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Effects of the methylene chain length of chemically introduced crosslinks on the properties of collagen

Kazuo Watanabe*, Junko Nakagawa, Tetsuya Ebihara and Yasushi Okamoto

Nippi Research Institute of Biomatrix, 1-1 Senju-Midoricho, Adachi-ku, Tokyo 120, Japan
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Crosslinkers containing aliphatic hydrocarbon chains of various lengths were introduced into solubilized collagen with lime (SCL) in order to stabilize its structure. The crosslinking resulted in an increase in the denaturation temperature and helix regeneration ratio of the modified SCL. The increase was prominent when a large fraction of the amino groups on the SCL side chains was modified with a crosslinker containing five or more methylene groups that can encompass more than one collagen helix pitch. These results suggest that the length of a crosslinker is important for the stabilization of the triple-helical structure of the SCL.

(Keywords: collagen; crosslink; chain length effect)

INTRODUCTION

Solubilized collagen with lime (SCL) is industrially produced from steer hide using an alkaline solution. One of the advantages of SCL is that it can be obtained in large quantities in comparison with other solubilized collagens, either acid-soluble or protease-treated. The amino acid composition of SCL is somewhat different from that of other collagens because some acid amides on the SCL side chains were hydrolyzed during the alkali treatment[1]. These altered amino acid compositions probably result in a lower denaturation temperature and a lower ratio of the triple helix regeneration after heat denaturation of the SCL. Thus, SCL is more unstable than the acid-soluble or protease-treated collagens. One way of stabilizing SCL is to introduce crosslinks. In our previous report[2], it was demonstrated that crosslinks consisting of an aliphatic hydrocarbon chain were more effective in the regeneration of the collagen structure than crosslinks containing one or two aromatic rings. In the present study, we examined the effects of the methylene chain lengths of the aliphatic crosslinking reagents on the stabilization of the triple-helical structure of the SCL.

EXPERIMENTAL

Materials

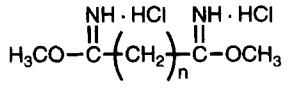
SCL made by Nippi Inc. was used for all experiments in the present study. The imidate crosslinking (ICL) reagents employed are as follows. Dimethyl adipimidate dihydrochloride (ICL4) was purchased from Nacalai Tesque Inc. Dimethyl pimelimidate dihydrochloride (ICL5) was obtained from Tokyo Kasei Kogyo Co. and dimethyl suberimidate dihydrochloride (ICL6) was from Wako Pure Chemical Industries. Dimethyl succinimidate dihydrochloride (ICL2), dimethyl glutarimidate dihydrochloride (ICL3) and dimethyl sebacimidate dihydrochloride (ICL8) were prepared by the Environmental Research Center[3,4]. The general formula of the ICL reagents is illustrated in *Scheme 1*. The maximum crosslinkable distance of the ICL reagents, which is calculated using the bond length and bond angle of the chemical bonds, is given in *Table 1*. The number shown in each abbreviation indicates the number of methylene groups contained in the backbone skeleton of the crosslinking reagent. In our previous report, ICL4 and ICL6 were labelled DMA and DMS, respectively[2].

Preparation of crosslinked collagens

The procedure for the preparation of the crosslinked collagens was previously described in detail[2]. In order to minimize intermolecular crosslinking, a 0.14% SCL solution, pH 10, was used for the crosslinking reaction[5]. A crosslinking reagent was added to the SCL solution at 4°C, and the mixture was then continuously stirred overnight. The crosslinking reaction was terminated by acidifying the reaction mixture with 1 M acetic acid. The acidic solution was dialysed against five changes of 20 l of 5 mM acetic acid and then lyophilized. The lyophilized product was stored below −20°C.

Quantitative analyses

The concentration of the crosslinked SCL was determined using the microbiuret method[6]. A crosslinked SCL solution was first heated at 100°C for 3 min,



Scheme 1 Structural formula of imidate crosslinking (ICL) reagent

* To whom correspondence should be addressed

Table 1 Maximum crosslinkable distance of ICL reagents

ICL reagents	Maximum crosslinkable distance (Å)
ICL1	4.996
ICL2	6.245
ICL3	7.494
ICL4	8.743
ICL5	9.992
ICL6	11.241
ICL7	12.490
ICL8	13.739

then 1 ml of 0.21% $CuSO_4$ in 30% NaOH was added to 2 ml of the heat-denatured SCL solution. The absorbance of the mixture was measured at 310 nm after 5 min. Water was mixed with 0.21% $CuSO_4$ instead of the protein solution as the blank for the microbiuret measurements.

The concentration of the free amino groups, which did not react with the crosslinking reagents, was quantified with trinitrobenzenesulfonic acid (TNBS)[7]. First, 0.5 ml of a crosslinked SCL solution was heated at 100°C for 3 min. After cooling, 0.5 ml of 0.5% aqueous TNBS solution was added to the heat-denatured SCL solution. The mixture was kept at 40°C for 4 h with mild shaking. The modification reaction was terminated by adding 1.5 ml of 12 N HCl to the reaction mixture, and the mixture was then autoclaved at 120°C for 1 h. The hydrolysate was diluted with 5 ml of water. In order to eliminate any excess of unreacted TNBS, the solvent extraction was carried out with three 10-ml portions of anhydrous ethyl ether. The remaining aqueous phase, after removing the ethyl ether, was heated in a hot-water bath for 15 min to evaporate the residual ether. The absorbance of the aqueous solution was measured at 346 nm, and the concentration of the amino groups combined with TNBS in the solution was calculated using the value of $1.46 \times 10^4\,l\,mol^{-1}\,cm^{-1}$, the molar extinction coefficient of trinitrophenyl L-lysine[8]. The blank sample was prepared by adding HCl to the SCL solution before the addition of TNBS to prevent any reaction of TNBS with SCL. The proportion of the modified amino groups in SCL with crosslinking reagents was obtained by comparing the total protein concentration in a crosslinked SCL solution with the free amino group concentration in the same solution.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) studies

SDS-PAGE was performed on a 5% polyacrylamide gel with a 3% stacking gel, using the method of Laemmli[9] with minor modifications. Collagen samples for SDS-PAGE were boiled for 3 min in a sample buffer solution. Proteins were stained in 0.25% Coomassie Brilliant Blue R-250 in 5/1/4 methanol/acetic acid/water and then destained in 2/3/35 methanol/acetic acid/water.

Gel permeation chromatography (g.p.c.) experiments

The procedure for g.p.c. experiments has been described earlier[2]. Three g.p.c. columns (Shodex OHpak SB-805, Shodex OHpak SB-804 and Shodex Protein KW-804) were employed in the present study to examine the molecular weight distribution of the crosslinked SCL in a high molecular weight region with high resolution. A g.p.c. sample was prepared by directly dissolving a lyophilized crosslinked SCL in hot water and then heating at 100°C for 3 min. The filtration of the solution was not carried out because the lyophilized sample completely dissolved in the boiling water.

Circular dichroism (c.d.) measurements

The helix-to-coil transition temperature of the crosslinked SCL and the triple helix regeneration ratio of the crosslinked SCL cooled after heat denaturation were determined by c.d. measurements, using a Jasco J-600 spectropolarimeter together with a Taitec EZL-80 constant temperature circulator equipped with a Taitec PU-9 thermocontrol unit to control the temperature of the crosslinked SCL solution. A 10-mm pathlength of the water-jacketed cell was employed in all c.d. measurements. A sample for spectroscopic measurements was prepared with cold water to avoid heat denaturation and then filtered to remove the insoluble moiety because a little insoluble matter may remain in the cold water. Other detailed conditions for the c.d. measurements for collagen have been described elsewhere[2,10].

RESULTS

The SDS-PAGE of the crosslinked SCLs, in which the proportion of the side chain amino group modification by ICL reagents with various chain lengths was about 20%, is shown in *Figure 1*. The α- and faint β-chain bands were observed in the uncrosslinked SCL, while the γ-chain band was not found. A faint γ-chain band appeared in the SCLs crosslinked with ICL2, ICL3 or ICL4. When SCL was crosslinked with an ICL reagent containing five or more methylene groups, the α- and β-chain bands disappeared, whereas the γ-chain band was clearly identified. Some unavoidable intermolecular polymerization also occurred during the crosslinking reaction; a polymer band was observed on the gel top of each lane.

In order to quantify the proportion of each chain fraction, g.p.c. experiments were performed. As a typical result, the elution patterns of the g.p.c. of the SCLs crosslinked with ICL4 or ICL5 are illustrated in *Figure 2*.

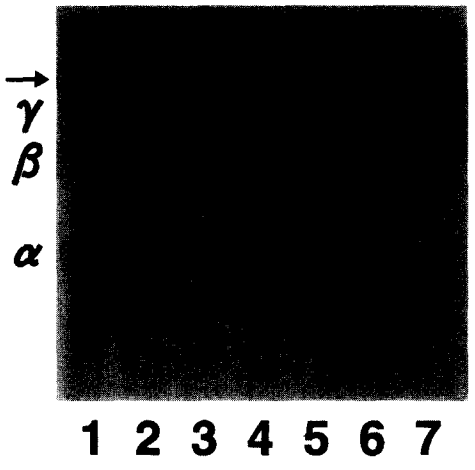


Figure 1 SDS-PAGE of SCLs crosslinked with the various ICL reagents: lane 1, uncrosslinked SCL; lane 2, ICL2; lane 3, ICL3; lane 4, ICL4; lane 5, ICL5; lane 6, ICL6; lane 7, ICL8. Arrow indicates the polymer fraction remaining on the gel top

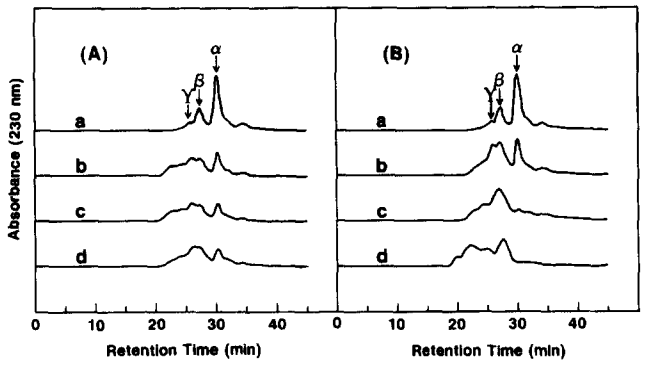


Figure 2 G.p.c. of SCLs crosslinked with ICL4 or ICL5. (A) ICL4–crosslinked SCL: the proportions of the modified amino group are (a) 0%, (b) 8.9%, (c) 12.1% and (d) 25.8%. (B) ICL5–crosslinked SCL: the proportions of the modified amino group are (a) 0%, (b) 9.7%, (c) 12.9% and (d) 28.2%

Table 2 Proportion of each g.p.c. fraction of the SCL crosslinked with ICl reagents

Crosslinking reagent on collagen	Ratio of each g.p.c. fraction (%)			
	α-chain	β-chain	γ-chain	Above γ-chain
SCL[a]	67.4	25.3	7.3	0
ICL2	49.8	28.8	16.1	5.3
ICL3	27.3	17.5	24.2	31.0
ICL4	24.2	23.5	26.5	25.8
ICL5	6.3	43.9	20.0	29.8
ICL6	7.3	43.0	19.3	30.4
ICL8	6.8	47.7	20.0	25.5

[a] Unmodified collagen

The g.p.c. elution pattern of the SCL crosslinked with ICL3 resembled that in *Figure 2A*, and the g.p.c. elution patterns of the SCLs crosslinked with ICL6 or ICL8 were similar to that in *Figure 2B*. The g.p.c. elution pattern of the SCL crosslinked with ICL2 resembled that of the uncrosslinked SCL rather than that of any crosslinked SCL. The proportion of the g.p.c. fractions above the α-chain fraction increased with an increase in the amount of crosslinks in the SCL. The peak of the α-chain on the chromatogram became smaller due to the introduction of crosslinks. The α-chain peak of the SCL crosslinked with ICL5 almost disappeared above 12% modification of the amino group of the SCL side chain, whereas the α-chain peak of the SCL crosslinked with ICL4 remained even when the proportion of the amino group modification of the SCL side chain was above 25%. The proportion of each g.p.c. fraction of the same samples as in *Figure 1* is shown in *Table 2*. The proportion of the α-chain fraction decreased with an increase in the methylene chain length of the ICL reagents and reached a minimum of about 7% by crosslinking the SCL with the reagents that contain five or more methylene groups. High molecular weight fractions above the γ-chain were observed in the crosslinked SCLs. This indicates that intermolecular crosslinking simultaneously occurred with intramolecular crosslinking during the crosslinking reaction. The proportion of the high molecular weight fraction of the SCL crosslinked with ICL2 was only 5%, but it was constantly about 30% when the SCL was crosslinked with ICL3–ICL8.

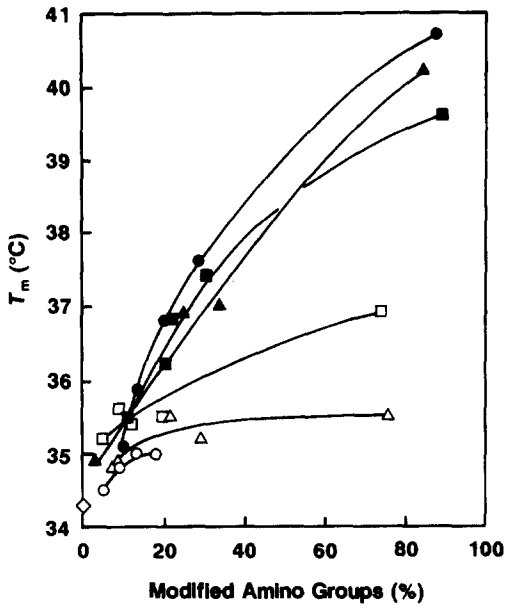


Figure 3 T_m of SCLs crosslinked by the ICL reagents with various methylene chain lengths as a function of the proportion of the modified amino group: (◇) SCL; (○) ICL2; (△) ICL3; (□) ICL4; (●) ICL5; (▲) ICL6; (■) ICL8

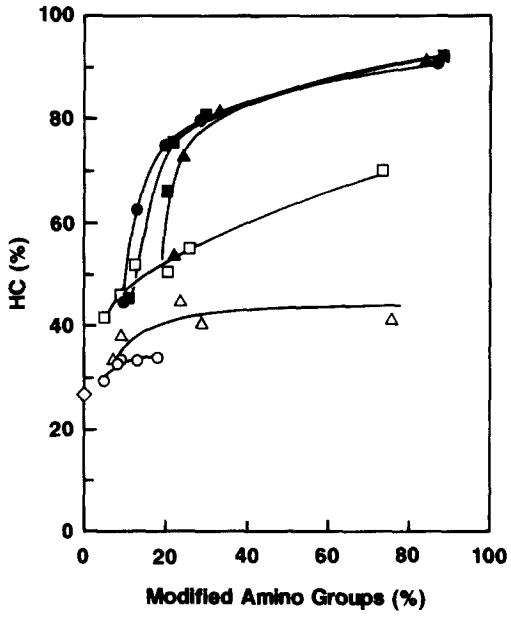


Figure 4 HC in the crosslinked SCLs regenerated from the heat-denatured state as a function of the proportion of the modified amino group: (◇) SCL; (○) ICL2; (△) ICL3; (□) ICL4; (●) ICL5; (▲) ICL6; (■) ICL8

Figure 3 represents the T_ms of the crosslinked SCLs as a function of the proportion of the amino group reacted with the crosslinking reagent. The T_ms of the crosslinked SCLs increased with an increase in the amount of the crosslinks contained in the SCL. The T_m of the acid-soluble collagen (ASC) in acidic solution was about 38°C[11]. The T_ms of the SCLs crosslinked with ICL2–ICL4 were still lower than the T_m of ASC, though they were higher than the T_m of the uncrosslinked SCL (*ca* 34°C). There was little difference among the T_ms of the SCLs crosslinked with ICL5, ICL6 or ICL8; the T_ms of these crosslinked SCLs were lower than the T_m of ASC

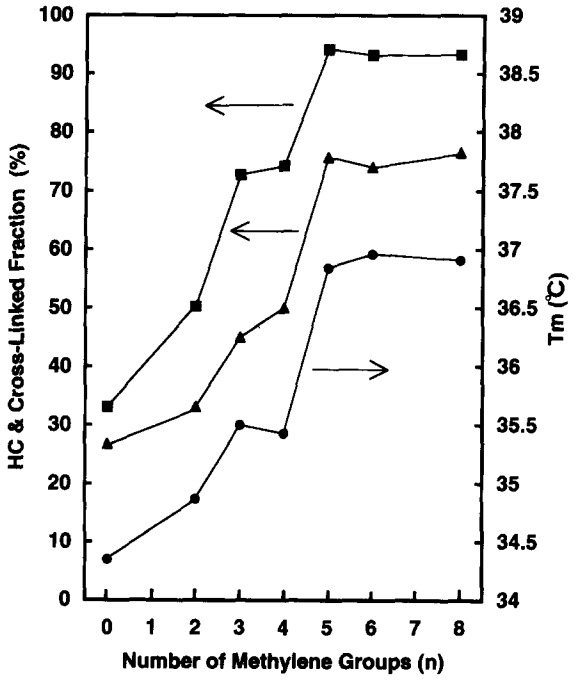


Figure 5 Plots of the properties of the crosslinked SCL as a function of the number of methylene groups in the crosslinker: (●) T_m; (▲) HC; (■) g.p.c. fraction above α-chain

when the proportion of the modified amino groups was below 40%, while they exceeded 38°C above 80%.

The helix content (HC) in the crosslinked SCLs regenerated from the heat-denatured state by cooling is shown in *Figure 4* as a function of the proportion of the amino group combined with the crosslinking reagent. The HC of SCL slightly increased due to the ICL2 crosslinking. The increase in the HC depended on the methylene chain lengths, and the HC of the SCL modified with crosslinkers having long methylene chain lengths, ICL5, ICL6 or ICL8, sharply increased when the proportion of the modified amino group increased and reached a maximum of about 90%.

Figures 3 and *4* indicate that the change in the T_m and HC of the crosslinked SCLs depends on the methylene chain length in a crosslink. Therefore, we replotted the values of the T_m and HC of the crosslinked SCLs of about 20% amino group modification *versus* the number of methylene groups contained in the ICL reagent, together with the proportion of the g.p.c. fraction above the α-chain, which contains at least one covalent crosslink. As shown in *Figure 5*, these three values showed a clear dependency on the methylene chain length of the crosslinker. Interestingly, the variation was rather small or did not increase between numbers 3 and 4, stepped up again between numbers 4 and 5, and then reached saturation.

DISCUSSION

Some SCL properties are different from those of ASC because of a change in the amino acid residues due to alkali treatment[1,2]. For example, the T_m of SCL (*ca* 34°C) is lower than the T_m of ASC (*ca* 38°C). In order to improve the physical properties of the SCL, the effect of the introduction of chemical crosslinks to the SCL on the T_m and HC of the SCL was examined.

As previously reported[2], the introduction of aliphatic crosslinks into the collagen molecule was more effective for the stabilization of the collagen triple-helical structure than that of the aromatic ones. Furthermore, the SCL crosslinked with the pyrene derivative had a T_m lower than the uncrosslinked SCL T_m[12]. These results suggest that the disintegration of the collagen triple helix was promoted by the steric hindrance due to the bulky backbone skeleton of the crosslinker. Therefore, aliphatic crosslinking reagents were employed in this study.

The change in the proportion of each chain constituting the collagen helix by introducing crosslinks was examined using the SDS-PAGE and g.p.c. methods. In the present experiments, the intramolecular crosslinking in a collagen molecule probably occurred using the ICL reagents because the γ-chain fraction appeared in all crosslinked SCLs. On the other hand, the intermolecular crosslinking between collagen molecules should have simultaneously occurred because some high molecular weight fractions above the γ-chain fraction were observed in the SCLs crosslinked with the ICL reagents other than ICL2; the crosslinkable distance of ICL2 might be too short to link between collagen molecules under the present experimental conditions. In order to suppress the intermolecular crosslinking between collagen molecules, a 0.14% SCL solution was used for the crosslinking reaction because Boedtker and Doty[5] demonstrated that the aggregation of native carp swim bladder ichthyocol could be avoided by suppressing the collagen concentration below 0.7%. Although the concentration of the SCL solution used was substantially lower than the concentration mentioned by Boedtker and Doty, intermolecular polymerization was to some degree unavoidable. However, the degree of intermolecular crosslinking was almost constant (25.5–31.0%) when the crosslinking reagents of ICL3–ICL8 were used for the crosslinking reaction. Therefore, the influence of intermolecular crosslinking on the several properties of the SCLs crosslinked with the ICl reagents other than ICL2 will never exceed 31.0% because Flory[13] theoretically predicted that intramolecular crosslinking is far more effective in the elevation of the melting point of fibrous proteins than intermolecular crosslinking.

As shown in *Figure 3*, it was found that the T_ms varied between about 34 and 40°C by introducing crosslinks and that the degree of the T_m increase depended on the methylene chain length of the crosslinker. These results indicate that the T_ms of SCL could be controlled by the introduction of aliphatic crosslinkers and that the crosslinkers with a long backbone length are more effective than the shorter ones in stabilizing SCL. The T_m of hemoglobin changed from 41 to 57°C by the introduction of a single crosslink[14], whereas the T_m increase of SCL was less than 7° even when a large amount of crosslinks was introduced into the collagen molecule. Although the precise mechanism of these phenomena is unknown at present, the effect of crosslinking on the T_m increase might depend on the molecular structure of each protein.

Three properties of the crosslinked SCL (i.e. T_m, HC and percentage of the g.p.c. fraction containing the crosslink) showed a clear dependence on the methylene chain length of the crosslinker. Interestingly, a remarkable difference in the properties of the crosslinked SCL was observed between the methylene chain numbers of 4 and 5, whereas the variation in the properties was rather small between the methylene chain numbers of 3 and 4.

Rich and Crick[15] have presented a molecular model of collagen. According to their model, the length of one collagen helix pitch is approximately 9.5 Å. As shown in *Table 1*, the maximum crosslinkable length of the crosslinkers is 8.743 Å for $n = 4$ and 9.992 Å for $n = 5$. The 9.5 Å falls between these two values. These results suggest the possibility that some properties of the SCL collagen markedly change when SCL is modified by a crosslinker that can span over one pitch of the collagen helix. Effects of the modification of other types of collagen by the crosslinkers employed in this study on several properties of the collagens remain to be elucidated. We are planning to examine the structure of the reformed crosslinked SCLs as well as their physical, chemical, and biological properties.

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REFERENCES

1. Fujii, T., *Hoppe-Seyler's Z. Physiol. Chem.*, 1969, **350**, 1257.
2. Watanabe, K., Nakagawa, J., Ebihara, T. and Okamoto, Y., *Polymer*, 1996, **37**, 1285.
3. McElvain, S. M. and Schroeder, J. P., *J. Am. Chem. Soc.*, 1949, **71**, 40.
4. Jenkins, A. D., Morrison, J. M., Mykytiuk, J., Trowbridge, L., Tsartolia, E. and Walton, D. R. M., *Makromol. Chem., Rapid Commun.*, 1991, **12**, 653.
5. Boedtker, H. and Doty, P., *J. Am. Chem. Soc.*, 1956, **78**, 4267.
6. Itzhaki, R. F. and Gill, D. M., *Anal. Biochem.*, 1964, **9**, 401.
7. Bubnis, W. A. and Ofner, C. M., III, *Anal. Biochem.*, 1992, **207**, 129.
8. Okuyama, T. and Satake, K., *J. Biochem.*, 1960, **47**, 454.
9. Laemmli, U.K., *Nature*, 1970, **227**, 680.
10. Hayashi, T., Curran-Patel, S. and Prockop, D. J., *Biochemistry*, 1979, **18**, 4182.
11. Na, G. C., *Biochemistry*, 1986, **25**, 967.
12. Watanabe, K., *Polym. J.*, 1997, **29**, 286.
13. Flory, P. J., *J. Am. Chem. Soc.*, 1956, **78**, 5222.
14. Yang, T. and Olsen, K. W., *Biochem. Biophys. Res. Commun.*, 1991, **174**, 518.
15. Rich, A. and Crick, F. H. C., *J. Mol. Biol.*, 1961, **3**, 483.