	$\alpha 2$ 283–309	GEVGLPLGSLGVPVGPVGNPANGLPAGK	2366.3	GEVGLPVGSLGVPVGPVGNPANGLPAGK	2352.2
14.	$\alpha 2$ 310–327	GAAGLPGVAGAPGLPGPR ^g	1514.9	GAAGLLGVAGAPGLPGPR ^g	1530.9
15.	$\alpha 2$ 328–342	GIPGPVGAAGATGAR	1251.7	GIPGPAGAAGATGAR	1223.7
16.	$\alpha 2$ 361–380	GEPGAVGQPPGPPGSGEEGK	1803.8	GEPGAAGPQPPGPPGSGEEGK	1775.8
17.	$\alpha 2$ 382–399	GSTGEIGPAGPPGPPGLR	1616.8	GPNGEVGSAGPPGPPGLR	1615.8
18.	$\alpha 2$ 414–423	AGVMGPAGSR	902.5	AGVMGPPGSR	928.5
19.	$\alpha 2$ 424–432	GATGPAGVR	785.4	GPTGPAGVR	811.4
20.	$\alpha 2$ 451–464	GFPSPGNIGPAGK	1255.6	GFPSPGNVGPAGK	1241.6
21.	$\alpha 2$ 465–476	EGPVGLPGIDGR	1166.6	EGPAGLPGIDGR	1138.6
22.	$\alpha 2$ 499–506	GPSGDGPK	714.3	GPTGDGPK	728.4
23.	$\alpha 2$ 520–543	GAPGPDGNNGAQPPGLQVQGGK ^g	2130.0	GAPGPDGNNGAQPPGPPQVQGGK ^g	2114.0
24.	$\alpha 2$ 544–569	GEQGPAGPPGFQGLPGPACTAGEAGK	2305.1	GEQGPAGPPGFQGLPGPACTAGEVCK	2333.2
25.	$\alpha 2$ 574–588	GIPGEFGLPGPAGAR	1395.7	GIPGEFGLPGPAGPR	1421.7
26.	$\alpha 2$ 592–609	GPPGESGAAGPTGPIGSR	1564.8	GPPGESGAAGPAGPIGSR	1534.8
27.	$\alpha 2$ 622–645	GEPGVGAPGTAGSPGSLPGER	2103.1	GEPVGLGAPGTAGSPGSLPGER	2117.1
28.	$\alpha 2$ 664–671	GDIGSPGR	758.4	GDVGSPPGR	744.4
29.	$\alpha 2$ 676–693	GAPGAIGAPGAPGANGDR	1505.8	GAPGAVGAPGAPGANGDR	1491.7
30.	$\alpha 2$ 748–777	GENGPVGTGPVGAAGPSPGPPGAGSR ^g	2565.3	GENGPVGTGPVGAAGPAGPPGPPGAGSR ^g	2549.3
31.	$\alpha 2$ 795–815	TGPPGSPGISGPPGPPGAPGK	1781.9	IGPPGSPGISGPPGPPGAPGK	1794.0
32.	$\alpha 2$ 831–846	SGETGASGPPGFVGEK	1476.7	TGETGASGPPGFAGEK	1462.7
33.	$\alpha 2$ 907–926	GPPGNVGNPVGAPGEAGR	1773.9	GPPGAVGNPVGAPGEAGR	1730.9
34.	$\alpha 2$ 927–938	DGNPNGDGPPGR	1152.5	DGNPDSGDGPPGR	1125.5
35.	$\alpha 2$ 939–945	DGQPGHK	738.4	DGQAGHK	712.3
36.	$\alpha 2$ 949–974	GYPGNAGPVGAAGAPGQVGPVGPVVK	2229.1	GYPGNPAGVGAAGAPGQVGPVGPVVK	2173.1
37.	$\alpha 2$ 979–996	GEPGPAGAVGPAGVGPGR ^g	1516.8	GEPGPAGSVGPAGVGPGR ^g	1532.8
38.	$\alpha 2$ 1024–1053	GHNLQGLPLAGHHGDQGAPGAVGPAGPR	2752.9	GHNLQGLPLAGHHGDQGAPVGPAGPR	2778.4
39.	$\alpha 2$ 1068–1080	IGQPGAVGPAGIR	1192.7	TGQPGAVGPAGIR	1180.6

^a The position of the peptide in $\alpha 1(I)$ or $\alpha 2(I)$ chain was obtained from the sequence alignment results of bovine and porcine collagen type I.

^b The sequence of bovine collagen type I was obtained from the Swiss-prot database.

^c The sequence of porcine collagen type I was obtained from reference 11.

^d The mass-to-charge ratio of the peptide was calculated based on the monoisotope and singly-charged state.

^e Only marker peptides within the collagen core sequence and with m/z ranging from 700 to 3000 were listed.

^f The differential amino acids are highlighted, and some proline residues in the peptides are hydroxylated.

^g Proline hydroxylation might cause bovine and porcine marker peptides generate similar MS/MS spectra.

2.2. Tryptic digestion

10 mg of bovine gelatin standard was dissolved in 5 ml of 0.05 mol/l NH_4HCO_3 (pH 8.0). The solution was filtered through a 0.22 μm syringe filter. 100 μl of gelatin solution was withdrawn and 20 μl of trypsin solution (1 $\mu\text{g}/\mu\text{l}$ in 0.05 mol/l NH_4HCO_3 , pH 8.0) was added. The mixture was incubated at 37 °C. Sample aliquots were withdrawn at time intervals of 120 min during the digestion process and analyzed by size exclusion chromatography (SEC). The porcine gelatin standard and bovine gelatins with different molecular weight ranges were dissolved and digested with the same method as described above.

2.3. SEC analysis of the digest mixture

The molecular weight range of the digest mixture was determined on a TSK G2000SW column (7.5-mm i.d. \times 300 mm length; particle size, 10 μm ; pore size 125 Å) using the Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was 87.5% 0.05 mol/l sodium phosphate buffer (pH 7.0):12.5% ACN (v/v). The flow rate was 0.5 ml/min. 10 μl of sample was withdrawn from the digest mixture and injected directly onto the column. The UV detection was recorded at 220 nm.

2.4. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

The molecular weight ranges of gelatins and the digest mixtures were determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Voyager DE Pro system, Applied Biosystems, Foster City, CA, USA). Sinapinic acid (10 mg/ml in 50% ACN containing 0.1% TFA) was used as matrix for gelatin analysis. α -cyano-4-hydroxycinnamic acid (5 mg/ml in 50% ACN containing 0.1% TFA) was used as a matrix for analysis of the digested gelatins. One microliter of sample was mixed with 2 μl of matrix solution, and 1 μl of the mixture was spotted onto a target plate. The mass spectrum was recorded in positive mode.

2.5. HPLC/MS analysis of the digested gelatins

The peptides in the digest mixture were analyzed by HPLC/MS. The on-line chromatographic separation was performed by reversed-phased chromatography on an Agilent Zorbax SB C18 column (2.1 \times 150 mm, 5 μm) using the Agilent 1100 system. Solvent A was water (containing 0.1% TFA), and solvent B was 60% ACN in water (v/v, containing 0.1% TFA). The gradient was 5–100% solvent B over 120 min. The flow rate was 0.2 ml/min. The outlet of the column was introduced into the ion source of an electrospray ionization mass spectrometer (LCQ DecaXP, Thermo Electron, San Jose, CA, USA). The spray voltage was set to 4.5 kV, and the heat capillary was kept at 300 °C. The data acquisition consists of three scan events, an MS scan followed by one zoomscan to determine the charge state of the ion, and MS/MS scan to provide an MS/MS spectrum. The MS scan range was set from m/z 400 to 900. The zoomscan and tandem mass spectrometry (MS/MS) functions were performed in data dependent mode. Dynamic exclusion was enabled with one count and a 0.5 min exclusion duration unit. The collision energy value was set as 33%. Each sample was injected again using the same method as above except that the MS scan range was changed to m/z 900 to 2000.

2.6. Database searching and data processing

Sequence information from MS/MS data was processed using Turbo SEQUEST algorithm in Bioworks 3.2 software (Thermo

Electron, San Jose, CA, USA). The database was created by extracting collagen entries from the Swiss-Prot/TrEMBL database (<http://www.expasy.org>, Jan 24, 2008). The $\alpha 1(I)$ and $\alpha 2(I)$ sequence of porcine collagen obtained from the Bell patent was added to the database (Bell et al., 2001). The database searches were performed with enzyme constraint (trypsin) and a differential modification of 16.0 for hydroxylation on the proline residue. Two missed cleavages were allowed. The following SEQUEST criteria were used as the peptide filter: Xcorr \geq 1.5 for +1 charged peptides; Xcorr \geq 2.0 for +2, Xcorr \geq 2.5 for +3 charged peptides. DeltaCn \geq 0.1 for all peptides. When a peptide listed in Table 1 was found among the search results, the peptide sequence was further verified based on the following criteria: (i) continuity of the b and y ion series, (ii) the quality of the MS/MS spectrum, and (iii) the number of ions in the MS/MS spectrum (Baldwin, 2004; Biemann, 1990; Romijn, Krijgsveld, & Heck, 2003).

3. Results and discussion

3.1. SEC analysis of gelatin and its tryptic digest mixtures

Fig. 1 shows the SEC chromatograms of undigested bovine gelatin and sample aliquots withdrawn during the digestion process. The undigested gelatin has a wide molecular weight range (Fig. 1 (A)). During the digestion process, the retention time of the digest mixture gradually shifted from left to right in the chromatogram, indicating that larger peptides in the gelatin were gradually degraded into peptides with lower molecular weight (Fig. 1 (B), (C) and (D)). After 10 h digestion, no remarkable changes in the elution profile was observed (Fig. 1 (E)). The molecular weight range of the digest mixture incubated at 37 °C for 10 h was analyzed using MALDI-TOF-MS. The mass spectrum in Fig. 2 shows that the molecular weights of peptides in the digest mixture are lower than 5000 Da, which is consistent with the molecular weight range of peptides resulting from the theoretical digestion of collagens. The chromatographic elution profile of porcine gelatin digested at 37 °C for 10 h is similar to that of bovine gelatin digested for 10 h.

3.2. HPLC-MS/MS analysis of the digested gelatins

Fig. 3 shows the total ion chromatograms (TIC) of the bovine and porcine gelatin digested at 37 °C for 10 h with scan range of m/z 400–900 and m/z 900–2000. The elution profiles indicate that the retention time scale of the peptides in the digested bovine gelatin is similar to that of the digested porcine gelatin. This might result from the high homology of $\alpha 1(I)$ and $\alpha 2(I)$ chains between bovine and porcine collagen, which was calculated as 97.1 and 95.3% respectively. Peptides in the digested samples generally show

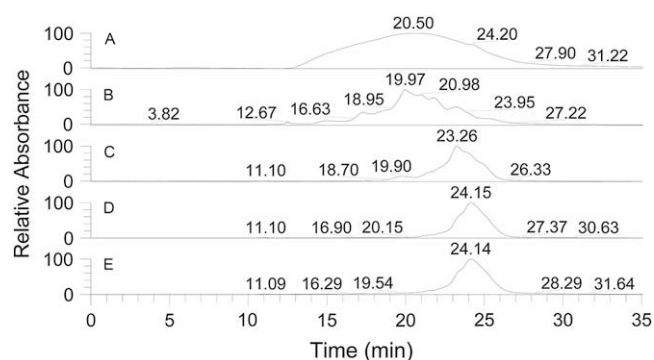


Fig. 1. Gel filtration chromatograms of bovine gelatin (A) and its digest mixtures incubated at 37 °C for 4 h (B), 8 h (C), 10 h (D) and 12 h (E).

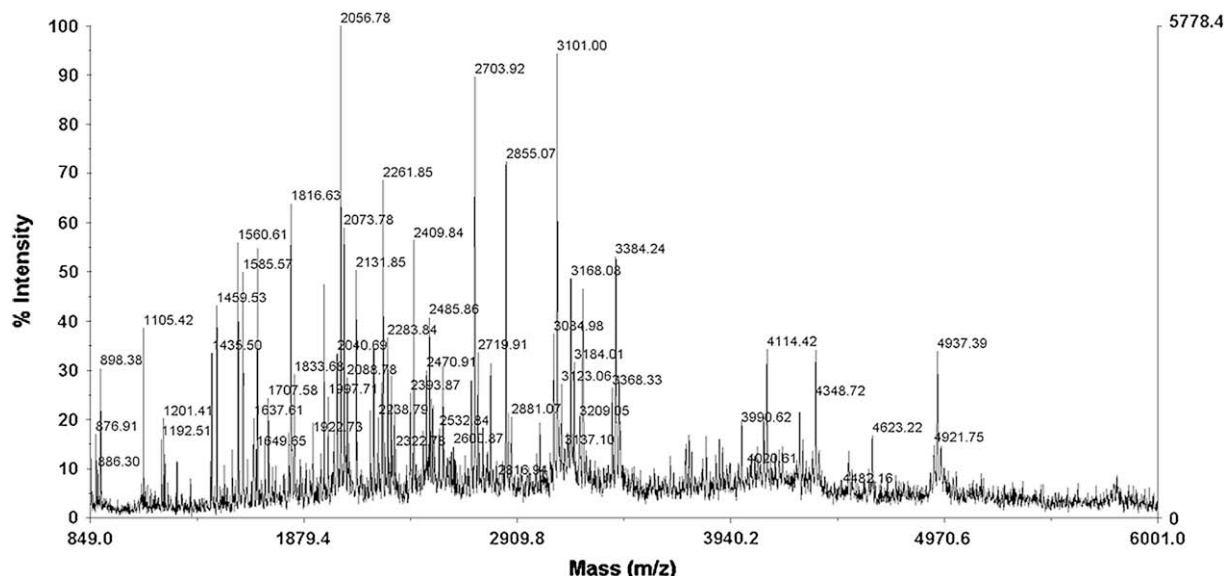


Fig. 2. MALDI-TOF mass spectra of bovine gelatin digested by trypsin at 37 °C for 10 h.

similar hydrophobicity/hydrophilicity due to the high content of glycine and proline. Thus, peptides in the digested gelatin were not easy to fully separate. To acquire as many MS/MS spectra as possible during HPLC–MS/MS analysis, one repeat count was used and the exclusion duration unit was decreased to 0.5 min in dynamic exclusion setting. Each sample was injected twice with the scan ranges from m/z 400 to m/z 900 and from m/z 900 to m/z 2000, respectively. In this way, HPLC–MS/MS might generate almost five thousand MS/MS spectra for one gelatin sample.

3.3. Identification of marker peptides in the digested bovine and porcine gelatins

The acquired MS/MS data were searched against the collagen database. When the peptide in the search results was among the peptides listed in Table 1, the MS/MS spectra was further verified. For example, the ion, m/z 924.0, was found in the digested bovine gelatin (Fig. 4(A)). Zoomscan spectra showed that this ion was doubly charged (Fig. 4(A) inset). Its MS/MS spectrum corresponded to the sequence, $T^{795}GP^*P^*GPSGISGPP^*GPPGP^*AGK^{815}$ (X_{corr} 2.96, ΔCn 0.22) in which four proline residues were hydroxylated (the hydroxylation sites were labeled by *). The partial sequence from $Pro^{*798}-Pro^{810}$ was derived from the observed y ions (Fig. 5(A)). The partial sequences, $T^{795}GP^*P^*$ and $G^{811}P^*AGK^{815}$, were confirmed

according to the b ions and doubly charged b or y ions. The sequence, $T^{795}-K^{815}$ is among the peptides listed in Table 1, and located in the $\alpha 2(I)$ chain of bovine collagen. This peptide was not found in the digested porcine gelatin. However, the ion, m/z 930.0 was found in the digested porcine gelatin (Fig. 4(B)). Zoomscan spectra showed that this ion was doubly charged (Fig. 4(B) inset). Its MS/MS spectrum corresponded to the sequence, $I^{795}GP^*P^*GPSGISGPP^*GP^*PGPAGK^{815}$ (X_{corr} 2.89, ΔCn 0.24). By a similar method, the partial sequence, $Pro^{*798}-Pro^{809}$ was derived from the observed y ions (Fig. 5(B)). The partial sequences, $T^{795}GP^*P^*$ and $P^{810}GP^*AGK^{815}$ were confirmed based on the b ions or doubly charged b or y ions. The sequence was located in the $\alpha 2(I)$ chain of porcine collagen. It is noticeable that some fragmental ions of m/z 924.0 were also observed in the MS/MS spectrum of m/z 930.0. These ions included a doubly charged y_{19} ion (m/z 844.7), and ions from y_7 (m/z 639.3) to y_{18} (m/z 1574.4) ions. The m/z difference between the m/z 924.0 and m/z 930.0 is consistent with the mass difference between thr and ile residues since these two ions were doubly charged. Thus, the peptide, $TGP^*P^*GPSGISGPP^*GPPGP^*AGK$, in the digested bovine

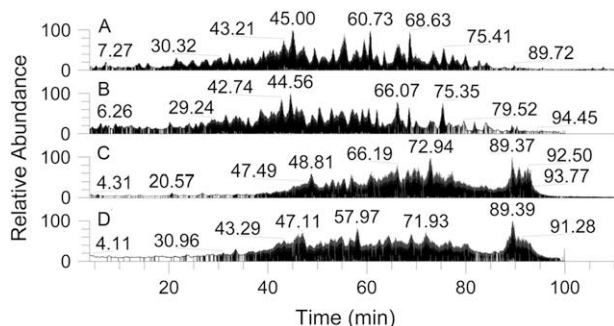


Fig. 3. Total ion chromatograms of the bovine and porcine gelatins digested at 37 °C for 10 h. (A) Bovine gelatin, m/z 400–900; (B) Porcine gelatin, m/z 400–900; (C) Bovine gelatin, m/z 900–2000; (D) Porcine gelatin, m/z 900–2000.

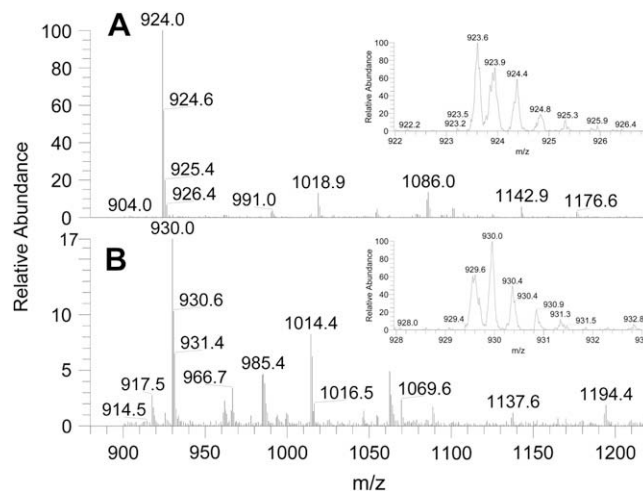


Fig. 4. Mass spectra and zoomscan spectra (inset) of the ion, m/z 924.0 detected in the digested bovine gelatin (A), and the ion, 930.0 detected in the digested porcine gelatins (B).

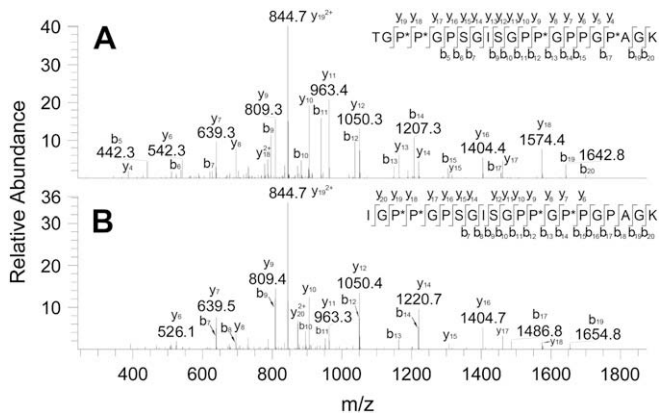


Fig. 5. MS/MS spectra of marker peptides detected from digested bovine and porcine gelatins. (A) The MS/MS spectrum of m/z 924.0 corresponds to the sequence, TGP*P*SGISGPP*GPPGP*AGK; (B) The MS/MS spectrum of m/z 930.0, corresponds to the sequence, IGP*P*GPGSISGPP*GP*PGPAGK; The hydroxylation sites of proline residues are labeled with *.

gelatin was confirmed, and the peptide, IGP*P*GPGSISGPP*GP*PGPAGK, in the digested porcine gelatin was also confirmed. The bovine and porcine gelatin were type B and type A respectively, suggesting that the marker peptides could be released by tryptic digestion in both the alkali and acid processed gelatins. In many cases, only one amino acid residue in a marker peptide specific for bovine gelatin was substituted by a different one in a marker peptide specific for porcine gelatin. Thus, it is necessary to verify the identified sequence since some bovine marker peptides show similar MS/MS spectra to those of porcine marker peptides.

3.4. The effect of proline hydroxylation on peptide identification

Collagen often undergoes several post-translational modifications by intra- and extracellular enzymes (Kagan, 2000). It is known that proline hydroxylation plays an important role for the collagen properties such as stability, antigenicity and mechanical properties (Arbogast et al., 1976; Mizuno, Hayashi, & Bachinger, 2003; Vitaliano, Berisio, Mazzarella, & Zagari, 2001). Proline constitutes almost 18% of the amino acids in collagens, thus, it is important to analyze the hydroxylation sites of proline for sequence verification. Fig. 6 shows two extracted ion chromatograms of m/z 738.4 and m/z 746.3 from digested bovine gelatin. Zoomscan showed that the two ions were both doubly charged (data not shown). MS/MS spectrum

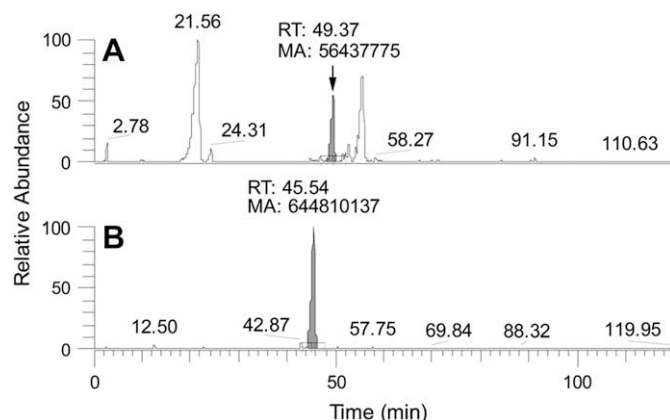


Fig. 6. Extracted ion chromatograms of m/z 738.4 (a) and m/z 746.3 (b) from digested bovine gelatin.

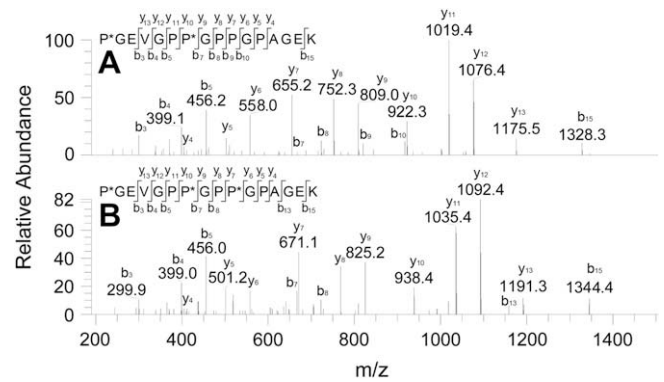


Fig. 7. MS/MS spectra of m/z 738.4 and m/z 746.3 detected in the digested bovine gelatin. (A) The m/z 738.4 was identified as the peptide, P*GEV[G]P[P]G[P]P[G]P[A]GE[K]; (B) The m/z 746.3 was identified as the peptide, P*GEV[G]P[P]G[P]P[G]P[A]GE[K]; The hydroxylation sites of proline residues are labeled with *.

indicated that the ion, m/z 738.4, was from the peptide, p^{921} *GEV[G]P[P]G[P]P[G]P[A]GE[K]⁹³⁶, in which two proline residues were hydroxylated (Fig. 7 (A)). The ion, m/z 746.3, originated from the peptide, p^{921} *GEV[G]P[P]G[P]P[G]P[A]GE[K]⁹³⁶ (Fig. 7 (B)). Hydroxylation of Pro⁹³⁰ resulted in the MS/MS spectrum of m/z 746.3, which is different from that of m/z 738.4. Compared to fragmental ions from m/z 738.4, the extra 16 mass units was observed in the Pro⁹³⁰*-containing fragmental ions from m/z 746.3. The fragmental ions lacking Pro⁹³⁰, such as m/z 300.0 (b_3), m/z 399.0 (b_4), m/z 456.2 (b_5), m/z 558.0 (y_6), m/z 501.1 (y_5) and m/z 404.1 (y_4) were found both in the MS/MS spectra of m/z 746.3 and m/z 738.4. The chromatographic peaks containing Pro⁹³⁰ and hydroxylated Pro⁹³⁰ were integrated. The result showed that 92% of Pro⁹³⁰ in PGEV[G]P[P]G[P]P[G]P[A]GE[K]⁹³⁰ were hydroxylated.

In some cases, hydroxylation of proline would make it more difficult to verify sequences that might be used as marker peptides. A particular example is the sequences in Table 1, G³¹⁰AAGLP³¹⁵GVA-GAPGLPGR³²⁷ and G³¹⁰AAGLL³¹⁵GVAGAPGLPGR³²⁷, which is located in the $\alpha 2(I)$ chain of bovine and porcine collagen respectively. The two sequences will produce similar MS/MS spectrum if Pro³¹⁵ is hydroxylated since the difference between Pro and Leu (16 mass units) is consistent with hydroxylation. Another similar case are the peptides, G¹¹⁴⁴PPGSAGSP¹¹⁵²GK¹¹⁵⁴ and G¹¹⁴⁴PPGSAGAP¹¹⁵²GK¹¹⁵⁴, located in the $\alpha 1(I)$ chain of bovine and porcine collagen, respectively. Hydroxylation of Pro¹¹⁵² will result in two peptides generating similar fragmental ions from b_1 to b_7 ions and/or from y_4 to y_{10} ions. Thus, the hydroxylation of Pro also makes the sequence identification more difficult if the hydroxylated Pro is adjacent to an amino acid residue listed in Table 2. In these cases, the sequence without Pro hydroxylation should be used as a possible marker peptide. For example, the single charged ion, m/z 911.6, was detected in digested bovine gelatin (Fig. 8 (A)). Its fragmental ions, m/z 854.2.0 (y_{10}), m/z 757.2 (y_9) and m/z

Table 2
Amino acid residues having a mass difference of 16 mass units.

Amino acids and their residue molecular weights ^a		Peptide number in Table 1		
Ala	71.04	Ser	87.03	3, 6, 11, 30, 37
Ser	87.03	Cys	103.01	n.f.
Pro	97.05	Ile/Leu	113.08	14, 23
Val	99.07	Asp	115.03	n.f.
Ile/Leu	113.08	Glu	129.04	n.f.
Asp	115.03	Met	131.04	n.f.
Met	131.04	Phe	147.07	n.f.
Phe	147.07	Tyr	163.06	n.f.

n.f.: not found between bovine and porcine type I collagen.

^a The molecular weights of the amino acid type I collagen were calculated based on the monoisotope.

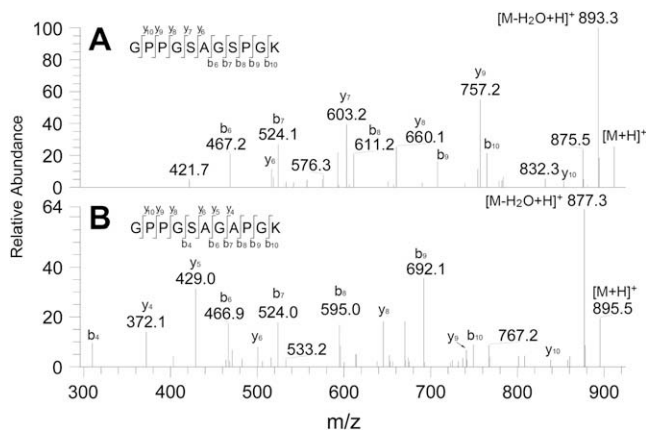


Fig. 8. MS/MS spectra of marker peptides detected in digested bovine and porcine gelatin. (A) The MS/MS spectrum of m/z 911.6 corresponding to the sequence, GPPGSAGSPGK (Bovine gelatin); (B) The MS/MS spectrum of m/z 895.4 corresponding to the sequence, GPPGSAGAPGK (Porcine gelatin).

660.1 (y_8), indicate that Pro¹¹⁴⁵ and Pro¹¹⁴⁶ are not hydroxylated. The eighth residue was confirmed as Ser according to the ions, m/z 534.1 (b_7) and m/z 611.2 (b_8). Thus, m/z 911.6 corresponds to the sequence, G¹¹⁴⁴PPGSAGSP¹¹⁵²GK (Fig. 8(A)). In digested porcine gelatin, the single charged ion, m/z 895.4, was observed. The fragmental ions, m/z 524.0 and m/z 595.0, were identified as the b_7 and b_8 ion, indicating that the eighth amino acid residue is Ala. Thus, m/z 895.4 was identified as G¹¹⁴⁴PPGSAGAP¹¹⁵²GK (Fig. 8(B)). The results suggest that the peptide hits in the search data should be further verified, and that the number and continuity of the b and y ion series in the MS/MS spectra are important for the identification of marker peptides.

3.5. The relative abundance of marker peptides in gelatins with different molecular weight ranges

The relative abundance of marker peptides was analyzed in bovine gelatins with different molecular weight (MW) ranges. The mean molecular weights of three gelatin samples were 29 kDa, 47 kDa and 62 kDa, respectively, as determined by MALDI-TOF-MS (data not shown). The gelatin samples were digested by trypsin at 37 °C for 10 h and the resulting peptides were analyzed using HPLC-MS/MS. The chromatographic peak containing the marker peptide, TGP⁷⁹P⁸⁰GP⁸¹SGISGPP⁸²GPPGP⁸³AGK⁸¹⁵, was integrated. The results indicate that the ratio of this peptide in the three digested bovine gelatins (29 kDa, 47 kDa and 62 kDa) was 1.0:1.3:1.6 (Fig. 9), suggesting that the content of this marker peptide was related to

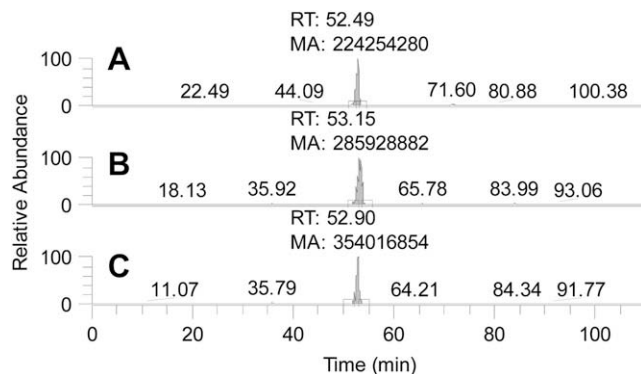


Fig. 9. Extracted ion chromatogram of m/z 924.0 corresponding to the peptide, T⁷⁹GP⁸⁰P⁸¹GP⁸²SGISGPP⁸³GPPGP⁸⁴AGK⁸¹⁵, detected in the digest mixture of bovine gelatin with mean molecular weight of 29 kDa (A), 47 kDa (B), and 62 kDa (C).

the original molecular weight of the digested gelatin. Gelatin is partially hydrolyzed collagen, and the sequence lengths of the peptides in undigested samples should be related to the MW range of the particular gelatin used. The potential to produce some marker peptides might be destroyed during the manufacturing process. Therefore, the higher the mean MW of the gelatin used, the more marker peptides could be detected. Concomitantly, the relative abundance of different marker peptides in the same gelatin should be related to the sequence length of each marker peptide. It is not easy to compare this value since the ionization efficiency of various peptides is different during the mass spectrometric analysis.

4. Conclusions

Despite the wide field of gelatin application, there are increasing concerns with the animal sources of gelatins. Throughout the Islamic world many countries severely restrict the importation or consumption of porcine-based products. US FDA prohibits using sources of animal by-products for gelatin manufacture if those sources were obtained from BSE positive countries. Some gelatins, such as tortoise-shell gelatin, deer-angle gelatin, and donkey-skin gelatin, can be used as Traditional Chinese Medicine for treatment of some diseases (Xu & Zhang, 1989). Therefore, it is important to establish a method to differentiate the various different kinds of gelatins. This study demonstrates that it is possible to differentiate bovine and porcine gelatin based on detection of marker peptides. HPLC-MS/MS analysis coupled with enzymatic digestion is a commonly used strategy for peptide or protein identification in proteomics. During MS/MS data processing, the threshold for specific peptide identification might be different from one species to another (Li et al., 2007; Shadforth, Dunkley, Lilley, Crowther, & Bessant, 2005). For degraded collagens, the case is more complex. Due to the very high homology between collagen sequences of mammals, detection of marker peptides in the digested collagens is a major challenge. Furthermore, proline hydroxylation makes peptide identification more difficult than for most other proteins. To increase the sequence reliability of the marker peptides in digested gelatin, the searching results should be further verified, and the number and continuity of the b and y ion series in the MS/MS spectra should be taken into account. The data obtained in this work indicate that it is possible to detect marker peptides in the bovine and/or porcine gelatins using HPLC-MS/MS. Marker peptide could be used as an index to differentiate bovine from porcine gelatins. Further experiments should be conducted using bovine and porcine gelatins from different geographical areas. The method presented in this research might also be helpful for the investigation of changes in collagen properties caused by proline hydroxylation in different animal tissues or during different growth stages.

Acknowledgements

We sincerely thank Prof. Jan-Christer Janson in Uppsala University of Sweden for reading the manuscript. This work was partly supported by the 973 project – the national development project on key basic research (No. 2007CB714305) and the 863 Hi-Tech research and development program of China (No. 2007AA021604).

References

- Arbogast, B. W., Gunson, D. E., & Kefalides, N. A. (1976). The role of hydroxylation of proline in the antigenicity of basement membrane collagen. *The Journal of Immunology*, 117(6), 2181–2184.
- Baldwin, M. A. (2004). Protein identification by mass spectrometry. *Molecular & Cellular Proteomics*, 3, 1–9.
- Bell, M. P., Neff, T. B., Polarek, J. W., & Seeley, T. W. (2001). Animal collagen and gelatins. *World Patent*, PCT/034647.

- Biemann, K. (1990). Nomenclature for peptide fragment ions. In J. A. McCluskey (Ed.), *Methods in enzymology*, Vol. 193 (pp. 886–888). Orlando: Academic Press.
- Cho, S. H., Jahncke, M. L., Chin, K. B., & Eun, J. B. (2006). The effect of processing conditions on the properties of gelatin from skate skins. *Food Hydrocolloids*, 20(6), 810–816.
- Cleary, E. (1996). Skin. In D. Wayne, & W. D. Comper (Eds.), *Extracellular matrix. Tissue function*, Vol. 1 (pp. 77–108). Amsterdam: Harwood Academic Publishers GmbH.
- Domon, B., & Aebersold, R. (2006). Mass spectrometry and protein analysis. *Science*, 312(5771), 212–217.
- Gomez-Guillen, M. C., Turnay, J., Fernandez-Diaz, M. D., Ulmo, N., Lizarbe, M. A., & Montero, P. (2002). Structural and physical properties of gelatin extracted from different marine species: a comparative study. *Food Hydrocolloids*, 16(1), 25–34.
- Hidaka, S., & Liu, S. Y. (2003). Effects of gelatins on calcium phosphate precipitation: a possible application for distinguishing bovine bone gelatin from porcine skin gelatin. *Journal of Food Composition and Analysis*, 16, 477–483.
- Johnston-Banks, F. A. (1990). Gelatin. In P. Harris (Ed.), *Food gels* (pp. 233–289). London: Elsevier Applied Science Publishers.
- Kagan, H. M. (2000). Intra- and extracellular enzymes of collagen biosynthesis as biological and chemical targets in the control of fibrosis. *Acta Tropica*, 77(1), 147–152.
- Kinter, M., & Sherman, N. E. (2000). *Protein sequencing and identification using tandem mass spectrometry*. New York: John Wiley & Sons Inc.
- Li, B., Chen, F., Wang, X., Ji, B. P., & Wu, Y. N. (2007). Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chemistry*, 102(4), 1135–1143.
- Mikulikova, K., Eckhardt, A., Pataridis, S., & Miksik, I. (2007). Study of post-translational non-enzymatic modifications of collagen using capillary electrophoresis/mass spectrometry and high performance liquid chromatography/mass spectrometry. *Journal of Chromatography A*, 1155(2), 125–133.
- Mizuno, K., Hayashi, T., & Bachinger, H. P. (2003). Hydroxylation induced stabilization of the collagen triple helix. *Journal of Biological Chemistry*, 278(34), 32373–32379.
- Nemati, M., Oveisi, M. R., Abdollahi, H., & Sabzevari, O. (2004). Differentiation of bovine and porcine gelatins using principal component analysis. *Journal of Pharmaceutical and Biomedical Analysis*, 34(3), 485–492.
- Ocana, M. F., Neubert, H., Przyborowski, A., Parker, R., Bramley, P., & Patel, R. (2004). BSE control: detection of gelatin-derived peptides in animal feed by mass spectrometry. *Analyst*, 129, 111–115.
- Romijn, E. P., Krijgsveld, J., & Heck, A. J. R. (2003). Recent liquid chromatographic – (tandem) mass spectrometric applications in proteomics. *Journal of Chromatography A*, 1000, 589–608.
- Shadforth, I., Dunkley, T., Lilley, K., Crowther, D., & Bessant, C. (2005). Confident protein identification using the average peptide score method coupled with search-specific, ab initio thresholds. *Rapid Communications in Mass Spectrometry*, 19(22), 3363–3368.
- Venien, A., & Levieux, D. (2005). Differentiation of bovine from porcine gelatins using polyclonal anti-peptide antibodies in indirect and competitive indirect ELISA. *Journal of Pharmaceutical and Biomedical Analysis*, 39, 418–424.
- Vitagliano, L., Berisio, R., Mазzarella, L., & Zagari, A. (2001). Structural bases of collagen stabilization induced by proline hydroxylation. *Biopolymers*, 58(5), 459–464.
- Xu, K. S., & Zhang, L. K. (1989). Comparison of amino acid composition of donkey-skin, porcine-skin, bovine-skin gelatin. *Chinese Journal of Pharmaceutical Analysis*, 9(3), 152–155.
- Yates, J. R., III (2004). Mass spectral analysis in proteomics. *Annual Review of Biophysics & Biomolecular Structure*, 33, 297–316.