Characterization of Collagen by Capillary Electrophoresis

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Characterization of pepsin-solubilized type I collagen was examined by a capillary electrophoresis using a capillary coated with α-dodecyl-ω-hydroxy poly(oxyethylene) to reduce protein adsorption and electroosmic flow. A good peak separation for each polypeptide was achieved at pH 5.6—6.5. The peaks were assigned to $\alpha 1$, $\alpha 2$, $\beta 11$, $\beta 12$, and γ by gel chromatography and reversed phase HPLC. It is found that each α chain signal split into several peaks, reflecting various residues of teropeptide.

Type I collagen, a major protein component of connective tissue, is prepared by extraction from animal tissue. The molecule is a heterotrimer consisting of two $\alpha 1$ which contains ca. 1000 amino acids (MW = ca. 100000) and one $\alpha 2$ which also contains ca. 1000 amino acids (MW = ca. 100000). These form a triple-helical region and two nonhelical regions at both ends (N-terminal and C-terminal) which are called teropeptide (Fig. 1).[1] Crosslinks of intra and inter molecules at teropeptide region make the collagen insoluble.[2] Although the triple helical region is stable to most proteolytic enzymes, teropeptide is not stable to proteolytic attack and pepsin digests teropeptide region.[1] As the results, crosslinks are broken and insoluble collagen turns into a pepsin-solubilized collagen (PSC) which keeps the triple-helical structure. As PSC is a biomaterial, it is expected to be a good biocompatible and biodegradable material.[3,4] Especially, it is expected as a good material for the medical field, because of its low antigenicity caused by digest of teropeptide.[5,6] However, as it is difficult to digest completely and a part of teropeptide remains, it is important to know the extent of such digest for medical use.

Usually sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),[7] gel chromatography,[8] and ion exchange chromatography[9] are used to analyze collagen but it is difficult to detect differences in teropeptide remaining in PSC precisely by them because there are very small differences in molecular weight of PSC. Recently capillary electrophoresis (CE) was developed for analysis of protein[10—12] and was expected to be able to detect small differences in teropeptide residues. In this study, analysis of PSC with CE was examined.

Experimental

Materials. PSC was purchased from Koken Co. and at first the commercial PSC(C-PSC) was used without further treatment. In some cases C-PSC was further digested with pepsin as follows to decrease the teropeptide region. The further digested PSC(FD-PSC) was analyzed to check our idea. C-PSC (0.5%) and pepsin (0.1 and 0.01%) were dissolved in 0.1 M acetic acid and the solution was placed in an incubator to digest teropeptide for 20 h at 35 °C keeping triple-helical structure followed by fractionation with GPC and reversed phase HPLC. All PSC samples were denatured at 70 °C for 3 min before analysis. Sodium phosphate and trichloro(octadecyl)silane and α-dodecyl-ω-hydroxy poly(oxyethylene) (Brij 35) were commercially obtained. Buffer solutions were prepared with double-deionized water passed through a 0.45 μm filter.

CE. CE was carried out with a Model CIA (Milipore Co.) equipped with UV detector (185 nm). A sampling vial was attached

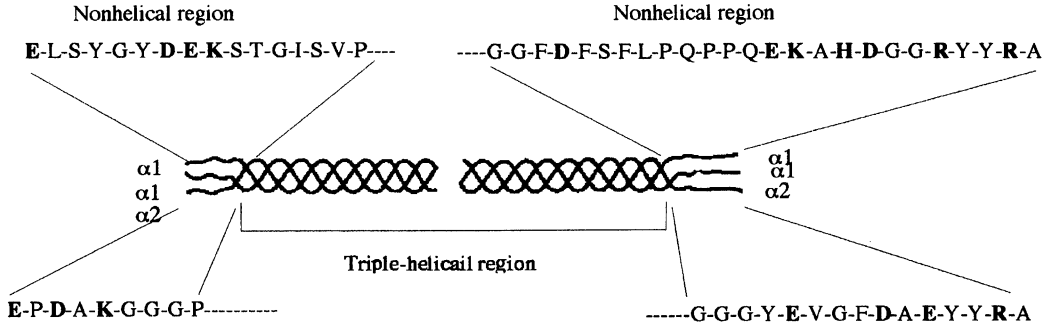

Fig. 1. Collagen molecule (type I) showing triple-helical region and nonhelical regions.

to the anode. Polyimide coated fused silica capillary (Milipore Co.) of 50 μm i.d. and 60 cm length (52 cm separation length) or modified capillary were used for CE. A modification of capillary wall was performed as follows.[13] Brij 35 solution (1% w/v) in buffer was pulled through a capillary for at least 1 h, and the then capillary was washed with Brij 35 solution (0.02% w/v) in buffer. PSC (1—5 mg ml^{-1}) was dissolved in separation buffer degassed under reduced pressure and was injected hydrodynamically for 40 s with 10 cm height differential. After analysis, the capillary was flushed with buffer for 4 min.

HPLC. A system of Waters Co. (multisolvent delivery system 600 and photodiode detector 996) with μ-Bondasphere C-18 reversed phase column (5 μm, 100 Å, 19×150 mm) was used. The flow rate was 10 ml min^{-1} and linear gradients were employed using water and 80% acetonitrile (A) containing 0.1% trifluoroacetic acid (gradient of A, 18% to 35% in 25 min).

GPC. A system for HPLC with Superdex 200HR 10/30 column (Pharmacia) was used for GPC analysis. The eluent was a buffer with 0.15 M sodium chloride (1 M = 1 mol dm^{-3}) and 0.05 M sodium phosphate at pH 6.8 and its flow rate was 0.75 ml min^{-1}.

Results and Discussion

PSC is not stable to heat and is denatured to random-coil gelatin of α1, α2, β11 (dimer of α1; MW = 200000), β12 (dimer of α1 and α2; MW = 200000), and γ (trimer of two α1 and α2; MW = 300000).[14] Those isoelectric points (pI) in a literature[15] are quoted in Table 1.

Initially C-PSC were analyzed with uncoated capillary at various pH values. As shown in Fig. 2, no signal can be found in the pH range of 4.9—9.4 because of adsorption of positively charged polypeptide onto negatively charged capillary wall. In the case of pH 11.9, one peak is found indicating coulombic repulsion between polypeptide and silanol group on the wall. However, it is impossible to characterize PSC by this peak.

In order to remove adsorption of polypeptide onto wall, the wall was coated with Brij 35. By using the coated column, analysis of C-PSC was examined at several pH values. As shown in Fig. 3, a good peak separation was achieved by using the Brij 35 coated capillary at pH 5.6—6.5 reflecting α1, α2, β11, β12, and γ. In the cases at pH 4.8—5.3, peak separation is not so good. The reason may be that all polypeptides are fully protonated in the pH range and thus the charge differences between polypeptides become small. In the case at pH 7.0, peak delay and broadening are found. The reason may be that polypeptides adsorb onto the wall at pH 7.0.

In order to assign the peaks obtained at pH 5.6—6.5, C-PSC was fractionated to α, β, and γ by GPC. Each fraction

Table 1. pI of Polypeptide Chains of PSC

Chain	pI
α2	10.2
β12	9.8
γ	9.7
α1	9.3
β11	9.3

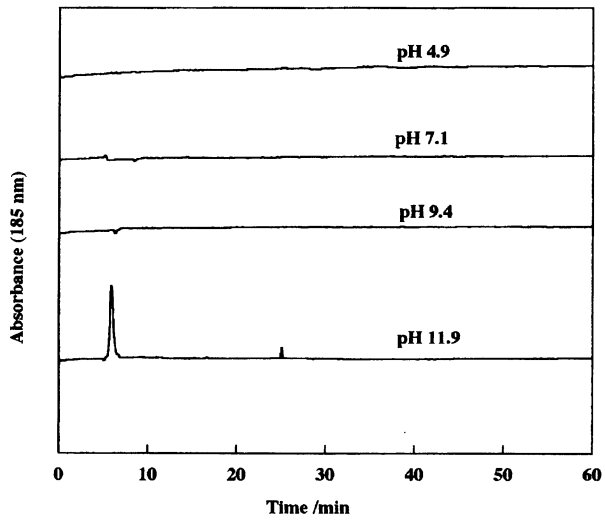

Fig. 2. Electropherograms of C-PSC at vatious pH. Conditions: capillary, 50 μm i.d.×60 cm length, 52 cm effective length; running buffer, 20 mM sodium phosphate; field, 420 V cm^{-1}; detection, 185 nm; temp, 35 °C.

was further fractionated to α1, α2, β11, β12, and γ by reversed phase HPLC, as shown in Fig. 4. (These peak assignments were done according to GPC and ion exchange chromatography). Then each fraction obtained by reversed phase HPLC was examined by CE, as shown in Fig. 5. As

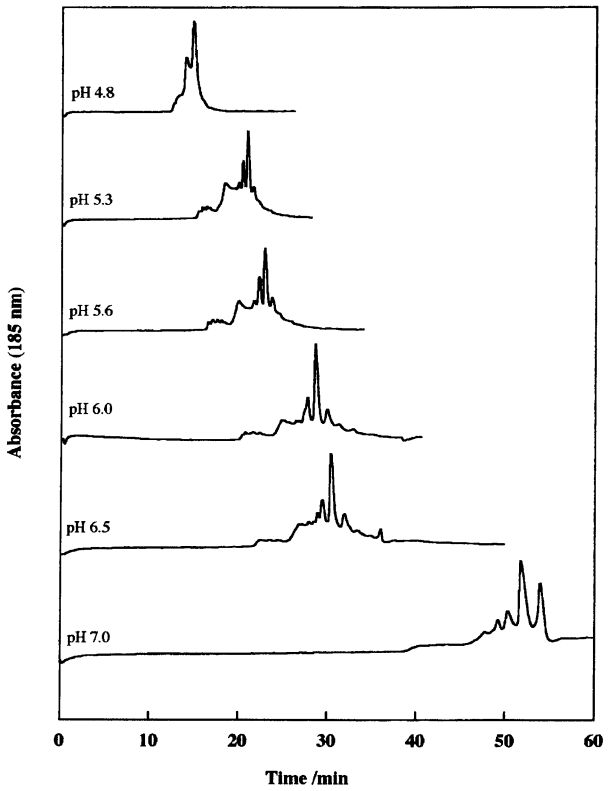

Fig. 3. Separation of C-PSC at several pH by using Brij 35 coated capillary. Conditions: capillary, 50 μm i.d.×60 cm length, 52 cm effective length; running buffer, 20 mM sodium phosphate and 0.02% Brij 35; field, 420 V cm^{-1}; detection, 185 nm; temp, 35 °C.

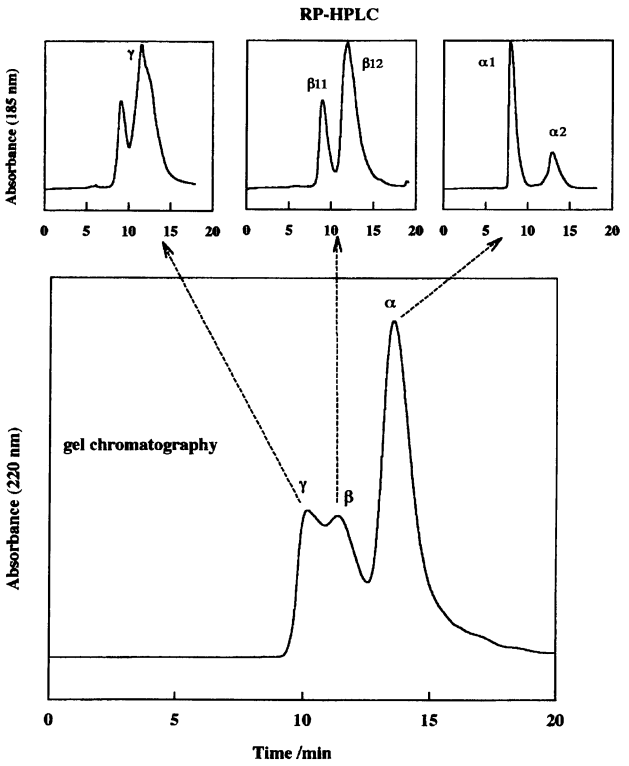

Fig. 4. C-PSC was fractionated to α, β, and γ by GPC, and each fraction was further fractionated to $\alpha 1$, $\alpha 2$, $\beta 11$, $\beta 12$, and γ by reversed phase HPLC.

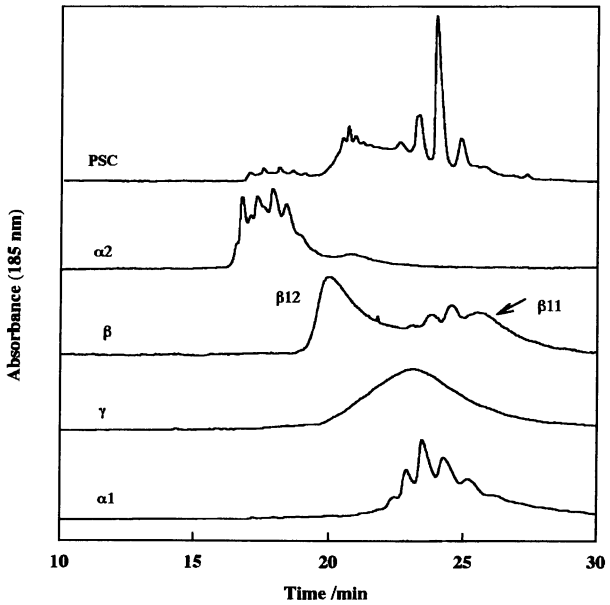

Fig. 5. Electropherograms of the polypeptide chains of C-PSC separated by gel chromatography and RP-HPLC. Conditions: capillary, 50 μm i.d. × 60 cm length, 52 cm effective length; running buffer, 20 mM sodium phosphate and 0.02% Brij 35, pH 5.6; field, 420 V cm^{-1}; detection, 185 nm; temp, 35 °C.

shown in Fig. 6, each electropherogram of C-PSC could be expressed as combinations of individual electropherograms for $\alpha 1$, $\alpha 2$, $\beta 11$, $\beta 12$, and γ. Although $\beta 11$ and γ (broken line in Fig. 6) are hidden below $\alpha 1$ and $\beta 12$ signals like baseline drift, these peaks could be treated as combinations of each curve, and $\alpha 1$, $\alpha 2$, and $\beta 12$ signals are found to be clearly separated. Hence CE is considered to be good for characterization of PSC.

Every signal except γ has fine structure, indicating the possibility of detection of slight differences in teropeptide residue and/or existence of various kinds of residues. We consider that it will be possible to estimate the extent of PSC digest or structure of residues from peak numbers and intensities in CE.

The reason of the fine structures of each peak is various residues generated by incomplete digest of teropeptides, which have various amino acids shown in Fig. 1, because various residues have various charges which influence migration of polypeptide in CE.

The mobility of polypeptide (m) was related to the valence (Z) and molecular weight (M) as Eq. 1 by Offord:[16]

$$m = k \cdot Z \cdot M^{-2/3}, \quad (1)$$

where k is a constant. In the case of $\alpha 1$ and $\alpha 2$, M differences among chains are very small and m is considered to depend on Z. Therefor variation of Z by digest should be taken into account. We considered the variation of Z as follows. Teropeptide of $\alpha 1$ and that of $\alpha 2$ consist of 11 isolated-amino acids (5 acidic and 6 basic) and 7 isolated-amino acids (2 acidic and 5 basic) before digest, respectively. Elimination of amino acids by digest takes place in various combinations and amino acids remain in various combinations at C- and N-terminal. At pH 5.6, acidic amino acids ionize to have negative charge and basic amino acids ionize

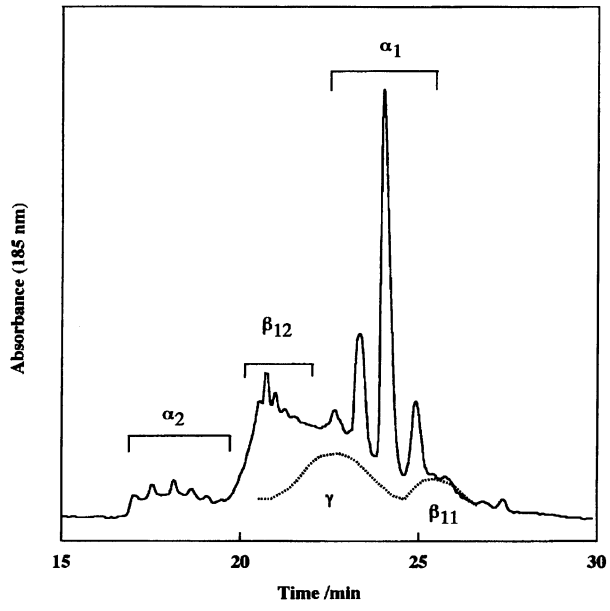

Fig. 6. Electropherograms of C-PSC. Conditions are the same as Fig. 5.

to have positive charge, and the balance of positive and negative charges decides *Z*. The mode of elimination, charges and charge balances were listed in Table 2.

As shown in Table 2, charge balances 4−—2+ in $\alpha 1$ and 4−—1+ in $\alpha 2$ were considered to appear by digest of teropeptide. As a voltage was applied at the ends of capillary (negatively at outlet), more positively charged chains migrate faster. Hence various charge balances were assigned to 7 peaks of $\alpha 1$ and 6 peaks of $\alpha 2$ signals of original PSC as shown in Table 3.

If teropeptides were digested completely, two single peaks by $\alpha 1$ and $\alpha 2$ should be observed at each 0 charge position in CE. Hence we tried further digest of C-PSC by treating with pepsin. FD-PSC were separated to $\alpha 1$ and $\alpha 2$ by GPC and

Table 2. Variation of Charges of Teropeptide by Pepsin Digest

Number of isolated-amino acid eliminated		Charge of $\alpha 1$			Charge of $\alpha 2$		
from N-terminal	from C-terminal	N-terminal	C-terminal	Balance	N-terminal	C-terminal	Balance
0	1	−2	+1	−1	−1	−2	−3
	2	−2	0	−2	−1	−3	−4
	3	−2	−1	−3	−1	−2	−3
	4	−2	0	−2	−1	−1	−2
	5	−2	−1	−3	−1	0	−1
	6	−2	−2	−4			
	7	−2	−1	−3			
	8	−2	0	−2			
1	1	−1	+1	0	0	−2	−2
	2	−1	0	−1	0	−3	−3
	3	−1	−1	−2	0	−2	−2
	4	−1	0	−1	0	−1	−1
	5	−1	−1	−2	0	0	0
	6	−1	−2	−3			
	7	−1	−1	−2			
	8	−1	0	−1			
2	1	0	+1	+1	+1	−2	−1
	2	0	0	0	+1	−3	−2
	3	0	−1	−1	+1	−2	−1
	4	0	0	0	+1	−1	0
	5	0	−1	−1	+1	0	+1
	6	0	−2	−2			
	7	0	−1	−1			
	8	0	0	0			
3	1	+1	+1	+2	0	−2	−2
	2	+1	0	+1	0	−3	−3
	3	+1	−1	0	0	−2	−2
	4	+1	0	+1	0	−1	−1
	5	+1	−1	0	0	0	0
	6	+1	−2	−1			
	7	+1	−1	0			
	8	+1	0	+1			
4	1	0	+1	+1			
	2	0	0	0			
	3	0	−1	−1			
	4	0	0	0			
	5	0	−1	−1			
	6	0	−2	−2			
	7	0	−1	−1			
	8	0	0	0			

reversed-phase HPLC. Then the $\alpha 1$ and $\alpha 2$ were analyzed by CE. As shown in Fig. 7, peaks of the $\alpha 1$ and $\alpha 2$ treated with 0.01% pepsin became more clear but those treated with 0.1% pepsin lost fine structure and a peak at 0 charge position became larger to indicate that the digest is proceeding. So the above mentioned assignment of peaks by charge balances is considered valid.

Conclusion

Polypeptide chains of PSC such as $\alpha 1$, $\alpha 2$, $\beta 11$, $\beta 12$, and γ were separated by CE with Brij 35 modified capillary. The best separation was performed at pH 5.6—6.5. Although $\beta 11$ and γ signals were hidden below $\alpha 1$ and $\beta 12$ signals like baseline drift, these peaks could be included as combinations of each curve. $\alpha 1$ and $\alpha 2$ signals split to several peaks which were related to various charge balances of polypeptide chains produced by various modes of digest of teropeptide. CE is considered to make it possible to determine the extent of digest of teropeptides which have not been analyzed by SDS-PAGE and GPC.

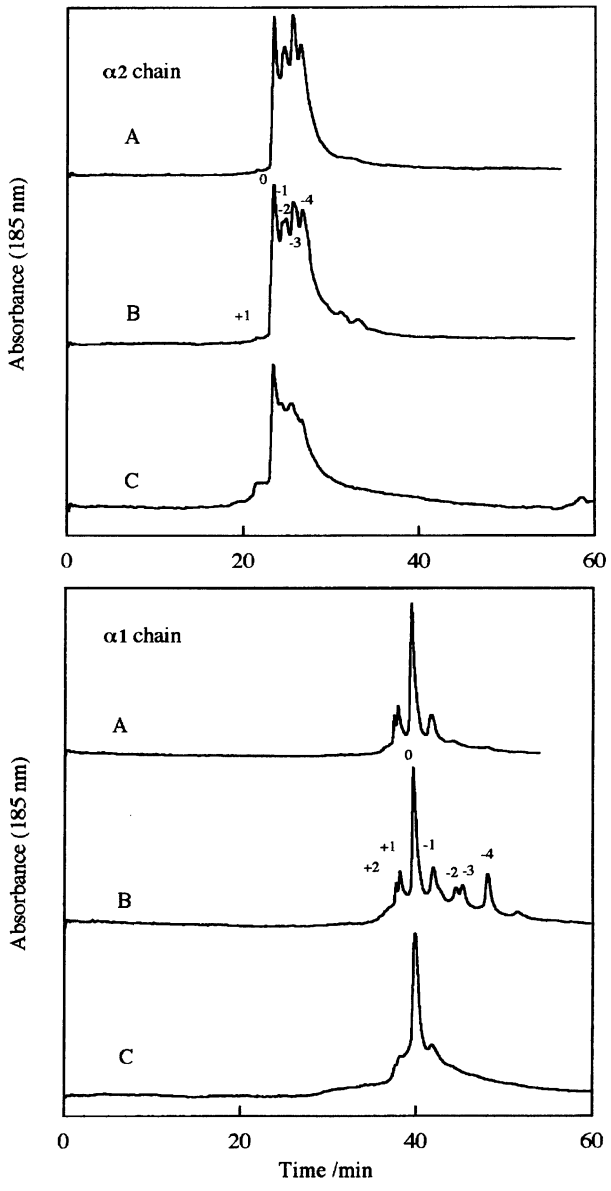

Fig. 7. Electropherograms of $\alpha 1$ and $\alpha 2$ treated with pepsin. A: untreatment, B: treatment with 0.01% pepsin at 35 °C, C: treatment with 0.1% pepsin at 35 °C. Condition are the same at Fig. 5. The number represent charge balances listed in Table 2.

Table 3. Number of Combinations of Charge Balance

Chain	Charge	No. of combination
$\alpha 1$	−4	1
	−3	4
	−2	8
	−1	11
	0	9
	+1	5
	+2	1
$\alpha 2$	−4	1
	−3	4
	−2	6
	−1	5
	0	3
	+1	1

Amino acid sequence of teropeptide limited to isolated amino acids are shown as below.

$\alpha 1$ $\overset{-}{\text{Glu}}-\overset{-}{\text{Asp}}-\overset{-}{\text{Glu}}-\overset{+}{\text{Lys}}$ ----------
$\overset{-}{\text{Asp}}-\overset{-}{\text{Glu}}-\overset{+}{\text{Lys}}-\overset{+}{\text{His}}-\overset{-}{\text{Asp}}-\overset{+}{\text{Arg}}-\overset{+}{\text{Arg}}$
$\alpha 2$ $\overset{-}{\text{Glu}}-\overset{-}{\text{Asp}}-\overset{+}{\text{Lys}}$ ---------- $\overset{-}{\text{Glu}}-\overset{-}{\text{Asp}}-\overset{-}{\text{Glu}}-\overset{+}{\text{Arg}}$

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