

Biochemical Behavior of the Depolymerized Product of Chondroitin Sulfate C by Ultrasonic Irradiation¹⁾

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Ultrasonically irradiated aqueous solution of chondroitin sulfate C was fractionated by the gel filtration method using Sephadex G 200, and the content of glucuronic acid in each fraction was measured. Only one new peak arose from the depolymerized product after irradiation. This new peak did not shift with prolonged irradiation. From these facts, chondroitin sulfate C in an aqueous solution was suggested to have been depolymerized not gradually, but directly into the species of limited molecular weight by ultrasonic irradiation.

From the result of gel filtration, K_{av} values of the original compound and the depolymerized product were found to be 0.08 and 0.24, respectively. The molecular weight estimated from the obtained K_{av} value was approximately equal to the limited molecular weight which was calculated by the authors' equation presented in the previous paper.¹⁾ The depolymerized product by ultrasonic irradiation can be an enzyme substrate against chondroitinase and chondro-6-sulfatase, like the original sample. It is supposed that the depolymerized product still maintains the characteristics of the original macromolecular compound.

It is well known that polymers in solution are readily depolymerized when treated by ultrasonic irradiation.³⁾ As the characteristics of ultrasonic depolymerization, the following items are pointed out: (1) there exists a lower limit of molecular weight for polymers resulted from ultrasonic irradiation regardless of the treatment time. (2) the distribution of molecular weight of depolymerized products is relatively narrow.^{3a)}

In the previous paper,¹⁾ the authors presented the equation for calculating the limited molecular weight in ultrasonic depolymerization, and described that the molecular weight of chondroitin sulfate C was reduced to 21800 from its original value of 69000 after ultrasonic irradiation in an aqueous solution.

In the case of chondroitin sulfate C, it is very interesting to see whether depolymerized products still maintain the same biochemical properties as that of the original compound. We have few reports on such a problem as biochemical behavior of ultrasonically depolymerized products, because most of researches on the depolymerization of macromolecular compounds by ultrasonics have been performed on synthetic polymers, such as polyvinylalcohol, polystyrene, *etc.*, in organic solvents.

But, in the case of natural high molecular weight compounds used as medicine, such as dextran⁴⁾ and polyvinylpyrrolidone,⁵⁾ it is well recognized that the difference of their molecular weight affects their physiological actions.

Therefore, the authors have planned to investigate biochemical properties of ultrasonically depolymerized products, and take up chondroitin sulfate C as a model compound.

- 1) This paper constitutes Part II of the series entitled Pharmaceutical Studies of Macromolecules. Part I: T. Tatsuhaara and S. Iguchi, *Yakuzaigaku*, **32**, 86 (1972).
- 2) Location: 1276, Katakasu, Higashi-ku, Fukuoka.
- 3) a) G. Schmid, C. Schneider, and A. Henglein, *Kolloid. Z.*, **148**, 73 (1956); b) G. Schmid and O. Rommel, *Z. Electrochem.*, **45**, 659 (1939); c) G. Schmid, *Z. Physiol.*, **A186**, 113 (1940).
- 4) G. Arturson and G. Wallenius, *Scand. J. Clin. Invest.*, **16**, 81 (1964).
- 5) H. Weese, *Deut. Med. Wochsch.*, **76**, 757 (1951).

In this paper, the distribution of the molecular weight of the depolymerized products after ultrasonic irradiation is presented at first, and then the result of biochemical investigation with isolated depolymerized products by the gel filtration method is described, in which the authors examined the possibility that the products may serve as the substrate against chondroitinase and chondro-6-sulfatase.

Experimental

Material and Reagents—Sodium chondroitin sulfate C was supplied by Kaken Yaku Kako Co., Ltd., and its characteristics were reported in the previous paper.¹⁾

Chondroitinase-ABC, and chondro-6-sulfatase which was obtained from *Proteus vulgaris*, NCTC 4636, were purchased from Seikagaku Kogyo Co., Ltd.

Tris buffer was prepared by dissolving 3 g of tris(hydroxymethyl)aminomethane (Sigma), 2.4 g of sodium acetate, 1.46 g of sodium chloride, and 50 mg of bovine serum albumin (Armour) in 100 ml of 0.13N hydrochloric acid. The pH was 8.0.

Tetraborate buffer (pH 9.0) was prepared by dissolving 5 g of potassium tetraborate in 100 ml of distilled water, and the pH was adjusted by potassium hydroxide.

p-(Dimethylamino)benzaldehyde reagent was prepared by dissolving 8 g of *p*-(dimethylamino)benzaldehyde in the mixture of 47.5 ml of glacial acetic acid (analytical grade) and 2.5 ml of concentrated hydrochloric acid (analytical grade).

Cetyl pyridinium chloride-hydrochloric acid reagent was prepared by dissolving 0.5 g of cetyl pyridinium chloride in 100 ml of 0.3N hydrochloric acid.

Barium chloride-gelatin reagent was prepared by dissolving 1.0 g of gelatin (Difco) in 100 ml of distilled water at 60° and allow to stand at 4° overnight. To this solution, 2.5 ml of 12N hydrochloric acid and 1.0 g of barium chloride were added. The solution gave an absorbance of *ca.* 0.07 at 360 m μ against a water blank when mixed with 0.15N hydrochloric acid in a proportion of 7:3 (v/v).

Depolymerization by Ultrasonic Irradiation—The apparatus and the condition of depolymerization by ultrasonic irradiation were the same as in the previous paper.¹⁾

Gel Chromatography⁶⁾—A column (2.6 \times 100 cm) with Sephadex G 200 was prepared and equilibrated with 1M sodium chloride. The sample was applied to the column at a rate of 10 ml per hour, and 5 ml of fractions were collected.

Determination of Chondroitin Sulfate C by Chondroitinase ABC⁷⁾—The mixtures, containing 0.1 ml of tris buffer, 0.1 ml of sample and 0.1 ml of chondroitinase ABC solution (0.05 unit), were prepared in test tubes immersed in an ice bath. The blank mixture differed in that it lacked the enzyme. After the incubation at 37° for 30 minutes, 0.5 ml of tetraborate buffer was added to each mixture. The tubes were heated in a boiling water bath for 7 minutes and cooled in the tap water. Four ml of glacial acetic acid and 1 ml of the *p*-(dimethylamino)benzaldehyde reagent were added and, immediately after mixing, the tubes were placed in a bath at 37°. After 20 minutes the tubes were cooled in the tap water and absorbance of the test solutions was immediately measured against the blank solution at 585 m μ .

Determination of Ester Sulfate by Chondro-6-sulfatase⁷⁾—The mixtures, containing 0.1 ml of tris buffer, 0.25 ml of sample, 0.1 ml of chondroitinase ABC solution (0.05 unit) and 0.05 ml of chondro-6-sulfatase were prepared in test tubes immersed in an ice bath. The blank mixture differed in that it lacked the enzymes. These mixtures were incubated at 37°, and the reaction was stopped after 30 minutes by the addition of 0.5 ml of the cetyl pyridinium chloride-hydrochloric acid reagent. The mixtures were allowed to stand for 10 minutes at 37°, and the precipitates, if any, were removed by centrifugation. An aliquot (0.9 ml) of the supernatant was pipetted into a test tube containing 2.1 ml of the barium chloride-gelatin reagent. After mixing, the whole was allowed to stand for 10 minutes at room temperature. The absorbance of the test solution was measured against the blank at 360 m μ .

Determination of Glucuronic Acid, Galactosamine and Ester Sulfate—The procedures for these measurements were the same as in the previous paper.¹⁾ Glucuronic acid was determined by the calbazole method of Dishe.⁸⁾ Galactosamine and ester sulfate were determined by the modified Elson-Morgan method of Strominger, Park and Thompson,⁹⁾ and by the Dodgson method,¹⁰⁾ respectively, after hydrolysis with 4N hydrochloric acid for 15 hours at 100°.

6) A. Wasteson, *Biochim. Biophys. Acta*, **177**, 152 (1969).

7) H. Saito, T. Yamagata, and S. Suzuki, *J. Biol. Chem.*, **243**, 1563 (1968).

8) Z. Dishe, *J. Biol. Chem.*, **167**, 189 (1947).

9) J.L. Strominger, J.T. Park, and R.E. Thompson, *Biol. Chem.*, **234**, 3263 (1959).

10) K.S. Dodgson, *Biochem. J.*, **78**, 312 (1961).

Result and Discussion

Separation of the Ultrasonically Depolymerized Products of Chondroitin Sulfate C by Gel Filtration Method

After the treatment of chondroitin sulfate C in an aqueous solution with ultrasonic irradiation, the gel filtration method was utilized to separate the depolymerized products and to investigate the molecular weight distribution. As the gel filtration technique, Wasteson's method,⁶ in which Sephadex G 200 and 1.0M aqueous sodium chloride solution as the eluent are employed, was adopted.

Fig. 1 is the gelchromatogram which shows the quantity of glucuronic acid in each fraction after ultrasonic irradiation on 0.25% aqueous chondroitin sulfate C solution followed by fractionation with the above method. It is noteworthy that the gelchromatogram of the original sample (non-irradiated) has only one peak, while that of the irradiated sample shows two peaks. With an increase of the ultrasonic irradiation time, the height of the first peak which was observed in the fraction of the non-irradiated sample decreased, and the second new peak heightened. Moreover, it was observed that the new peak did not shift and any other peak did not appear even after increasing the irradiation time. This new second peak corresponds to the species of limited molecular weight produced in the ultrasonic depolymerization reaction. From this result, it was concluded that chondroitin sulfate C was depolymerized not gradually, but directly to the species of a certain molecular weight, the limited molecular weight, by ultrasonic irradiation.

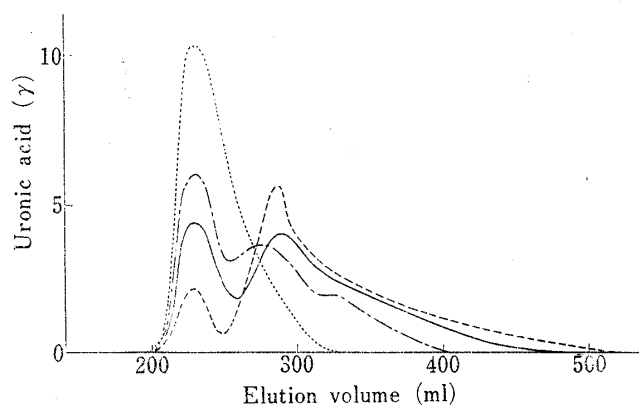


Fig. 1. Elution Diagrams of Depolymerized Product of Chondroitin Sulfate C in an Aqueous Solution after Ultrasonic Irradiation

Each sample was chromatographed individually on Sephadex G 200 column (26.4 × 1000 mm) and eluted with 1.0M NaCl solution.
 -----: non-irradiated, - - - - -: 15 minutes irradiated,
 -----: 30 minutes irradiated, - · - · - ·: 60 minutes irradiated

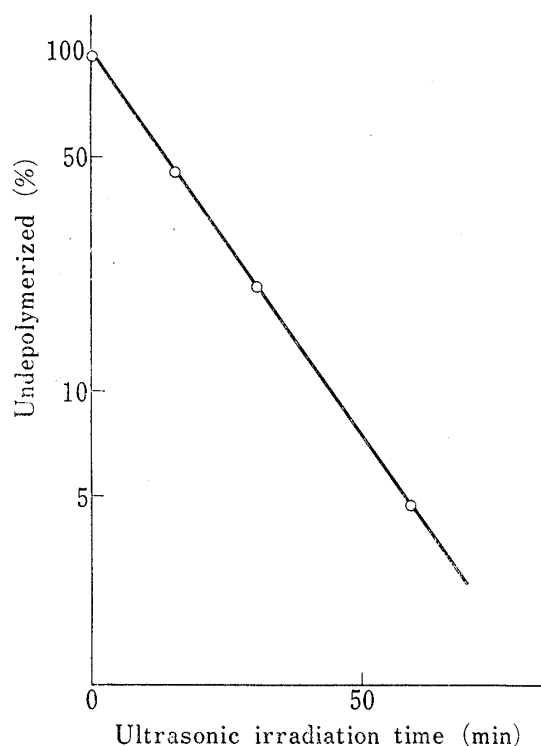


Fig. 2. Relationship between the Ultrasonic Irradiation Time and the Undepolymerized Chondroitin Sulfate C in 0.25% Aqueous Solution

If the peak of the depolymerized product is observed at the settled position in the gel filtration, the amounts of the original sample and the depolymerized product can be estimated from Fig. 1. According to this concept, the logarithmic plot of the area due to the undepolymerized part against the irradiation time is illustrated in Fig. 2, which shows a good straight

line. From this relation, it is estimated that about 95% depolymerization yields after 60 minute irradiation.

Molecular Weight of the Depolymerized Product by Ultrasonic Irradiation

In gel filtration, it is generally recognized that the plot of K_{av} ¹¹⁾ value versus the logarithm of molecular weight is on a straight line. Wasteson already reported a similar result with the gel filtration of chondroitin sulfate. The K_{av} values of the original sample and the new depolymerized product in the gel chromatogram were approximately 0.08 and 0.24, respectively. The relationships between these K_{av} values and the original molecular weight (69200) or the limited molecular weight (21800, calculated by the authors' equation) were identical with those obtained from Wasteson's K_{av} -log MW profile.

Behavior of the Main Depolymerized Product against Chondroitinase

The intact fractions obtained by gel filtration were too dilute for any quantitative test.

The main depolymerized product was investigated with the view that it may be an enzyme substrate by the method of Saito, Yamagata and Suzuki⁷⁾ using chondroitinase or chondro-6-sulfatase. In this case, the intact fractions obtained by gel filtration were too dilute for quantitative test and contained phosphate ions which interfered the analysis. Therefore, samples were prepared as follows. At first the fractions were dried by lyophilization, and then dissolved into distilled water to give solutions of proper concentration. And these solutions were eluted through Sephadex G 75 column to desalt, and were used as the samples.

On the contents of galactosamine and sulfate in each fraction, the ratios of the values obtained by chemical and enzyme methods were shown in Table I.

TABLE I. The Contents of Galactosamine and Sulfuric Acid in Gel Filtrated Fractions of an Aqueous Solution of Chondroitin Sulfate C^{a)} after Ultrasonic Irradiation

Ultrasonic irradiation times (min)	Fraction (ml)	Galactosamine (γ)			Sulfuric acid (γ)		
		Modified Elson-Morgan's method (A)	Chondroitinase ABC method (B)	Ratio (B/A)	Dodgson method (C)	Chondro-6-sulfatase method (D)	Ratio (D/C)
0	220—240	301	295	0.98	225	230	1.02
15	220—240	165	149	0.90	131	119	0.91
15	280—320	156	136	0.87	124	88	0.71
30	220—240	120	110	0.92	89	80	0.90
30	280—320	210	185	0.88	154	137	0.89
60	220—240	52	39	0.77	37	34	0.92
60	280—300	159	130	0.82	118	86	0.73
60	300—400	105	98	0.93	78	66	0.85

a) 0.25%

As shown in Table I, the depolymerized products of the samples with different irradiation times can also be enzyme substrate as is the non-depolymerized fraction.

Infrared Spectrum

Infrared (IR) absorption spectroscopy was used to investigate the decomposition of chondroitin sulfate C molecule by ultrasonic irradiation (ex. the elimination of sulfate and acetyl group).

11) $K_{av} = (V_e - V_o) / (V_t - V_o)$; V_e , V_o , and V_t are elution volume, void volume, and total volume, respectively.

As shown in Fig. 3, no apparent difference was detected between the IR spectrum of the original sample and that of the depolymerized product. Therefore, the principal structure of the macromolecular compound is unlikely to have been influenced by ultrasonic irradiation.

It is interesting to note here that the biomacromolecular compound was depolymerized into a lower molecular weight species by ultrasonic irradiation without destroying its main functional groups, and thus the depolymerized product maintained the same biochemical characteristics as those of the original. When macromolecular compounds are reduced to lower molecular weight species, the solubility and transport of the latter are usually increased. Therefore, it appears certain that considerably lower molecular compounds are made from macromolecular compounds. Ultrasonic irradiation may be a useful means for obtaining depolymerized products with a relatively narrow molecular weight distribution from macromolecular compounds.

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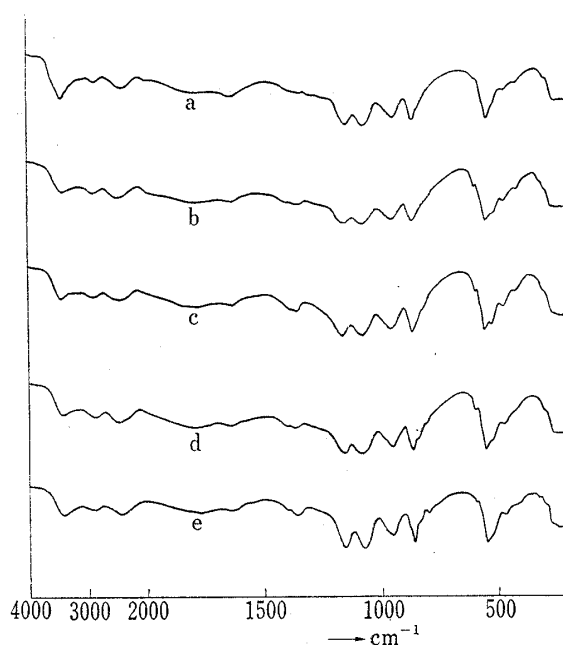


Fig. 3. Infrared Spectra of Depolymerization Product of Chondroitin Sulfate C^{a)} in an Aqueous Solution after Ultrasonic Irradiation (Nujol)

a : non-irradiated d : 45 minutes irradiated
b : 15 minutes irradiated e : 60 minutes irradiated
c : 30 minutes irradiated
a) 0.25%