Reduction of Tetrazolium Salt XTT by Aminoreductone Formed during the Maillard Reaction of Lactose

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Lactose, a reducing disaccharide abundant in milk, reacts extensively with the amino groups of protein through the Maillard reaction. The Maillard reaction products showed 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]bis(4-methoxy-6-nitro)benzensulfonic acid hydrate (XTT) reducibility. The objective of this study was to clarify the Maillard reaction products responsible for the XTT reducibility. When lactose and butylamine were heated at 100 °C, the characteristic UV maximum at 320 nm was recognized and the relationship between the XTT reducibility and the compound with absorption maximum at 320 nm was investigated. The time course and the dependence on the heating temperature of the formation of the compound with absorption maximum at 320 nm were similar to those of the XTT reducibility. Their relationship showed a significant correlation (r = 0.967, n = 19). Furthermore, the spectrum change of the heated model solution by the addition of XTT suggested that the compound with absorption maximum at 320 nm would be involved in the reduction of XTT. Because the compound with absorption maximum at 320 nm was identified as an aminoreductone, 1-(butylamino)-1,2-dehydro-1,4-dideoxy-3-hexulose, by NMR analysis, it can be concluded that this was the main XTT-reducing substance.

Keywords: *Maillard reaction; lactose; XTT; aminoreductone*

INTRODUCTION

The Maillard reaction, a chemical reaction between amino groups and reducing sugars, is very significant for foods because it strongly affects qualities such as flavor and color. At the same time, the Maillard reaction leads to the loss of nutritional values and the formation of mutagens. Especially during milk processing the Maillard reaction causes problems due to browning and the generation of off-flavor. Therefore, detection of Maillard reaction products is important for the quality evaluation of foods (Weenen, 1998; Friedman, 1996).

Recently, we proposed an assay method for determining the ability of ultrahigh-temperature (UHT)-treated milk to reduce 3′-[1-[(phenylamino)carbonyl]-3,4- tetrazolium]bis(4-methoxy-6-nitro)benzensulfonic acid hydrate (XTT) as a method of evaluating the extent of the Maillard reaction (Ukeda et al., 1996, 1998). Compared with the previously reported methods such as quantification of lactulose (Geier and Klostermeyer, 1980), hydroxymethylfurfural (HMF) (Keeney and Bassette, 1959), and furosine (Finot et al., 1981; Resmini et al., 1990), the XTT assay is a rapid and convenient method and is found to be applicable for estimating not only the extent of thermal treatment but also the storage conditions because the ability decreased depending on the storage period and temperature (Ukeda et al., 1995, 1996). Moreover, it was also suggested that the XTT assay is applicable to the estimation of the extent of heat treatment of foods other than milk (Ukeda et al., 1998). However, the Maillard reaction product that is responsible for the reduction of XTT was not established. In this investigation, we tried to clarify the XTT-reducing substance, which is generated during the Maillard reaction of lactose.

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MATERIALS AND METHODS

Reagents. XTT was purchased from Sigma Chemical Co. (St. Louis, MO), and lactose monohydrate was from Nacalai Tesque, Inc. (Kyoto, Japan). Butylamine was obtained from Wako Pure Chemical Industries (Osaka, Japan). Chloroform-*d* ($CDCl_3$) was purchased from Cambridge Isotope Laboratories, Inc. (Cambridge, MA). All other reagents were of the highest grade commercially available. Milli-Q water was used in all procedures.

Heating of Sample Solution. Lactose monohydrate (262 mM) and butylamine (1.16 M) were dissolved in 1.28 M phosphate buffer (pH 7.0), and the pH was adjusted to 7.0 with phosphoric acid. The sample solution (1.2 mL) was put into a 1.5-mL polypropylene tube with a stopper and heated at 80−100 °C for 30 min with a dry heater (Dry Thermo Unit DTU-1C, Taitec Co., Saitama, Japan). Immediately after heating for an indicated time, the sample solution was cooled by ice. Lactose monohydrate (127 mM)-glycine (15 mM) in 20 mM phosphate buffer (pH 6.7) was also used in this experiment as the model solution of milk.

Preparation of Aminoreductone. The preparation of aminoreductone was carried out according to the method of Pischetsrieder et al. (1998). Briefly, lactose monohydrate (262 mM)-butylamine (1.16 M) in 1.28 M phosphate buffer (pH 7.0) was heated at 100 °C for 15 min. Immediately after heating, the sample solution was cooled by ice. It was extracted three times with 2.4 mL of ethyl acetate. The solvent was evaporated in a water bath at 70 °C under nitrogen atmosphere. The extracted residue (crude aminoreductone) was dissolved in an appropriate solvent and used in the subsequent experiment.

XTT Assay Procedure. The assay was performed in a 96-well microtiter plate using an eight-channel adjustable volume pipettor according to our previous paper (Ukeda et al., 1996). Each well contained 60 μL of 0.5 mM XTT solution prepared

10.1021/jf000653z CCC: $19.00
Published on Web 10/05/2000

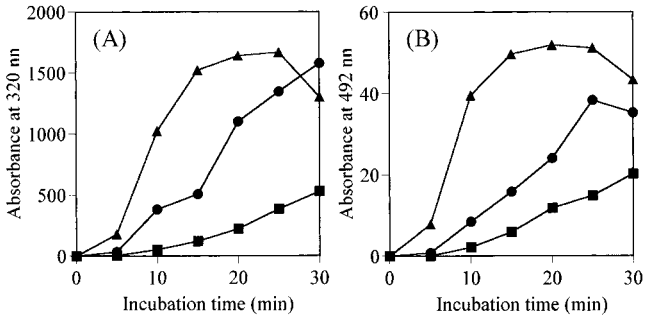

Figure 1. Effect of heating temperature on the absorbance at 320 nm and the XTT reducibility. Lactose (262 mM) and butylamine (1.16 M) in 1.28 M phosphate buffer (pH 7.0) were heated at 80 °C (■), 90 °C (●), and 100 °C (▲).

with 0.2 M potassium phosphate buffer (pH 7.0) containing menadione at the saturation level. The menadione was added as a redox mediator (Ukeda et al., 1995). Then 40 μL of sample was added into the well. After it was mixed in a microplate shaker for 15 s at a speed of 500 rpm, the difference in the absorbance between 492 and 600 nm (as the reference) was read on a microplate reader MPR A4i (Tosoh, Tokyo, Japan) as the absorbance at 0 min. Again, after 20 min at room temperature, the absorbance difference was read, and the increase in the 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 an appropriate dilution.

^{1}H NMR and ^{13}C NMR Analyses. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), and DEPT data for aminoreductone in $CDCl_3$ were measured with a JEOL Lambda 400 spectrophotometer (Tokyo, Japan).

UV Spectroscopy. UV spectra were obtained by means of a Pharmacia Biotech Ultrospec 3000 spectrophotometer (Uppsala, Sweden).

RESULTS AND DISCUSSION

Recently we suggested that nitrogen-containing heterocyclic compounds might be involved in the generation of XTT reducibility in the amino−carbonyl reaction of D-glucosamine, glutaraldehyde, glyceraldehyde, etc. (Ukeda et al., 1998). However, the XTT-reducing substance derived from a disaccharide or hexose was not investigated in detail. To clarify the XTT-reducing substance, first, the spectrophotometric analysis of a model solution of milk heated at 130 °C for 15 min was done. In the model solution of lactose-glycine the characteristic UV maximum at 318 nm was recognized. Because other UV absorption maxima were not observed, the compound with absorption maximum at 318 nm was supposed to be the main Maillard reaction product derived from lactose. Thus, we investigated the relationship between XTT reducibility and the compound showing the characteristic absorption maximum using lactose-butylamine.

The solution of lactose and butylamine was heated at 80−100 °C for 30 min. The heated lactose-butylamine solution exhibited an absorption maximum at 319.5 nm. During the heating, the change in absorbance at 320 nm (Figure 1A) and XTT reducibility (Figure 1B) was monitored. Both increased gradually in accordance with the rise in temperature and heating time. This result indicates that the compound with the absorption maximum at 320 nm was formed by the heating. Moreover, the behavior of increase in absorbance at 320 nm correlated with that of the XTT reducibility. Because a similar trend was recognized in the time course of the XTT reducibility and the absorbance at 320 nm, the XTT reducibility was plotted against the absorbance at 320 nm when the solution consisting of lactose and butylamine was heated at 80−100 °C for 30 min (Figure 2). As shown in Figure 2, a linear relationship between them ($r = 0.967$, $n = 19$) was recognized. The regression equation was as follows; $y = 0.0287x + 1.64$, where x and y represent the absorbance at 320 nm and the XTT reducibility, respectively. From this result it was found that the compound with the absorption maximum at 320 nm might be responsible for the reduction of XTT.

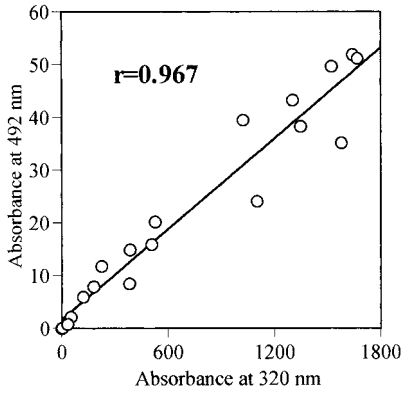

Figure 2. Relationship between the absorbance at 320 nm and the XTT reducibility. Lactose (262 mM) and butylamine (1.16 M) in 1.28 M phosphate buffer (pH 7.0) were heated at 80−100 °C.

To further elucidate the participation of the compound with absorption maximum at 320 nm in the reduction of XTT, the spectrum change of heated solution by addition of XTT was examined. The solution containing lactose and butylamine was heated at 100 °C for 15 min, and then the heated model solution was diluted 1000-fold (3 mL), followed by the addition of 0.5 mM XTT (0.5 mL). The spectrum change was recorded at intervals of 5 min for 20 min. Before the addition of XTT, only the absorption maximum at 320 nm was recognized. The absorption at 320 nm decreased gradually after the addition of XTT, whereas the absorption at 470 nm increased (data not shown). When XTT is reduced, the corresponding water-soluble formazan shows an absorption maximum at 470 nm. The spectrum changes after the addition of XTT therefore suggest that the compound with the absorption maximum at 320 nm was oxidized by XTT and XTT was simultaneously reduced by the compound with the absorption maximum at 320 nm. It can be clearly assumed that the compound with the absorption maximum at 320 nm contributed to the reduction of XTT.

Schoetter et al. (1994) reported that the aminoreductone which was formed during the Maillard reaction of maltose and butylamine showed an absorption maximum at 322 nm in methanol. Moreover, Pischetsrieder et al. (1998) reported that the structure of the aminoreductone from lactose and butylamine was identical with that of the aminoreductone from maltose and butylamine. Because the characteristics of aminoreductone reported previously were similar to those of the compound with an absorption maximum at 320 nm in this study, we tried to extract the compound with an absorption maximum at 320 nm from a lactose-butylamine solution heated at 100 °C for 15 min according to the method reported by Pischetsrieder et al. (1998). The extracted compound with the absorption maximum at 320 nm had the ability to reduce XTT, and the slope of the regression curve between the XTT reducibility

Table 1. ^{13}C and ^{1}H NMR Assignments of the Compound from Lactose-butylamine in $CDCl_3$[a]

no.	δ_C	δ_H	no.	δ_C	δ_H	no.	δ_C	δ_H
1	13.6	0.90	5		4.95	9	36.2	2.57
2	21.0	1.33	6	134.0	6.73	10	69.6	4.07
3	33.1	1.53	7	131.0		11	66.0	3.50
4	48.0	3.19	8	187.2				

[a] Chemical shifts are given in parts per million.

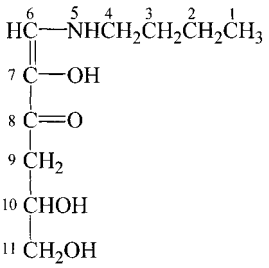

Figure 3. Structure of aminoreductone formed from lactose and butylamine.

and the absorbance at 320 nm was 1.33 times that of the heated lactose-butylamine solution. From this result, the participation of the extracted compound with the absorption maximum at 320 nm in the reduction of XTT can be deduced. The ^{13}C and ^{1}H NMR assignments are shown in Table 1. The signals of the compound with an absorption maximum at 320 nm could be assigned to the aminoreductone, 1-(butylamino)-1,2-dehydro-1,4-dideoxy-3-hexulose, shown in Figure 3, and the result agreed with that reported by Schoetter et al. (1994). From these results, it can be concluded that the aminoreductone formed during the Maillard reaction of lactose was mainly responsible for the reducibility of XTT.

In our previous work, the XTT assay could differentiate UHT-treated milks by the extent of thermal treatment; that is, the more severely thermally treated milk showed the higher XTT reducibility. Also, the XTT reducibility of the UHT-treated milk was reported to gradually decrease, dependent on storage conditions such as the time and temperature (Ukeda et al., 1996). On the basis of the result obtained by this investigation, it can be assumed that the aminoreductone was produced by the thermal treatment and that during the storage the aminoreductone was oxidized by Cu^{2+} or oxygen in milk. Consequently, the decrease in XTT reducibility during storage may be associated with the oxidation of aminoreductone in milk. Further experiments would be needed to confirm this assumption.

Moreover, in dairy products, free amino acids occur only in low amount and most of the amino groups are bound to protein such as a lysine side chain or the N terminus. It is presumed, therefore, that the aminoreductone was formed by the reaction between lactose and the ϵ-amino group of protein-bound lysine or the N terminus. It is well-known that the blockage of the lysine residue causes loss of nutritive value because the modified lysine is no longer available for digestion (Van Boekel, 1998). As described above, the aminoreductone may play a very important role in the quality of milk, and the XTT assay may be considered to be a promising method for evaluating the quality of dairy products.

ACKNOWLEDGMENT

We are grateful to Dr. C.-S. Kim of the Faculty of Agriculture, Kochi University, for the NMR analysis.

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Received for review May 26, 2000. Revised manuscript received August 15, 2000. Accepted August 16, 2000. This study was partially supported by a Grant-in-Aid from The Food Science Institute Foundation, Japan.

JF000653Z