
Maillard Reaction in Milk – Effect of Heat Treatment

Tomoko Shimamura and Hiroyuki Ukeda

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/50079>

1. Introduction

Milk is usually subjected to heat treatment to ensure microbiological safety before retail and consumption. There are three types of heat treatment; (1) low temperature long time (LTLT) pasteurization, (2) high temperature short time (HTST) pasteurization, and (3) ultra-high temperature (UHT) treatment. In all types of heat treatment, the Maillard reaction occurs in milk.

The Maillard reaction (nonenzymatic glycation) is a chemical reaction between amino group and carbonyl group; it is the extremely complex reaction that usually takes place during food processing or storage. In the case of milk, lactose reacts with the free amino acid side chains of milk proteins (mainly ϵ -amino group of lysine residue) to proceed to early, intermediate, and advanced stages of Maillard reaction and forms enormous kinds of Maillard reaction products. The reactions of lactose and milk proteins have been frequently investigated and the formations of various Maillard reaction products in milk during heat treatment have been demonstrated [1]. In the general Maillard reaction, firstly an Amadori product is generated, and it progresses to the 3-deoxyosone or 1-deoxyosone route depending on the reaction pH. In the case of the Maillard reaction of disaccharides such as lactose, there is a third reaction route. It is the 4-deoxyosone route. A main carbohydrate in milk is lactose. Thus, the Maillard reaction in milk progresses via the above described three routes. Finally, the Maillard reaction results in the formation of melanoidins (browning compounds).

2. Effect of the Maillard reaction on milk proteins

The Maillard reaction shows various effects on milk proteins such as bioavailability, solubility, forming property, emulsifying property, and heating stability [1-4]. In addition,

the formation of flavor compounds and browning compounds is caused as the consequences of the Maillard reaction between lactose and milk proteins [1, 5].

As for the effect of the Maillard reaction on the bioavailability of milk proteins, various studies were performed. Generally, in the Maillard reaction in milk, lactose mainly reacts with ϵ -amino group of lysine residue of milk proteins. Thus, the lysine loss by the Maillard reaction increases with a severity of heat treatment. The modified lysine cannot be available as a nutrient any more. For example, steam injection process (direct heating) generated 3.6% (120°C for 400 sec) and 6.8% (130°C for 290 sec) of the blocked lysine in whole milk. The indirect heating at 115°C for 10 to 40 min increased the modified lysine from 11.0 to 13.0% [6]. In addition, it was revealed that the lysine residues in skim milk powder were more susceptible to heating than those in skim milk [7].

Le *et al.* [3] recently suggested that the Maillard reaction was responsible for the solubility loss in milk protein concentrate powder. It was also reported that the glycated β -lactoglobulin was more stable at acidic pH and more stable against heating. The glycation of β -lactoglobulin, moreover, could improve its forming and emulsifying properties [4]. These results suggested the usefulness of the Maillard reaction for enabling milk proteins to have different properties.

3. Monitoring of the Maillard reaction of milk using XTT assay

The Maillard reaction has a lot of effects on the function of milk proteins and sensory property of milk and dairy products as described above. Particularly, in the manufacturing of milk, the excess progress of Maillard reaction and the formation of melanoidins are undesired, because a commercial value of milk is drastically decreased by them. Therefore, the detection of the Maillard reaction products is important for the quality control of milk. So far, several heat-induced markers have been proposed to control and check the heat treatment given to milk and dairy products. For example, furosine, hydroxymethylfurfural (HMF), and lactulose concentrations have been recognized to be the most promising indicators, since these concentrations increase with the heat treatment [1]. In Japan, protein reducing substance value (PRS) obtained by a ferricyanide assay is also widely-used conventional indicator [8]. It is based on the detection of reducing substances such as sulfhydryl group which are generated by heating in the fraction of acid-precipitated milk protein. These method, however, are generally time-consuming and complicated.

We proposed an assay method for determining the ability of milk to reduce 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT: Figure 1) as a method of evaluating the extent of the Maillard reaction [8-11]. The tetrazolium salt XTT is reduced to water-soluble formazan which is suitable for the spectrophotometric measurement. Taking account into an economical view and a rapidity of assay, we tried to develop a microplate assay. The assay conditions were as follows: XTT concentration, 0.5 mM; reaction pH, 7.0; menadione concentration, saturation level (ca. 0.55 mM); reaction temperature, room temperature; volume of XTT solution, 60 μ L; volume of sample solution, 40 μ L; detection wavelength, 492 nm; reference wavelength, 600 nm;

reaction time, 20 min. The procedure of the XTT assay was as follows: the XTT solution was added into each well in a microplate. Afterward, the sample solution was added to the well. After mixing on a microplate shaker at 500 rpm for 15 sec, a difference in the absorbance between 492 and 600 nm was read on a microplate reader as the absorbance at 0 min. After 20 min, the absorbance difference was read again. An increase in the absorbance for 20 min was recorded as the ability of the sample to reduce XTT (XTT reducibility).

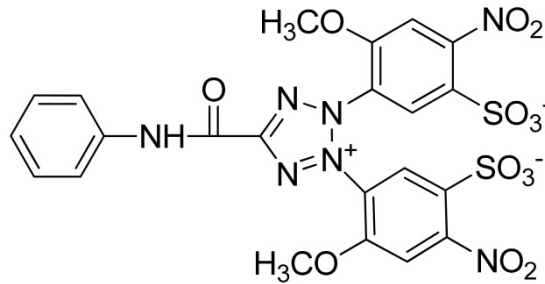


Figure 1. Structure of tetrazolium salt XTT.

Using the XTT assay described above, the XTT reducibility of milk was examined (Figure 2). In this test, an LTLT milk (Milk A: 65°C for 30 min) and two kinds of UHT milk (Milk B: 130°C for 2 sec, Milk C: 140°C for 3 sec) were used. When the milk was mixed with XTT solution, intense orange color which was derived from the XTT formazan was recognized. As shown in Figure 2, Milk C showed the highest XTT reducibility and the order of XTT reducibility was Milk C, B, and A. At the same time, the HMF content in Milk B and C was also determined as the conventional indicator of the Maillard reaction, because it was reported that the HMF content clearly increased with the severity of the heating treatment of milk [12, 13]. As a result, the content of HMF in Milk C (12.6 μM) was higher than that of Milk B (8.95 μM). From these results, it was revealed that the XTT assay could estimate the degree of thermal stress delivered to the milk as well as the HMF value.

In addition, the changes in the XTT reducibility during storage of UHT milks (Milk B and C) were investigated. For purpose of comparison, the PRS of Milk B and C were also examined by the ferricyanide assay [8]. The UHT milks were stored at 4°C and 37°C for about 4 weeks (Figure 3, 4). The XTT reducibility of Milk B and C gradually decreased depending on the storage period and the rate of decrease in the XTT reducibility was clearly larger at higher storage temperature (Figure 3). This result strongly suggested that, when the XTT assay is applied to the milk heated under a given condition, the result can serve to estimate not only the heating condition but also the storage period after heat treatment if the storage temperature is known or if storage period is known.

On the other hand, the PRS of Milk B and C were almost constant at all storage conditions (Figure 4). This result showed that the ferricyanide-reducing substances might be stable unlike the XTT-reducing substance. In addition, we found that the HMF value of Milk C did not significantly change during its storage for 60 days at 5°C [11]. It was in accordance with

the result of Fink and Kessler about HMF [13]. They also reported that the lactulose concentration of UHT milk was constant throughout the storage period for 70 days at room temperature [13]. It could be concluded, therefore, that the XTT assay is applicable for the estimation of storage conditions which was impossible by the conventional method.

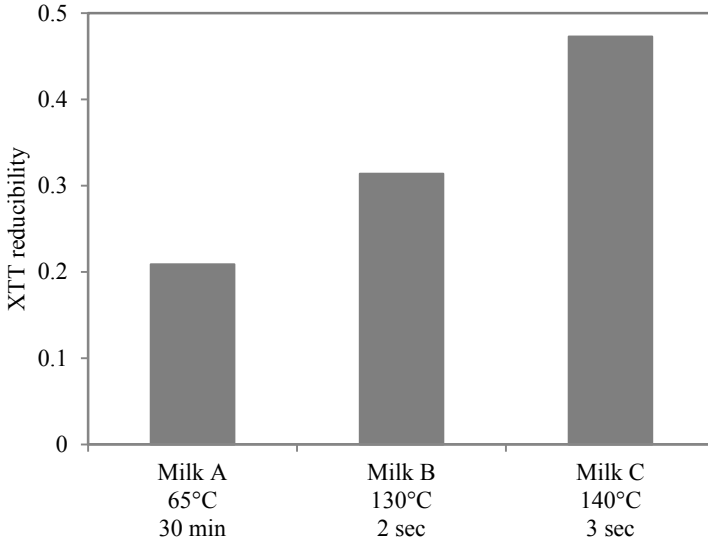
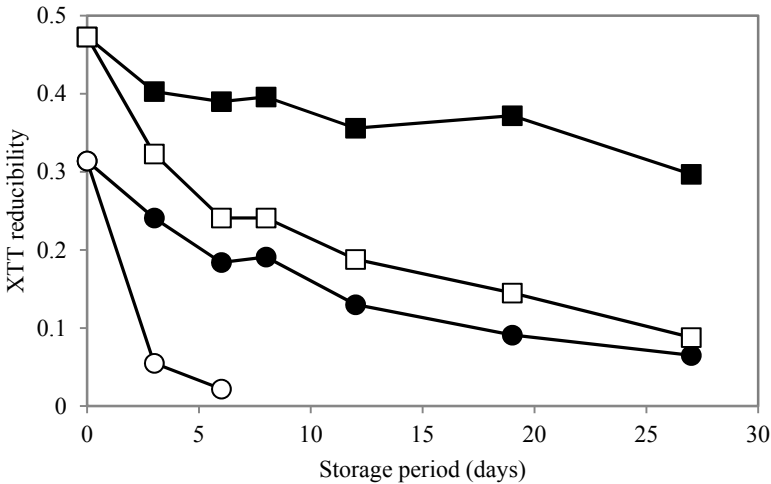
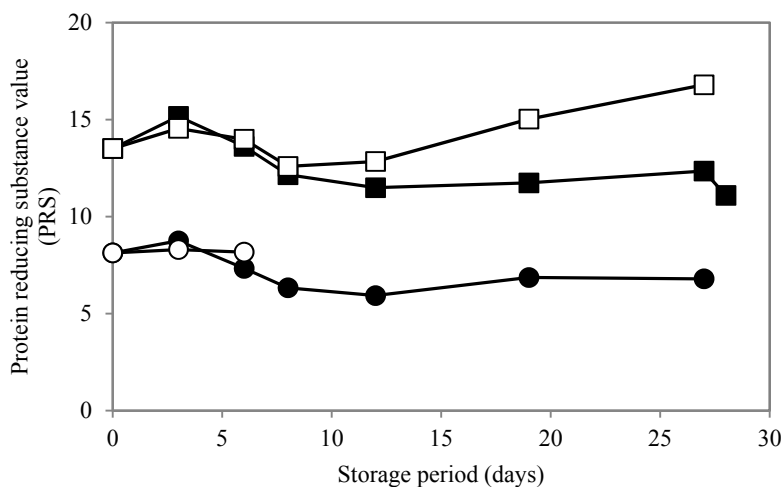


Figure 2. Comparison of XTT reducibility of LTLT milk (Milk A) and UHT milks (Milk B and C).



Milks were stored at 4°C (●, Milk B; ■, Milk C) and 37°C (○, Milk B; □, Milk C).

Figure 3. Changes in XTT reducibility during storage.



Milks were stored at 4°C (●, Milk B; ■, Milk C) and 37°C (○, Milk B; □, Milk C).

Figure 4. Changes in PRS during storage.

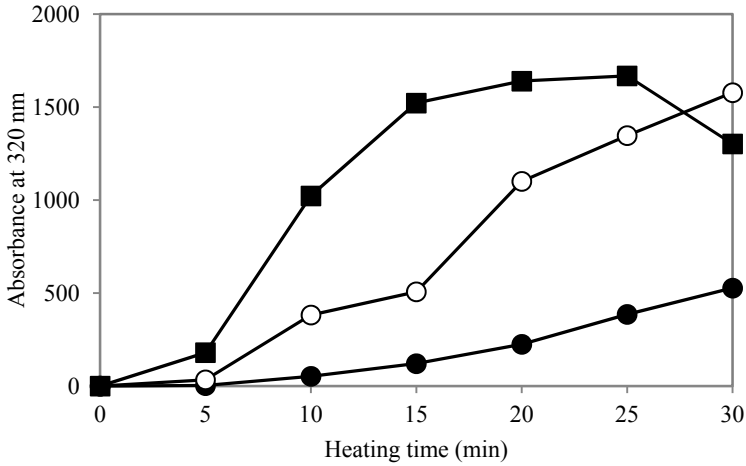
4. Demonstration of the presence of aminoreductone formed during the Maillard reaction in milk

4.1. In the model system of lactose and butylamine [9]

In order to clarify the XTT-reducing substance that is formed during the Maillard reaction in milk, we firstly used a model system consisting of lactose and butylamine, and then performed the spectrophotometric analysis of the heated model solution. The model solution of lactose-butylamine heated at 80-100°C for 0-30 min showed a characteristic UV absorption maximum at 320 nm. During the heating at 80-100°C for 30 min, the changes in the absorbance at 320 nm (Figure 5) and the XTT reducibility (Figure 6) were investigated. As a result, both indices increased gradually in accordance with the rise in temperature and heating time. This result indicated that the compound with the absorption maximum at 320 nm was formed by heating. Moreover, the behavior of increase in absorbance at 320 nm was similar to that of the XTT reducibility. Since the similar trend was recognized in the time course of both indices, the XTT reducibility was plotted against the absorbance at 320 nm. In consequence, a significant relationship between them was recognized with a correlation coefficient of 0.967 ($n = 19$, $p < 0.001$). From these results it was found that the compound with the absorption maximum at 320 nm might be responsible for the reduction of XTT.

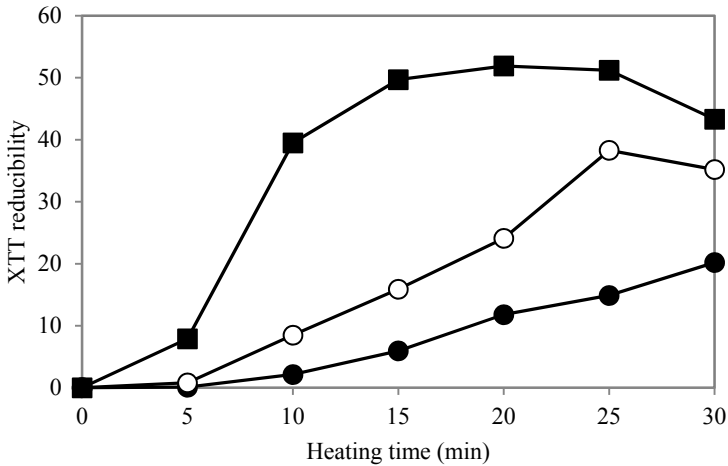
The ^{13}C - and ^1H -NMR analyses of the compound with the absorption maximum at 320 nm which was extracted from lactose-butylamine model solution heated at 100°C for 15 min were performed. The signals of the ^{13}C - and ^1H -NMR could be assigned the compound as the aminoreductone, 1-(butylamino)-1,2-dehydro-1,4-dideoxy 3-hexulose (Figure 7). This compound was reported as the Maillard reaction product formed in the 4-deoxyosone route.

It was also reported as the characteristic compound in the Maillard reaction of disaccharides [14]. In addition, we demonstrate a linear relationship between the XTT reducibility and the amount of aminoreductone which was determined more specifically by HPLC [10]. These results strongly indicated that the aminoreductone formed during Maillard reaction of lactose was mainly responsible for the reduction of XTT.



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 (■).

Figure 5. Effect of heating temperature and time on the absorbance at 320 nm.



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 (■).

Figure 6. Effect of heating temperature and time on the XTT reducibility.

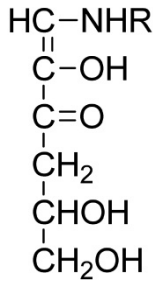


Figure 7. Structure of aminoreductone generated by the Maillard reaction of lactose and butylamine. (R = butyl group)

From these results, we presumed that the aminoreductone is formed by the Maillard reaction between lactose and ϵ -amino groups of milk proteins, and then it is responsible for the reduction of XTT. However, at that time, there was no report to prove the presence of aminoreductone in milk. Thus, we tried to demonstrate it using model system consisting of lactose and milk proteins and UHT milk.

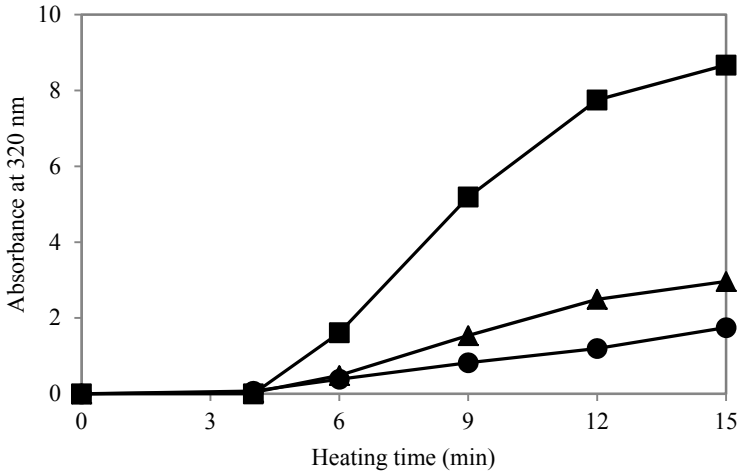
4.2. In the model system of lactose and milk proteins

As a model system of milk, the solution consisting of lactose (4.6%) and casein (2.6%), α -lactalbumin (0.12%), or β -lactoglobulin (0.32%) was used and heated at 130°C for 15 min. After heating, the characteristic absorption maximum or shoulder at 320 nm was recognized. In addition, the changes of the absorbance at 320 nm (Figure 8) and the XTT reducibility (Figure 9) were investigated. In all model systems, the increases in the absorbance at 320 nm and the XTT reducibility depended on the heating time. Because similar tendencies were observed between two indices in all model systems, correlations were examined. Consequently, there were significant linearities as follows: casein ($r = 0.993$, $n = 6$, $p < 0.001$), α -lactalbumin ($r = 0.996$, $n = 6$, $p < 0.001$), and β -lactoglobulin ($r = 0.975$, $n = 6$, $p < 0.001$). From these results, it was suggested that aminoreductone is generated in the Maillard reaction between lactose and milk proteins and it is responsible for the reduction of XTT.

4.3. In milk [15]

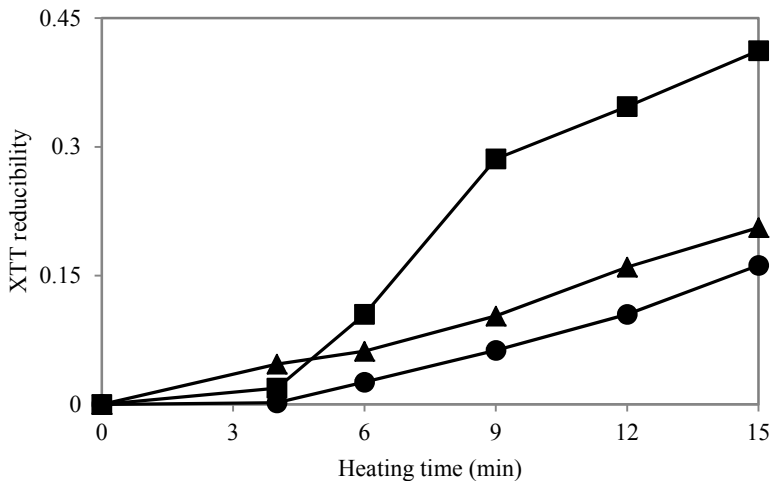
As described above, a possibility of the formation of aminoreductone on the milk proteins during the Maillard reaction with lactose was clearly shown. However, direct demonstration of the presence of aminoreductone in milk had not been accomplished because of a difficulty in isolation of an intact aminoreductone from milk proteins. For instance, aminoreductone is labile and hence not suitable for enzyme hydrolysis and multiple extraction steps. To achieve the practical application of the XTT assay in food industries including dairy products, it was essential to demonstrate the presence of aminoreductone in milk. Because of this background, we attempted to isolate aminoreductone from milk proteins using 2,4-dinitrophenylhydrazine (DNP), a common labeling reagent for the carbonyl group, and Cu^{2+} [16]. A mechanism of derivatization of

aminoreductone in milk is shown in Figure 10. In this derivatization step, Cu^{2+} plays as an oxidizing agent against aminoreductone, and the oxidized aminoreductone (OAR) has two or three carbonyl groups. Finally, it was assumed that two or three carbonyl groups in OAR are derivatized by DNP (OAR-DNP).



Lactose (4.6%) and casein (■: 2.6%), α -lactalbumin (●: 0.12%), or β -lactoglobulin (▲: 0.32%) in 20 mM phosphate buffer (pH 6.7) were heated at 130°C.

Figure 8. Effect of heating time on the absorbance at 320 nm.



Lactose (4.6%) and casein (■: 2.6%), α -lactalbumin (●: 0.12%), or β -lactoglobulin (▲: 0.32%) in 20 mM phosphate buffer (pH 6.7) were heated at 130°C.

Figure 9. Effect of heating time on the XTT reducibility.

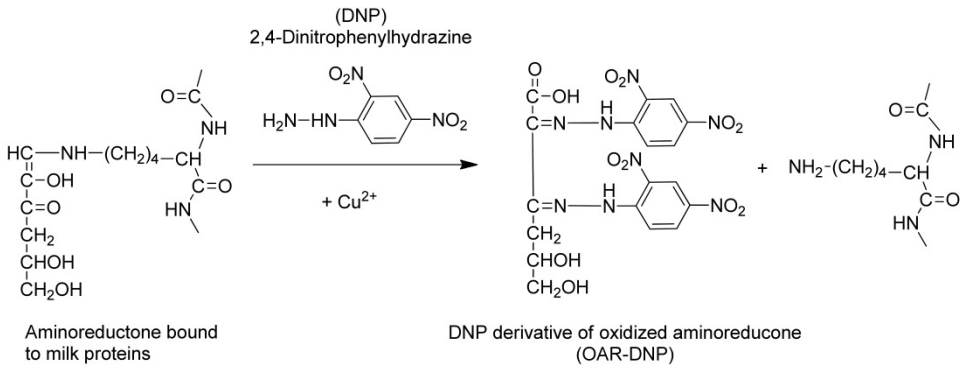


Figure 10. Derivatization mechanism of aminoreductone in milk using DNP and Cu^{2+} .

The derivatization using DNP and Cu^{2+} was applied to aminoreductone in UHT milk (140°C , 3 sec). In this study, the UHT milk was reheated at 130°C for 15 min in order to increase the content of aminoreductone, because the original content of aminoreductone in the commercially available UHT milk was not so high. As a result, the reheating of UHT milk could increase the content of DNP derivative by 40 times. The DNP derivative which was thought to be corresponding to OAR-DNP in the reheated UHT milk was purified by preparative normal-phase HPLC and preparative reversed-phase HPLC. Finally, the purified compound (4.2 mg) was obtained from 980 mL of UHT milk and analyzed by ^{13}C - and ^1H -NMR. The NMR signals of the DNP derivative from UHT milk could be assigned to the structure of OAR-DNP shown in Figure 10. In addition, the NMR signals of DNP derivative from UHT milk were nearly the same as those of the OAR-DNP from lactose-butylamine model system. These results demonstrated that aminoreductone was formed by the Maillard reaction on the milk proteins and present in milk.

Therefore, considering the above, the principle of the present XTT assay can be concluded as follows (Figure 11): (1) Lactose and ϵ -amino groups of lysine residue in milk proteins react non-enzymatically to form the Amadori product by the heating process. (2) Aminoreductone structure is formed on the milk proteins after elimination of galactose moiety from lactose through 4-deoxyosone pathway. (3) Aminoreductone is oxidized by XTT, whereas XTT is simultaneously reduced to the corresponding water-soluble formazan.

It would be thought that the above mentioned steps (1) and (2) progresses depending on the time and temperature of heating process in milk production, so the XTT assay can differentiate the extent of heat treatment of milk. Based on the study using model system of lactose and butylamine, the relationship between aminoreductone concentration and XTT reducibility was examined. As a result, there was a good linearity was recognized ($r = 0.98$) and a regression equation was $y = 0.606x + 0.046$, in which x and y represented the concentration of aminoreductone (mM) and the XTT reducibility [17]. Based on this equation, the concentration of aminoreductone in UHT milk could be estimated as 0.44 mM.

aminoreductone was reported [19]. From these results, it was suggested that the aminoreductone formed by the Maillard reaction would contribute to keep the nutritional value and sensory quality of milk.

Furthermore, aminoreductone showed antimicrobial activity against *Helicobacter pylori* (*H. pylori*). *In vitro* it effectively inhibited the growth of 24 kinds of *H. pylori* strains including antibiotic-resistant strains and had a bactericidal activity [20, 21]. The Killing ability was observed even in acidic condition. In addition, aminoreductone also had the antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) [21, 22]. These results indicated that foods containing aminoreductone, such as milk and dairy products, have a potential health benefits in medical practice.

6. Conclusion

In this chapter, the demonstration of the presence of aminoreductone formed by the Maillard reaction in milk and the specific assay method for aminoreductone were focused and introduced. Since the novel functionality of aminoreductone have come out one after another, the information of aminoreductone obtained by the XTT assay would gain importance in the quality control in milk and dairy products.

Author details

Tomoko Shimamura* and Hiroyuki Ukeda
Faculty of Agriculture, Kochi University, Nankoku, Japan

7. References

- [1] van Boekel M. A. J. S. (1998) Effect of heating on Maillard reactions in milk. *Food Chem.* 62: 403–414.
- [2] Evangelisti F., Calcagno C., Nardi S., Zumin P. (1999) Deterioration of protein fraction by Maillard reaction in dietetic milks. *J. Dairy Res.* 66: 237-243.
- [3] Le T. T., Bhandari B., Deeth H. C. (2011) Chemical and physical changes in milk protein concentrate (MPC80) powder during storage. *J. Agric. Food Chem.* 59: 5465-5473.
- [4] Augustin M. A., Udabage P. (2007) Influence of processing on functionality of milk and dairy proteins. In: Taylor S. L., editor. *Advances in Food and Nutrition Research*. Amsterdam: Elsevier. pp. 1-38.
- [5] Freedman M. (1996) Food browning and its prevention: an overview. *J. Agric. Food Chem.* 44: 631-653.
- [6] Finot P. A., Deutsch R., Bujard E. (1981) The extent of the Maillard reaction during the processing of milk. *Prog. Fd. Nutr. Sci.* 5: 345-355.
- [7] Henle T., Walter H., Klostermeyer H. (1991) Evaluation of the extent of the early Maillard-reaction in milk products by direct measurement of the Amadori-product lactuloselysine. *Z. Lebensm. Unters. Forsch.* 193: 199-122.

* Corresponding Author

- [8] Shimamura T., Ukeda H., Sawamura M. (2001) Quality evaluation of milk using XTT assay –comparison with ferricyanide assay. *Nippon Shokuhin Kagaku Kogaku Kaishi* (in Japanese) 48: 840-843.
- [9] Shimamura T., Ukeda H., Sawamura M. (2000) Reduction of tetrazolium salt XTT by aminoreductone formed during the Maillard reaction of lactose. *J. Agric. Food Chem.* 48: 6227-6229.
- [10] Shimamura T., Ukeda H., Sawamura M. (2004) Relationship between the XTT reducibility and aminoreductone formed during the Maillard reaction of lactose: the detection of aminoreductone by HPLC. *Food Sci. Technol. Res.* 10: 6-9.
- [11] Ukeda H., Goto Y., Sawamura M., Kusunose H., Kamikado H., Kamei T. (1995) Reduction of tetrazolium salt XTT with UHT-treated milk: its relationship with the extent of heat treatment and storage conditions. *Food Sci. Technol. Int.* 1: 52-57.
- [12] Kessler H. G., Fink R. (1986) Changes in heated milk and stored milk an interpretation by reaction kinetics. *J. Food Sci.* 51: 1105-1111.
- [13] Fink R., Kessler H. G. (1988) Comparison of methods for distinguishing UHT treatment and sterilization of milk. *Milchwissenschaft* 43: 275-280.
- [14] Pischetsrieder M., Schoetter C., Severin T. (1998) Formation of an aminoreductone during the Maillard reaction of lactose and *N*^α-acetyllysine or proteins. *J. Agric. Food Chem.* 46: 928-931.
- [15] Shimamura T., Kurogi Y., Katsuno S., Kashiwagi T., Ukeda H. (2011) Demonstration of the presence of aminoreductone formed during the Maillard reaction in milk. *Food Chem.* 129: 1088-1092.
- [16] Shinohara K., Fukumoto Y., Tseng Y.-K., Inoue Y., Omura H. (1974) Action of amino reductones on nucleic acids. *Nippon Nogei Kagaku Kaishi* (in Japanese) 48: 499-506.
- [17] Trang V. T., Kurogi Y., Katsuno S., Shimamura T., Ukeda H. (2008) Protective effect of on the photo-degradation of riboflavin. *Int. Dairy J.* 18: 344-348.
- [18] Ukeda H., Shimamura T., Hosokawa T., Goto Y., Sawamura M. (1998) Monitoring of the Maillard reaction based on the reduction of tetrazolium salt XTT. *Food Sci. Technol. Int. Tokyo* 4: 258-263.
- [19] Pischetsrieder M., Rinaldi F., Gross U., Severin T. (1998) Assessment of the antioxidative and prooxidative activities of two aminoreductones formed during the Maillard reaction: effects on the oxidation of β -carotene, *N*^α-acetylhistidine, and *cis*-alkenes. *J. Agric. Food Chem.* 46: 2945-2950.
- [20] Trang V. T., Takeuchi H., Kudo H., Aoki A., Katsuno S., Shimamura T., Sugiura T., Ukeda H. (2009) Antimicrobial activity of aminoreductone against *Helicobacter pylori*. *J. Agric. Food Chem.* 57: 11343-11348.
- [21] Takeuchi H., Shimamura T., Kudo H., Ukeda H., Sugiura T. (2012) Antimicrobial activity of natural products and food components. *Current Research in Agriculture and Food Chemistry* 1: 1-12.
- [22] Trang V. T., Takeuchi H., Kudo H., Katsuno S., Shimamura T., Kashiwagi T., Son V. H., Sugiura T., Ukeda H. (2011) *In vitro* antimicrobial activity of aminoreductone against the pathogenic bacteria methicillin-resistant *Staphylococcus aureus* (MRSA). *J. Agric. Food Chem.* 59: 8953-8960.