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XTT (3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(methoxy-6-nitro)benzenesulfonic acid hydrate) was reduced by incubated glucosamine hydrochloride. The XTT reducibility by incubated glucosamine was linearly related with the DNA-breaking activity. In order to elucidate the reaction mechanism, the glucosamine derivatives formed during the incubation process were separated by HPLC, and the compound responsible for the reduction was analyzed. Among the incubated products, fructosazine and deoxyfructosazine were identified by LC-MS, FAB-MS, and ^{1}H- and ^{13}C-NMR. These products showed no XTT reducibility, but an unstable intermediate with a molecular weight of 322 displayed reducibility. Since the intermediate gave fructosazine by oxidation with XTT and was a precursor of deoxyfructosazine, we conclude that the intermediate could have been dihydrofructosazine. Therefore, the XTT reducibility by incubated glucosamine was based on dihydrofructosazine formed by the condensation of two molecules of glucosamine.

Key words: glucosamine; DNA-breaking activity; fructosazine; XTT

Glucosamine hydrochloride is currently used for the treatment of degenerative joint disease in small animals and presents a novel approach for the treatment of degenerative conditions. Glucosamine, an amino monosaccharide, is a natural component of glycoproteins found in connective tissues and gastrointestinal mucosal membranes. It is a precursor of the disaccharide unit of glycosaminoglycans which are the building blocks of the articular cartilage, the proteoglycans. Numerous reports have recently focused on the utility of glucosamine for the treatment of osteoarthritis.[1–4]

Glucosamine has also been shown to have high DNA-breaking activity, and this activity is promoted more by the presence of Cu^{2+}.[5] Yamaguchi *et al.*[6,7] have suggested that glucosamine was unstable in the neutral pH range, being converted into certain intermediates such as a dihydropyrazine compound which generated a carbon-centered radical. These intermediate compounds were regarded as DNA-breaking substances. Therefore, monitoring those biologically active intermediates is important for the further application of glucosamine as a drug or supplement.

We have recently found a good relationship between the DNA-breaking activity of incubated glucosamine and the XTT (3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(methoxy-6-nitro)benzenesulfonic acid hydrate) reducibility and suggested that the XTT assay based on this reducibility may be applicable for monitoring those biologically active products.[8] In order to confirm the usefulness of the XTT assay to monitor the biologically active products derived from glucosamine, we elucidate in the present investigation the reaction mechanism for XTT reduction by glucosamine.

Materials and Methods

Reagents. D-Glucosamine hydrochloride was purchased from Nacalai Tesque (Kyoto, Japan). XTT (3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(methoxy-6-nitro)benzenesulfonic acid hydrate) and diethylenetriaminepentaacetic acid (DTPA) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of the highest grade available and were used without further purification. All solutions were prepared with water purified by a Milli-Q system (Millipore, Tokyo, Japan).

Incubation of glucosamine. Glucosamine (0.5 M) dissolved in a 50 mM sodium phosphate buffer (pH 7.4) containing 5 μM DTPA was incubated at 37°C. After incubating for the indicated period, the reaction mixture was used in the subsequent experiment.

XTT assay procedure. The assay was performed in a 96-well microtiter plate according to the method previously described.[8] With the standard procedure, each well contained 60 μl of 0.5 mM XTT prepared with a 0.2 M potassium phosphate buffer (pH 7.0)

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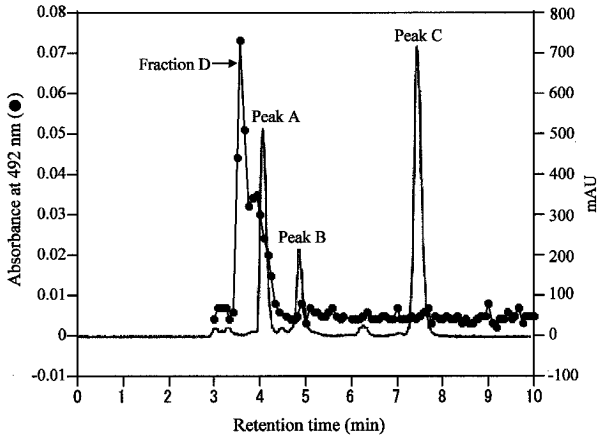

Fig. 1. HPLC Chromatogram of the D-Glucosamine Derivatives and XTT Reducibility of the Fractionated Eluate (● absorbance at 492 nm).

D-Glucosamine (0.5 M) in a 50 mM sodium phosphate buffer (pH 7.4) containing 5 μM DTPA was incubated at 37°C for 4 h. HPLC conditions are described in the Materials and Methods section. The XTT reducibility was measured after the eluate had been fractionated from 3.00 to 10.00 min at intervals of 5 s.

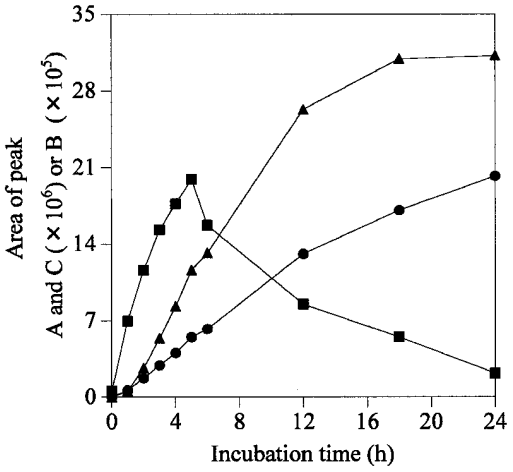

Fig. 2. Effect of Incubation Time on the Area of Peak A (●), B (■) and C (▲).

D-Glucosamine (0.5 M) in a 50 mM sodium phosphate buffer (pH 7.4) containing 5 μM DTPA was incubated at 37°C for 24 h. At the indicated time, the incubated solution (5 μl) was injected for an HPLC analysis under the conditions described in the Materials and Methods section.

saturated with menadione. A 40-μl amount of the sample was dropped into a well. After it had been mixed by a microplate shaker at a speed of 500 rpm for 15 s, the difference in the absorbance between 492 nm and 600 nm (as the reference) was read by an MPR A4i microplate reader (Tosoh, Tokyo, Japan) as the absorbance at 0 min. After 20 min at room temperature, the absorbance difference was again read, and the increase in absorbance difference was recorded as the ability of the sample to reduce XTT (XTT reducibility). When the XTT reducibility of a sample solution was too high, it was subjected to the assay after appropriate dilution.

Separation of incubated glucosamine by HPLC. The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-10A pump combined with Shimadzu SPD-M10A photodiode array detector and a Cosmosil HPLC column (5C_{18}-MS; 4.6 mm i.d. × 150 mm). The separation conditions were as follows: detection, 274 nm (200–400 nm); eluent, Milli-Q water; flow rate, 0.5 ml/min; injection volume, 5 μl. The data were analyzed by Shimadzu CLASS VP-5 software.

Analytical methods. LC-(ESI)MS analyses were run on a Jasco (Tokyo, Japan) PV-1580 HPLC system coupled with a Finnigan LCQ Duo mass spectrophotometer equipped with an electrospray (ESI) interface (Thermo Quest, Tokyo, Japan). The HPLC conditions were the same as those just described. FAB mass spectra were obtained with a JEOL (Tokyo, Japan) MS route JMS-600 W instrument, using glycerol as the matrix. NMR spectra were measured with a JEOL LNM-LA400 spectrometer in D_2O.

Results

When 0.5 M glucosamine was incubated at 37°C, the solution showed XTT reducibility depending on the incubation time. The maximum reducibility appeared after 4 h of incubation and thereafter gradually decreased in accordance with the results in our previous paper.[8] The glucosamine solution that had been incubated for 4 h was analyzed by HPLC (Fig. 1). Three major peaks were detected in the chromatogram when monitoring at 274 nm that were characteristic of fructosazine derived from glucosamine.[9] These peaks are designated as A, B and C in order of their elution time. Peaks A, B and C showed absorption maxima at 272, 272 and 276 nm, respectively. In order to know the time-course characteristics of these peaks during the incubation process, glucosamine was incubated for 24 h and each peak area was plotted against the incubation time (Fig. 2). Peaks A and C progressively increased with incubation time, whereas peak B reached a maximum after 5 h and thereafter decreased. The eluate corresponding to each peak was collected and incubated again at 37°C. When the incubated solution was applied to the HPLC analysis, the areas of both peaks A and C did not change in spite of the re-incubation. Peak B decreased with incubation time, and no new peaks appeared in the chromatogram. This result indicates that both peaks A and C were stable end-products derived from glucosamine and that peak B was an intermediate during the incubation process.

In order to identify which peak was related to the XTT reduction, the eluate from 3.00 to 10.00 min was collected every 5 s, and the XTT reducibility of each fraction was measured (Fig. 1). Unexpectedly,

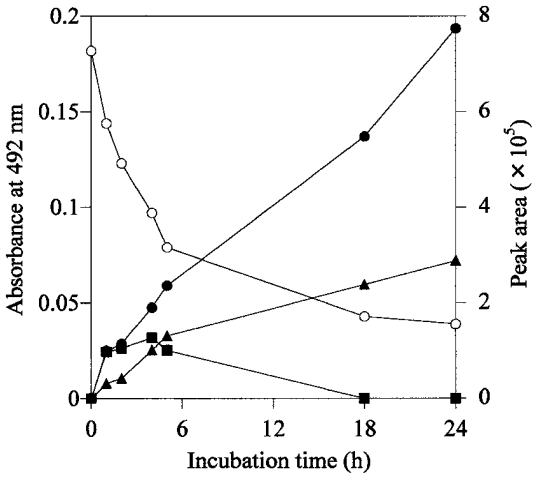

Fig. 3. Effect of Re-incubation Time on the XTT Reducibility of Fraction D (○) and on the Area of Peak A (●), B (■) and C (▲).

Collected fraction D was re-incubated at 37°C for 24 h. At the indicated time, re-incubated fraction D was subjected to the XTT assay and HPLC analysis. HPLC conditions are described in the Materials and Methods section.

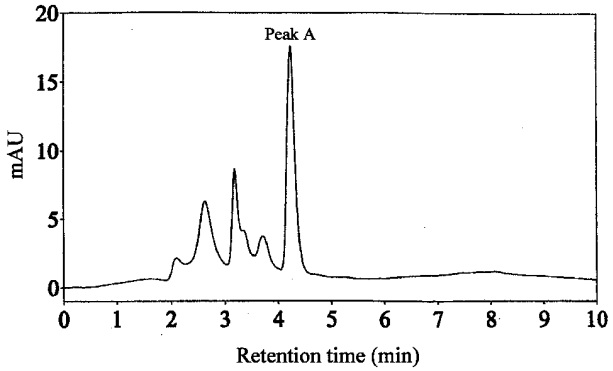

Fig. 4. HPLC Chromatogram of the Reaction Mixture of Collected Fraction D and the XTT Solution.

Collected fraction D was reacted with XTT for 20 min and then applied to an HPLC analysis under the conditions described in the Materials and Method section.

XTT reducibility was only observed in the eluate with a retention time of 3.50–4.25 min, different from peaks A, B and C. The fraction from 3.58 to 3.91 min having high XTT reducibility is designated as fraction D. The XTT reducibility of fraction D depended on the incubation time of glucosamine and reached a maximum value at 5 h. In spite of the appearance of XTT reducibility, no peak was recognizable in fraction D. Thus, the substance responsible for the XTT reducibility seemed to have a relatively low absorption at 274 nm.

Although the formation of glucosamine derivative has already been reported,[5,9,10] no systematic investigation on the formation mechanism has previously been made. To clarify the relationship between fraction D and other intermediate products, fraction D was again incubated at 37°C, and the XTT reducibility and HPLC pattern of the incubated solution were examined (Fig. 3). The XTT reducibility decreased with incubation time and, at the same time, peaks A, B and C appeared in the chromatogram. Peak B was formed *via* fraction D and changed to other substances. After the XTT reducibility of fraction D had been measured, the reaction mixture was applied to an HPLC analysis. Multiple peaks were apparent in the chromatogram but the major one was peak A whose absorption maximum was 272 nm (Fig. 4), while no peak appeared with a retention time corresponding to that of peak B or C. It is clear, therefore, that fraction D was mainly oxidized to peak A with XTT and that peaks B and C were not the oxidized form of fraction D.

We next tried to elucidate the structures of peaks A, B and C and fraction D after separating from the reaction mixture by HPLC. By monitoring with LC-MS, A, B, C and D showed $(M+H)^+$ at m/z of 321, 305, 305, and 323, respectively. Stable peak A and C fractions were collected by preparative HPLC and lyophilized. The FAB-MS spectrum of isolated peak A showed $(M+H)^+$ at $m/z=321.0$, in accordance with that obtained by LC-MS. The ^{1}H-NMR spectrum exhibited the following signals [δ_H (D_2O)]: 3.53 (q, 2H, $J=6.4$ Hz, H-3′, H-3″), 3.71 (m, 6H, H-2′, H-2″, H-4′, H-4″), 5.04 (s, 2H, H-1′, H-1″) and 8.60 (s, 2H, H-3, H-6). The ^{13}C-NMR spectrum showed following signals [δ_c(D_2O)]: 65.8 (C-4′, C-4″), 73.9 (C-3′, C-3″), 74.3 (C-1′, C-1″), 76.2 (C-2′, C-2″), 144.8 (C-3, C-6) and 157.9 (C-2, C-5). These data coincide with those of fructosazine shown in Fig. 5.[10] The FAB-MS spectrum of isolated peak C showed $(M+H)^+$ at m/z 305.0, in accordance with that obtained by LC-MS. The ^{1}H-NMR spectrum exhibited the following signals [δ_H (D_2O)]: 2.82 (dd, 1H, $J=13.9$ and 10.9 Hz, H-1″b), 3.07 (d, 1H, $J=$ 14.1 Hz, H-1″a), 3.52 (m, 3H, H-2″, H-3′, H-3″), 3.70 (m, 4H, H-4′, H-4″), 3.88 (m, 1H, H-2′), 5.00 (s, 1H, H-1′), 8.38 (s, 1H, H-6) and 8.56 (s, 1H, H-3). The ^{13}C-NMR spectrum showed the following signals [δ_c(D_2O)]: 40.4 (C-1″), 65.3 (C-4″), 65.8 (C-4′), 73.9 (C-3′), 74.15 (C-2″), 74.20 (C-1′), 76.3 (C-2′), 77.3 (C-3″), 145.0 (C-3), 147.0 (C-6), 156.1 (C-5) and 156.9 (C-2). These data coincide with those of deoxyfructosazine (Fig. 5) described by Sumoto *et al.*,[10] except for the assignment of H-2′ and H-2″. Since H-2′ has fewer neighboring hydrogens and an excess hydroxyl group instead of one hydrogen, the proton should appear in a lower magnetic field and give a more complex signal. Therefore, peak C could be identified as deoxyfructosazine as shown in Fig. 5. Similarly, peak B and fraction D were separated and lyophilized. Due to the instability of these compounds, $(M+H)^+$ obtained by FAB-MS failed to agree with that by LC-MS. Therefore, it was impossible to identify these compounds by ^{1}H- and ^{13}C-NMR. Fraction D had a molecular weight of 322

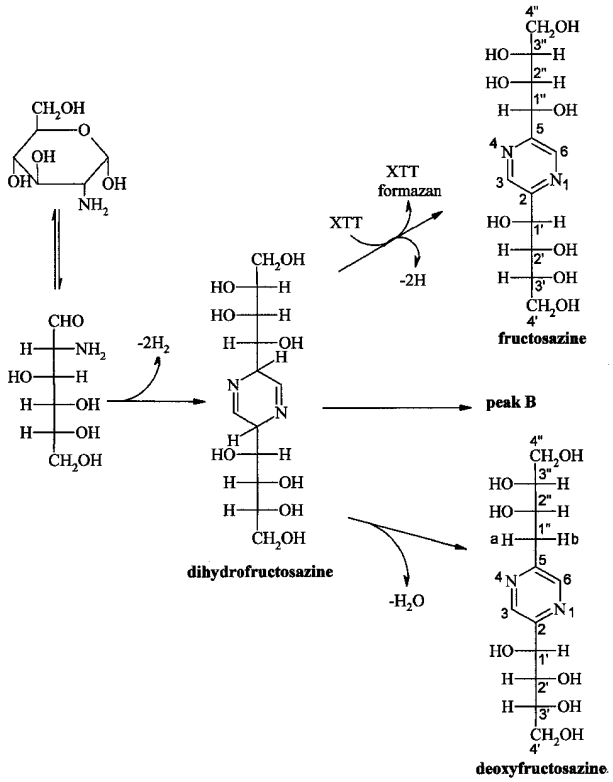

Fig. 5. Reduction Mechanism for XTT by D-Glucosamine Derivatives.

according to the LC-MS analysis. Oxidation of the fraction with XTT clearly gave fructosazine. It was also a precursor of deoxyfructosazine. These results strongly suggest that fraction D could have been dihydrofructosazine as shown in Fig. 5. The possibility of generating dihydrofructosazine in a dehydrochlorinated glucosamine solution has been reported by Kashige *et al.*[5] The results of this study confirmed this by an analysis of the product separated by HPLC. Since there is no information on a glucosamine derivative having a molecular weight of 304 other than deoxyfructosazine, the structural elucidation of peak B remains for a future investigation. These findings show that the reaction mechanism for the reduction of XTT with glucosamine can be explained as indicated in Fig. 5; glucosamine generated dihydrofructosazine by the condensation of two molecules during the incubation process. Dihydrofructosazine could be oxidized to fructosazine with dissolved oxygen, but when XTT was present, it could be more quickly oxidized; at the same time, XTT was reduced to the corresponding formazan. Although deoxyfructosazine and peak B (unknown structure) were also generated from dihydrofructosazine, their formation did not result from the oxidation reaction.

Discussion

The results of our previous study show that the incubated glucosamine had XTT reducibility that was related to its DNA-breaking activity.[8] However, the XTT-reducing substance which was generated during the incubation of glucosamine was not clarified. In this study, the mechanism for the reduction of XTT with incubated glucosamine was investigated by using an HPLC analysis with an ODS column. When the incubated glucosamine was subjected to HPLC and monitored at 274 nm, which is the characteristic absorption maximum of a glucosamine derivative, three peaks (A, B and C) could be detected on the chromatogram. These peaks, however, had no ability to reduce XTT. Contrary to our expectation, XTT reducibility was only observed in the eluate with a retention time of 3.50–4.25 min. The fraction of 3.58–3.91 min showing high XTT reducibility was designated as fraction D. The molecular weight of fraction D was determined as 322 by the LC-MS analysis. Although an NMR analysis of fraction D could not be performed because of its instability, it was revealed that fraction D was a precursor of peak A (fructosazine), peak B (an unknown product) and peak C (deoxyfructosazine). It was also found that fraction D was oxidized by XTT to fructosazine. Judging from these results, we conclude that fraction D was dihydrofructosazine and that dihydrofructosazine was the XTT-reducing substance in the incubated glucosamine. It has been reported that dihydrofructosazine showed DNA-breaking activity by the generation of active oxygen and a carbon-centered radical.[5,7,10] Therefore, the XTT assay, which can detect dihydrofructosazine, can be employed for evaluating the DNA-breaking activity of glucosamine. Since amino sugars such as mannosamine and galactosamine, apart from glucosamine described here, have shown DNA-breaking activity in the same manner and the XTT reducibility,[8,10–12] the dihydrofructosazine type of compound could also be involved in the reduction of XTT with those amino sugars. An investigation of these amino sugars is now in progress. The DNA-breaking activity of amino sugars has recently been suggested to be related to the apoptosis of cancer cells.[13] Based on this finding, the XTT assay might be of value to present the diverse biological activities that amino sugars show.

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