Oxidative Degradation of β-Cyclodextrin Induced by an Ascorbic Acid–Copper Ion System

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Autoxidation of ascorbic acid (AsA) in the presence of Cu²⁺ generates some oxygen radicals, which induce the cleavage of glucosidic linkages of β-cyclodextrin (β-CD). The oxidative cleavage of β-CD gave several kinds of oligosaccharides and their reducing terminal sugars were mainly d-glucose. D-Erythrose, D-threose, D-arabinose, and D-xylose were also detected as minor reducing terminals by GLC and GC-MS analyses. From the experiments using several radical scavengers, the hydroxyl radical (OH radical) was identified as the main active radical.

During this reaction, 2-thiobarbituric acid reactive substances (TBARS) were produced by the oxidative degradation of β-CD, but their chemical structures and formation mechanism are still unknown.

Some active oxygen radicals formed in biological systems and the oxidation of some organic compounds may be involved in cancer, aging, the deterioration of biological constituents and the like. Many chemical compounds such as antitumor antibiotics,¹⁻³ sugar derivatives,⁴⁻⁵ and Maillard reaction products⁶ generate oxygen radicals that cause scission of DNA strands, oxidative degradation of biopolymers, and lipid peroxidation.

On the other hand, it has been reported that ascorbic acid (AsA)-transition metal ion systems generate superoxide (O₂⁻) and promote the oxidative depolymerization of several polysaccharides such as hyaluronate and alginate under physiological conditions.⁷⁻⁸ In such degradations, the main active species may not be O₂⁻ but the OH radical derived from O₂⁻, but it has remained unclear how OH radical is generated in AsA-metal ion systems and how they interact with polysaccharides.

We have studied the interaction between an AsA–Cu²⁺ system and β-CD as a model of polysaccharides under physiological conditions and now report on the oxidative degradation of β-CD by the action of OH radical generated in the AsA–Cu²⁺ system.

MATERIALS AND METHODS

Materials. β-CD was purchased from Hayashibara Biochemical Lab. Ascorbic acid and other reagents used were all of guaranteed grade.

Preparation of the interaction system. The reactions were done in 25 ml of 1/15 M phosphate buffer (pH 7.2) containing of 2 mM β-CD, 0.66 mM AsA and 3.3 µM CuSO₄ at room temperature.

The course of the degradation of AsA catalyzed by Cu²⁺ was followed by UV absorption at 265 nm. Oxidative degradation of β-CD induced by AsA–Cu²⁺ system was measured by the Park–Johnson method as the increase of reducing sugars.

Preparation of thiobarbituric acid reactive substance (TBARS) was measured by the method of Waravdekar et al.⁹ To 0.5 ml of reaction mixture, 0.5 ml of periodic acid (IO₃⁻) solution was added and the reaction mixture was left at room temperature for 20 min. After addition of 1 ml of sodium arsenite (NaAsO₂) solution to stop the reaction, 4 ml of TBA reagent was added and the mixture was kept for 10 min in a boiling water bath. After this was cooled, the absorption was measured at 532 nm.

Fractionation of the oxidized β-CD. Fractionation and characterization of the oxidized β-CD were done on a β-CD–AsA–Cu²⁺ system containing 2 mM β-CD, 0.66 mM AsA and 3.3 µM Cu²⁺. After incubation for 24 hr, the
reaction mixture was fractionated using an Amberlite XAD-7 column (2.0 x 26 cm) with water and water-methanol as eluates, followed by a Toyo Pearl HW-40 superfine column (2.7 x 40 cm) with water. The fractions obtained from Amberlite XAD-7 and Toyo Pearl HW-40s columns had strong reducing power and were concentrated to dryness.

Structural analyses of oligosaccharides from the oxidized β-CD. Alditol acetates. The fractions F-1 and F-2 obtained from gel filtration were reduced with NaBH₄, treated with Amberlite IR-120 (H⁺) and concentrated. The reduced products were hydrolyzed in 20 ml of 0.2 n HCl at 120°C for 75 min in an autoclave. The hydrolyzates were neutralized by Dowex 1 x 8 (CO₃⁻), dried, and then acetylated with acetic anhydride-anhydrous pyridine at 70°C for 2 hr.

Preparation of 2-aminopyridine derivatives. 2-Aminopyridine derivatives of the oligosaccharides in F-1 and F-2 were prepared and hydrolyzed by the method of Hase et al.10

To the fractions F-1 and F-2 solutions (50 mg/ml of water), 910 mg of 2-aminopyridine, 550 mg of sodium cyanoborohydride, 0.4 ml of acetic acid, and 3.6 ml of methanol were added. The mixtures were heated in a sealed tube at 75°C for 7 hr, then the products were absorbed with Dowex 50 x 2 (H⁺) and the resin was washed with water. 2-Aminopyridine derivatives were eluted with 0.6 n aqueous ammonia, concentrated, and chromatographed on a Toyo Pearl HW-40s gel column to remove the excess of 2-aminopyridine. 2-Aminopyridine derivatives eluted with 0.02 n acetic acid were hydrolyzed with 2 n HCl at 100°C for 4 hr, neutralized with Dowex 1 x 8 (CO₃⁻) and concentrated to dryness.

GLC and GC-MS analyses. GLC. A Shimadzu gas-chromatograph GC-4BMPF was used under the following conditions: Injection and detection temp. 280°C, carrier gas (N₂) 30 ml/min, columns; 5% silicone GE XE-60 on Chromosorb W (AW) 0.3 x 200 cm, temp. 180~230°C 5°C/min for alditol acetates, 1.5% silicone OV-17 on Shimalite W (AW) 0.3 x 200 cm, temp. 180~250°C 5°C/min for TMS ether of 2-aminopyridine derivatives.

GC-MS. A JEOL model JMS D-100 Mass Spectrometer with same kinds of glass column was used under the following conditions: Ionizing voltage 28 eV, ionizing current 300 μA; ion source temperature 250°C.

RESULTS

Oxidative cleavage of β-CD induced by AsA–Cu²⁺ system

AsA itself was rather unstable in neutral conditions in phosphate buffer (pH 7.2) and oxidized gradually without metal ions. Moreover, AsA is quickly degraded to C-5 or other smaller compounds in the presence of trace amounts of metal ions. Figure 1 showed that autoxidation of AsA proceeded in the presence of trace amounts of Cu²⁺ as represented by a decrease of the absorption at 265 nm. With increases in the concentration of Cu²⁺ from 3.3 to 10 μM, the rate of AsA degradation was accelerated and AsA was decomposed completely on incubation for a few hours. Other metal ions such as Fe²⁺ and...
Fe$^{3+}$ had the same activity as Cu$^{2+}$, but the rate of AsA degradation was lower than with Cu$^{2+}$ (data not shown).

Large increases of reducing power in the reaction mixtures composed of AsA–Cu$^{2+}$ and β-CD were observed, and they were also proportional with the concentration of Cu$^{2+}$ (Fig. 2). These results suggested the oxidative cleavage of glucosidic linkages or the decomposition of glucose in β-CD. These changes in the oxidized β-CD may be due to some oxygen radical species such as O$_2^-$ or OH radical generated by the AsA–Cu$^{2+}$ system.

**Effects of chelating agents and radical scavengers**

From the results shown in Figs. 1 and 2, oxygen radicals seemed to induce the oxidative cleavage of β-CD, and therefore the effects of some enzymes relating to the decomposition of active oxygens, chelating agents, and scavengers were examined.

As shown in Table I, oxidative degradation of β-CD induced by the AsA–Cu$^{2+}$ system was inhibited completely by catalase, EDTA, and OH radical scavengers such as thiocyanate, t-butanol, and sodium formate, but superoxide dismutase (SOD) which catalyze the disproportionation reaction of O$_2^-$ was not very effective in this system. Therefore, the result suggests that the oxidative cleavage of β-CD was due to the action of OH radical generated from H$_2$O$_2$ through O$_2^-$ during the autoxidation of AsA.

**Fractionation of oxidized β-CD**

β-CD was incubated with the AsA–Cu$^{2+}$ system at room temperature for 24 hr and the reaction mixture was freeze-dried and resolved with 5 ml of water (insoluble matter was removed by centrifugation). The sample was

**Table I. Effects of Chelating Agents and Radical Scavengers on Oxidative Degradation of β-CD**

<table>
<thead>
<tr>
<th>Scavengers</th>
<th>Conc.</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>40 μM</td>
<td>83</td>
</tr>
<tr>
<td>KSCN</td>
<td>1 mM</td>
<td>98</td>
</tr>
<tr>
<td>t-BuOH</td>
<td>1 mM</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>100</td>
</tr>
<tr>
<td>Na-formate</td>
<td>1 mM</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>86</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.15 mg/50 ml</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>0.15 mg/50 ml</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>0.75 mg/50 ml</td>
<td>84</td>
</tr>
<tr>
<td>SOD</td>
<td>0.15 mg/50 ml</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>and SOD respectively</td>
<td>100</td>
</tr>
</tbody>
</table>

![Fig. 3. Elution Pattern on a Column of Amberlite XAD-7 of Oxidized β-CD.](image)

Symbols: —●—; reducing power, —○—; total sugar.
fractionated through an Amberlite XAD-7 column with 200 ml of water and 400 ml of water–methanol (1:1, v/v) as eluents to give two fractions (Fig. 3). The first fraction (F-A) passed through the XAD column is a minor part composed of oxidized β-CD with strong reducing power (yield 17.4%). The second one (F-B) was the main fraction containing unreacted β-CD and decomposition products of AsA.

The fraction F-A was concentrated and chromatographed on a Toyo Pearl HW-40s gel column; it gave three fractions (Fig. 4). However, fraction F-3 was mixture of the degradation products of β-CD and AsA. Therefore, the reducing terminals of F-1 and F-2 were analyzed.

*Structural analyses of reducing terminals in the fraction F-1 and F-2*

Fractions F-1 and F-2 were reduced with NaBH₄, hydrolyzed, and acetylated. These acetates were mixtures of glucose acetate from nonreducing parts and alditol acetates from reducing terminals. As shown in Fig. 5, many small peaks of alditol acetate other than glucose acetate (peak E) were detected and identified as erythritol, threitol, arabinitol, xylitol, mannitol, and glucitol by comparison with authentic samples on GLC and GC-MS. Fragmentation of peak H on GC-MS seemed to be deoxy unsaturated hexitol, but its actual structure is still unknown.

2-Aminopyridine derivatives of fractions F-

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**Fig. 4.** Gel-chromatogram of the Fraction F-A Using a Toyo Pearl HW-40s Column.

**Fig. 5.** Gas-chromatogram of Alditol Acetates Derived from Fractions F-1(A) and F-2(B). Symbols: A; erythritol, B; threitol, C; arabinitol, D; xylitol, E; glucose, F; mannitol, G; glucitol.
1 and F-2 were prepared to detect the reducing terminal sugars by the method of Hase. After the preparation of 2-aminopyridine derivatives from the fractions, each sample was analyzed by GLC in the form of its TMS ether (Fig. 6). The retention time of 2-aminopyridine derivatives was observed beyond 10 min. Peaks 1 and 2 were identified as 2-aminopyridine derivatives of glucose and arabinose, respectively, from their retention times and GC-MS analyses (Figs. 7 and 8). On the other hand, deoxy unsaturated hexose was not detected clearly in this experiment. The peaks other than peaks 1 and 2 have not been identified.

2-Thiobarbituric acid reactive substances

As mentioned above, the formation of deoxy unsaturated hexitol was presumed from the mass fragmentation of its alditol acetates, and a similar compound had also been detected in the oxidative degradation of β-CD induced by lipid peroxidation. However, its structure and formation mechanism were not postulated.

To detect deoxy or deoxy unsaturated sugars, IO₄⁻ treatment then thiobarbituric acid
DISCUSSION

Our results suggest that the oxidative degradation of \( \beta \)-CD is caused mainly by the action of OH radical generated by the autooxidation of AsA in the presence of \( \text{Cu}^{2+} \). A mechanism for the formation of oxygen radicals in AsA-metal ion system is proposed in Scheme 1. It may be considered that \( \text{O}_2^- \) is generated through an AsA-metal ion complex, followed by the formation of hydrogen peroxide from the disproportionation of \( \text{O}_2^- \), and metal ion is reduced to \( M^{(n-1)+} \) by the action of AsA or \( \text{O}_2^- \). Consequently, OH radical is generated by the reaction of hydrogen peroxide and \( M^{(n-1)+} \) (Fenton reaction).

On the other hand, the reaction mechanism between sugar and oxygen radicals in the AsA–Cu\(^{2+}\) system was estimated to be the same as the results obtained by \( \gamma \)-radiolysis of sugars in aerated conditions as reported previously.\(^{12,13}\) The reaction was initiated by hydrogen abstraction from glucose molecules to give sugar radicals that must be transformed to some oxidative products or to some degradation products by C–C bond scission through their hydroperoxy radicals. And furthermore, the unstable glucosidic linkages of these oxidized products must be hydrolyzed secondarily to form D-glucose and other pentoses as reducing terminals. \( \beta \)-CD must be degradated to smaller oligosaccharides through this pathway.

As for TBARS in the \( \beta \)-CD–AsA–Cu\(^{2+}\) system, it seemed to be derived from the interaction between sugar and oxygen radicals. The formation of TBARS may occur by the radical dehydration reaction in glucose of \( \beta \)-
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Scheme 1. Oxygen Radical Formation from the Interaction between AsA, Metal Ions, and Oxygen.

CD with OH radical. However, its structure and formation mechanism remains unknown.

Since the concentration of AsA and Cu²⁺ are both in the physiological range in this experiment, oxygen radicals derived from this system may cause the depolymerization of several biopolymers such as nucleic acids, proteins, and polysaccharides in foods and living systems. We are now investigating the interaction between oxygen radicals and several polysaccharides.

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REFERENCES