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Analysis of Low Molecular Weight Collagen by Gel Permeation Chromatography

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Abstract : Collagen, which accounts for one-third of human protein, is reduced due to human aging, and much attention is focused on making collagen into food to prevent such aging. Gel permeation chromatography with Reflective Index (RI) detection (GPC/RI) was chosen as the most suitable instrument to confirm molecular weight distribution, and we explored the use of this technique for analysis of collagen peptide molecular sizes and distributions. Data reliability was verified by matrix-assisted laser desorption/ionization coupled to time-of-flight (MALDI-TOF) mass spectrometric analysis. The data were considered meaningful for comparative analysis of molecular weight distribution patterns.

Keywords : Collagen, GPC, molecular weight distribution, refractive Index (RI)

Introduction

There is particular interest in functional foods that are said to prevent skin aging, prevent joint weakness, and maintain body health. Among such foods, various synthetic collagens[1-3] are available in the marketplace. However, the effectiveness of a particular collagen may depend on the raw material source, synthesis method, and enzyme treatment.[4] A larger amount of collagen absorbed by the body might be expected to have a more robust effect. However, a method to objectively verify the efficacy of a particular collagen has not yet been developed.

Collagen is a fibrous protein that is present in most connective tissues in the body, including skin and cartilage in most animals (especially mammals).[2,5,6] Collagen resembles a rope in which three polypeptide molecules are twisted into a triple helix.[3,7] In each polypeptide chain, glycine and proline are alternately arranged, with several different amino acids inserted between (e.g., hydroxylysine and hydroxyproline). These amino acids have important roles in the creation of a quaternary structure in which the chains are tightly connected via hydrogen bonds. Although collagen is very strong under tension, it decomposes at approximately 37°C because of weakened linkages between polypeptides.[8]

Collagen is synthesized as a polypeptide precursor molecule (procollagen) in the blast cells of connective tissue (e.g., fibroblasts and chondroblasts) that have been affected by transforming growth factor-beta signaling substances activated by thrombospondin. In these cells, a signal is transmitted to the nucleus by Smad proteins; three procollagen chains are then individually and simultaneously synthesized in the cell. Peptide residues at the end of each procollagen are placed outside the cell membrane, and strong collagen fibers are then formed via intermolecular hydrogen bonds. There are 29 types of fibrous and non-fibrous collagens comprising subtypes I through VIII.[3,9]

The unique structure of collagen makes it reasonably stable, but decomposition is promoted in vivo by various factors, such as natural aging.[10,11] Ultraviolet (UV) light is another such factor.[12] When exposed to UV light, fibroblasts in the skin stop synthesizing collagen. UV light also promotes the synthesis of matrix metalloproteinase, which is a proteolytic enzyme that breaks down collagen.[4] As skin ages, the amount of matrix metalloproteinase synthesized increases and the rate of collagen decomposition increases accordingly. Collagen contained in food, hydrolyzed collagen, and collagen peptide have molecular weights of approximately

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290,000, 5,000, and < 500 Da, respectively. It has been reported that the absorption rate in the body, antioxidant activity, and anti-aging activity all increase with decreasing molecular size.[10,11,13]

Molecular weight measurement of polymers is one of the most basic and important analytical techniques.[8] The mean molecular weight and shape of the molecular weight distribution greatly influence the physical properties of a polymer.[14] Gel permeation chromatography (GPC) is a key technique for measuring the molecular weight of a sample relative to the weight of a standard material.[15,16] Briefly, GPC uses the pore size of packing particles inside the column to allow low molecular weight substances to remain in the column for longer times,[8,16] while large molecular weight substances are eluted quickly. The molecular weight distribution is calculated relative to the chromatogram of a reference sample with a known molecular weight. GPC is a very useful technique for measuring the molecular weight and composition of high to low molecular weight substances.[9,17] Application to polymers is particularly important for quality control purposes and for adjustment of polymerization conditions.[3,18]

In this work, GPC and matrix-assisted laser desorption/ionization coupled to time-of-flight (MALDI-TOF) mass spectrometry[19] were used to investigate the molecular weight distributions of collagen samples. [1,5,6,13,14]

Experimental

Materials

The six fish collagen peptide products were supplied by the analytical sponsor, are made by different manufacturers, and all have different expected molecular weights. Details of the corresponding collagens are given in Table 1. These samples were prepared at a concentration of 5 mg/mL in pure distilled water to make a standard solution, filtered through a PVDF membrane filter (0.45 um, 13 mm), and injected to obtain a chromatogram.

In addition, The standards were used to provide molecular weight standards: poly(ethylene glycol), poly(ethylene glycol), and pullulan. All these standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Collagen sample details.

No.	Name	Expected M.W.	From
1	A	300	India
2	B	500	China
3	C	1,000	Korea
4	D	3,000	Korea
5	E	500	Korea
6	F	1,000	Korea

GPC

Molecular weight measurements were performed using a GPC-6000 instrument (Futecs, Korea) equipped with two columns. One is OHpak SB-802HQ HPLC column (particle size 6 μm, 8.0 × 300 mm), and the other is OHpak SB-802.5HQ HPLC column (particle size 8 μm, 8.0 × 300 mm). These are from Shodex™ (Showa Denko K.K., Japan). Using these two columns, it is capable of separations up to 10,000 Da. The mobile phase was distilled/deionized water at a flow rate of 1.0 mL/min; the molecular weight distributions of the collagen peptides were monitored using a refractive index detector (GPC/RI) at 40°C. A mixed molecular weight standard composed of poly(ethylene glycol) (520 Da), poly(ethylene glycol) (3,450 Da), and pullulan (6,600 Da) was used to calibrate the instrument (Figure 1).

MALDI-TOF mass spectrometry

All experiments were performed using an UltrafleXtreme MALDI-TOF mass spectrometer (Bruker) equipped with a 337-nm N2 laser operating in positive-ion reflectron mode. The matrix of α-cyano-4-hydroxycinnamic acid was manually deposited onto the MALDI target from a 70:30:0.1 (v/v/v) acetonitrile/water/trifluoroacetic acid solution. The analyte and matrix were mixed in equal volumes; 1-μL aliquots

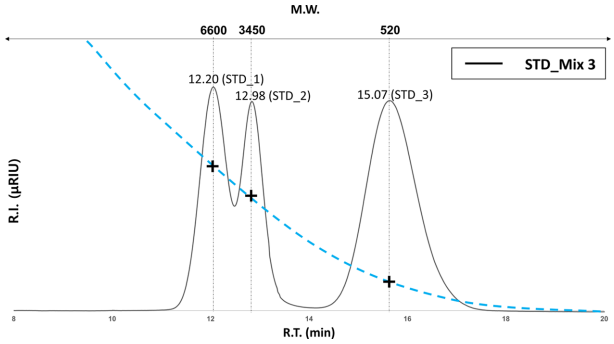

Figure 1. Chromatogram of the reference standard.

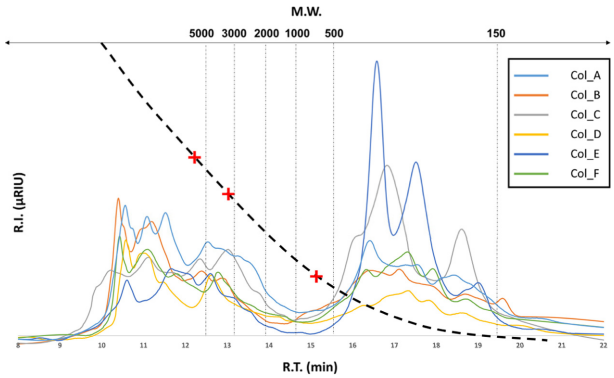

Figure 2. GPC molecular weight distributions of collagen samples.

were then spotted on top of the target plate using the dried droplet method.[19]

Results

The mean molecular weight distributions of the six collagen samples (A to F) determined by GPC/RI are presented in Figure 2 and Table 2. Their MALDI-TOF mass spectra are presented in Figure 3.

The molecular weight distributions presented in Figure 2 were determined for samples prepared at the same concentration. Increasing retention time on the column corresponds to decreasing molecular weight; retention times ranged from approximately 10 to 20 min. Greater signal intensity indicates greater content of the corresponding molecular weight fraction. The curve derived from the three standards was used to estimate the mean molecular weight of a sample. The molecular weight distribution was estimated from the peak areas (%). In this manner, the GPC method was used for quantitative analysis of mean molecular weight and molecular weight distribution.

MALDI-TOF mass spectrometry was used to confirm the intrinsic molecular weight of a sample, and the findings were compared with the results of GPC analysis. However, the GPC/RI analysis was limited to samples with masses < 5,000 Da because of ion condensation (adsorption).

The GPC/RI results are presented in Table 2 where "Ver_I (All)" refers to all data and "Ver_II (Part)" refers to the subset excluding samples with molecular weights > 5,000 Da. The data corresponding to Ver. II (Part), which are considered more reliable, provided a number mean molecular weight (Mn) and weight mean molecular weight (Mw) of 253 and 514 Da, respectively, for collagen E; the proportion of the peak area value occupied by the low molecular weight band in the range of 150 to 500 Da was 83.93% (i.e., the highest proportion) of the total. Collagen E contained the lowest molecular weight material (< 500 Da), which was consistent with the Ver_I (Raw) data.

Table 2. Molecular weight distributions of collagen samples A to F.

Sample	Spec	Ver_I (All)			Ver_II (Part)		
		*Mn	**Mw	<500Da Area (%)	Mn	Mw	<500Da Area (%)
A	300	337	5013	35.62	278	723	55.21
B	500	393	6935	34.26	261	897	66.67
C	1,000	324	4535	52.41	283	647	72.41
D	3,000	332	4952	34.35	333	1022	56.74
E	500	279	2581	64.82	253	514	86.63
F	1,000	304	3967	45.88	301	741	70.05

*Mn : Total Number average molecular weight
**Mw : Total Weight average molecular weight

Discussion

The Ver_II (Part) GPC data of the six collagen samples (A–F) were considered more reliable for molecular weight details below approximately 5,000 Da. The data above 5,000 Da were excluded from the complete data set for the following reasons:

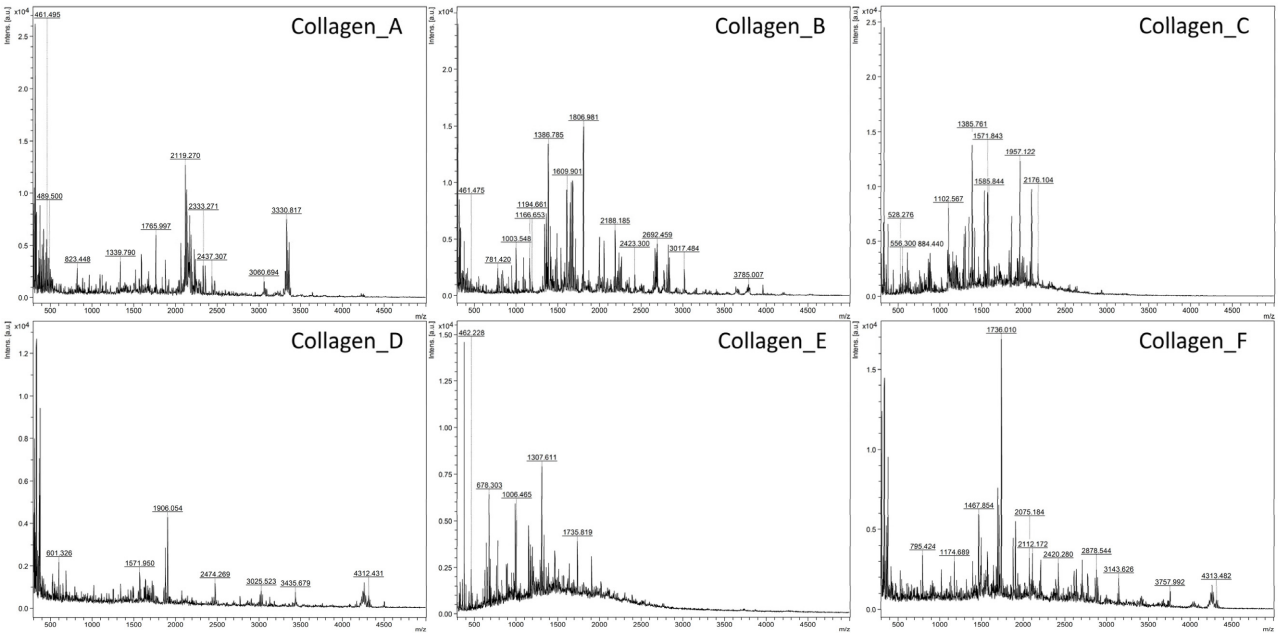

Figure 3. MALDI-TOF mass spectra of collagen samples A to F.

1) MALDI-TOF mass spectral analysis did not reveal any peaks at approximately 5,000 Da or higher. Thus, the likelihood of sub-5,000 Da material in a sample analyzed by GPC was considered low.

2) In the GPC/RI data, the possibility that signals were affected by ion condensation (adsorption) cannot be excluded.

3) The upper molecular weight limit of the GPC column was 10,000 Da. Accordingly, there is little confidence in data exceeding this limit.

4) This sample is a peptide in which amino acids are sequenced, and comparative analysis of UV and RI detectors under the same conditions may be meaningful. It is considered that additional experiments are necessary.

It is important to recognize the limitations of using GPC for the molecular weight analysis of polymers. First, the molecular weight of a polymer is a comparative value that is determined relative to molecular weights of various reference materials; it is difficult to define the true value for a sample. Second, although the reproducibility of molecular weight measurements by GPC is very good within a laboratory, reproducibility between laboratories is poor. Notably, the results depend on the analytical conditions, such as the type of column. Furthermore, GPC analysis is a time- and quantity-intensive technique that requires several standard substances to be analyzed together to estimate the molecular weight of a single sample.

Conclusions

For polymeric materials, GPC and MALDI-TOF mass spectrometry provide complementary molecular weight and molecular weight distribution data. The MALDI-TOF technique provides true, rather than comparative, molecular weight information; however, it is not suitable for all polymers because of the ion adsorption issue. An alternative GPC method for absolute molecular weight measurements uses multi-angle light scattering detection. Advances in these techniques will undoubtedly lead to more accurate molecular weight data that can be applied to quality control of biopolymers, such as collagen.

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