Galactose Is the Limiting Factor for the Browning or Discoloration of Cheese during Storage

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Summary The browning or discoloration of cheese is often observed during long-time ripening or aging. In the present study, we identified galactose as a limiting factor for the browning, and clarified the involvement of the Maillard reaction for the discoloration. A precursor of browning of Cheddar cheese was isolated by procedures of solvent extraction and chromatography. D-Galactose and d-lactose were identified as a precursor of browning of Cheddar cheese A and B, respectively. Cheddar cheese (A, B, and C), sugar-added cheese, and nine kinds of retail cheese were stored at 4 to 70°C for 0 to 10 d, before the L*-values, a*-values, and b*-values and sugar contents of each sample were measured. Cheese to which galactose was added turned brown more intensively during storage than the non-added control and the other sugar-added cheese. The more galactose was added, the more intensive the browning of the cheese appeared. The decrease in galactose correlated with the ΔL*-values, Δa*-values, Δb*-values, and ΔE-values indicating the browning or discoloration of cheese samples. The decrease in sugars of nine kinds of retail cheese during storage also correlated with the ΔL*-values, Δa*-values, and ΔE-values of these cheese samples. These results clearly indicate that sugars, especially galactose, in cheese are an important factor for the browning of cheese during storage. In general, a high amount of amino acids, peptides, and proteins exists in ripe or mature cheese. Therefore, sugars, especially galactose, were considered to be the limiting factor for the Maillard reaction causing the browning of ripe or mature cheese during storage.

Key Words cheese, browning, Maillard reaction, galactose, storage

Cheese is high in calories, and is also rich in protein, fat, vitamins, and calcium. Cheese is a popular food because of its excellent preservability and palatability. One of the reasons for its high palatability is proteolysis during cheese ripening.

Proteolysis by starter bacteria and rennet during cheese ripening results in the increase in amino acids. In particular, glutamic acid is one of the most abundant amino acids in ripened cheese (1–7) and is important for the umami taste of cheese (8). In addition to taste, the characteristic cheese flavor and texture are produced during ripening. For the development of acceptable Cheddar cheese flavor, a well-balanced breakdown of the curd protein, casein, into small peptides and amino acids is necessary (9). These products of proteolysis contribute directly to flavor (10) or act as precursors of flavor compounds. Furthermore, the texture of cheese depends on the cheese composition and the extent of biochemical changes during ripening (9).

On the other hand, the browning or discoloration of cheese is often observed during long-time ripening or aging. For example, pink discoloration has been reported in ripened cheese varieties such as Cheddar, Swiss, Grana and Italian types (11–14). Major studies related to pink discoloration have been focused on cheese starter cultures, particularly thermophilic lactobacilli (15). Martley and Michel (13) suggested that pink discoloration in Cheddar cheese is associated with three factors: the presence of galactose in cheese, formation of low molecular weight nitrogen compounds due to proteolysis, and the concentration of oxygen in the cheese bag. Paramita and Broome (16) suggested that α-dicarbonyl compounds produced by lactic acid bacteria cause pink discoloration by the Maillard reaction at low temperature in Romano-type cheese. The Maillard reaction is the non-enzymatic browning that occurs between the carbonyl group of reducing sugars and the amino group of free amino acids, peptides, or proteins. It is known that the Maillard reaction plays an important role in the formation of the brown color and flavor of various heated or stored foods. However, the relationship between pink color development in cheese and the Maillard reaction is chemically unclear.

Brown discoloration of cheese is often observed when it is cooked at high temperature and stored for long time at room temperature or low temperature. As for the Cheddar cheese, Wang and Sun (17) demonstrated a linear relationship between browning intensity and baking temperature from 70 to 130°C. Matzdorf and Cuppett Hutkins (18) observed the increases in baking temperature and baking period accompanied with the decrease in L*-values (a measure of lightness), Spanneberg et al. (19) showed that the decrease of methyl-
glyoxal, the Maillard reaction intermediate, was highly correlated with the increase of $N\varepsilon$-carboxylethyllysine, the advanced glycation endproduct, during storage of Harzer cheese or acid curd cheese. The authors said that methylglyoxal and $N\varepsilon$-carboxylethyllysine could be markers of the degree of maturation. Divine et al. (20) proposed that the condensation of aminoacetone derived from methylglyoxal causes browning at low temperature in Parmesan cheese.

In this way, studies on the discoloration of cheese during storage strongly suggested that the Maillard reaction contributes to the browning of cheese. However, there is no report to indicate direct chemical evidence for the relationship between the browning of cheese and the Maillard reaction. Further, although we often observe definite differences in susceptibility to the browning during storage among Cheddar cheese samples, we don’t know the reason for the difference. The objectives of this study were to identify a limiting factor for the discoloration or browning of cheese during storage and to clarify the relationship between the browning of cheese and the Maillard reaction.

**MATERIALS AND METHODS**

*Materials.* Cheddar cheese samples (A, B, and C) were obtained from a Japanese food maker from 2012 to 2014 (Natori Co., Tokyo, Japan). Two Camembert cheeses, two Gouda cheese, two Mozzarella cheeses, and three processed cheese were purchased at a local market in Tokyo in 2016.

*Addition of sugars to cheese.* Cheddar cheese B (10 g) was chopped and heated at 70˚C for 10 min. One milliliter of 100 mg/mL $D$-galactose, $D$-glucose, or $D$-lactose solution, or 10, 20 or 50 mg/mL $D$-galactose solution was, respectively, added to it, and stirred. As a control, 1 mL of water was added to the heated cheese.

*Storage of cheese.* Cheddar cheese (A, B, and C), the sugar-added cheese, and nine kinds of retail cheese (10 g) were sealed in food grade plastic films (Hiryu N-4, Asahi Kasei Pax Co., Tokyo, Japan) using a heat sealer (SQ-303, Asahi Kasei Pax). The crusts of Camembert cheese were removed before packing. They were stored at 4, 20, 30, 40, 50, 60, and 70˚C for 0 to 10 d. The $L^\ast$, $a^\ast$, and $b^\ast$-values of each sample were measured with a color meter (NF333, Nippon Denshoku Industries, Tokyo). Samples were tested in triplicate. The $\Delta L^\ast$, $\Delta a^\ast$, $\Delta b^\ast$, and $\Delta E$-values of each sample between before and after storage were calculated. $\Delta E$-value, the color difference, was calculated by the following equation:

$$\Delta E = (\Delta L^\ast)^2 + (\Delta a^\ast)^2 + (\Delta b^\ast)^2)^{1/2}$$

*Extraction of cheese with solvents to search for a precursor of browning.* Cheddar cheese A (20 g) was chopped before being soaked in hexane (20 mL) for 10 min with occasional shaking. After filtration, the hexane extract was obtained. This procedure was further repeated twice. The three hexane extracts were combined. The residue after hexane extraction was similarly extracted with ethyl acetate (20 mL) three times. The three ethyl acetate extracts were combined. The residue after ethyl acetate was further extracted with ethanol (20 mL) three times. The three ethanol extracts were combined. The residue after ethanol extraction was soaked in methanol (20 mL), sonicated for 30 min, and filtered. This procedure was repeated a further two times using 20 mL of methanol. The three methanol extracts were combined (Methanol ext. I, Fig. 1A). The extracts and the residues were stored at 70˚C for 3 d and for 7 d, respectively. Cheddar cheese A, B, and C were also similarly fractionated using hexane, ethyl acetate, and methanol (Methanol ext. II) without ethanol. Three kinds of Methanol ext. II of Cheddar cheese A, B, and C were stored at 70˚C for 3 d.

*Isolation of a precursor of browning from Cheddar cheese A.* Methanol ext. I obtained as described above from Cheddar cheese A (200 g) was concentrated in vacuo and dissolved in 400 mL water. This extract was applied to a column of ODS (300 mL, Chromatorex, Fuji Silysia Chemical, Kasugai, Japan). Non-adsorbed fractions were
collected and applied to a column of cation exchange resin (250 mL; Amberlite IR120B [H⁺], Organo, Tokyo). Non-adsorbed fractions were collected and concentrated to 1 mL in vacuo, to which 60 mL acetonitrile was added. As a precipitate was formed, the supernatant was obtained by centrifugation at 1,600 × g for 10 min at 4°C and concentrated in vacuo. The obtained paste (ca. 100 mg) was then applied to a column of silica gel (i.d. 14 mm × 100 mm; silica gel 60, Merck KGaA, Darmstadt, Germany), which was developed with 1-butanol : acetic acid : water (12 : 1 : 1, v/v/v). Fractions containing an imperceptible compound were collected and concentrated in vacuo, before white powder (3 mg) was obtained. During these procedures, each fraction was monitored with thin layer chromatography (TLC). The obtained powder was analyzed using NMR (AVANCE III 600, Bruker Biospin, Karlsruhe, Germany). TLC, and high-performance liquid chromatography (HPLC).

TLC. TLC conditions were as follows: TLC phase, silica gel F254 (Merck KGaA); mobile phase, 1-butanol : acetic acid : water (8 : 3 : 1, v/v/v); detection reagent, 10% sulfuric acid. The Rf values of D-galactose, D-glucose, and D-lactose were 0.3, 0.4, and 0.1, respectively.

HPLC. HPLC conditions were as follows: column, HILICpak VG-50 4E (i.d. 4.6 mm × 250 mm, Showa Denko, Tokyo) equipped with a guard column HILICpak VG-50G 4A (i.d. 4.6 mm × 10 mm, Showa Denko); eluent, acetonitrile : methanol : water (85 : 5 : 10, v/v/v); flow rate, 1 mL/min; column temp., 60°C; detector, Alltech 3300 ELSD (Alltech, Grace, IL). HPLC analyses were performed in duplicate for all samples. The retention times of D-galactose, D-glucose, and D-lactose were 9, 10, and 18 min, respectively.

Search for precursors of browning from three Cheddar cheese samples. Methanol ext. II obtained from Cheddar cheese (A, B, and C) and the stored ones at 70°C for 3 d were subjected to TLC. Spots with Rf values of 0.3 of Cheddar cheese A and 0.1 of Cheddar cheese B were scraped from TLC and extracted by methanol. These extracts were compared with an authentic sample of D-galactose, D-glucose, and D-lactose using TLC and HPLC.

Extraction and measurement of sugars. Sugars were determined by the internal standard method for HPLC analysis according to Mullin and Emmon (21) with some modifications. Cheese was cut into small cubes. Fifty milliliters of 0.5 mg/mL xylose (an internal standard) solution was added to 5 g of cheese, which was homogenized for 5 min. The homogeneous mixture was stirred at 50°C for 15 min, before being centrifuged at 700 × g for 5 min at 4°C. The supernatant was filtered (Advantec No. 2, Tokyo), before the filtrate was concentrated to about 5 mL. The concentrate was further filtered with Advantec No. 5C filter paper and Chromatodisc 25A (0.45 μm, Kurabo, Osaka, Japan). One milliliter of the filtrate was loaded on a cation exchanger cartridge (Bond Elut Jr SCX, Agilent Technologies, Santa Clara, CA). After neutralization of the non-absorbed fraction using 0.1 M NaOH, 1 mL of acetonitrile was added to 1 mL of it, which was subjected to an HPLC device equipped with an evaporative light-scattering detector.

Visual estimation of cheese samples. Samples of Cheddar cheese A, B, and C (ca. 10 g) were packed in food grade plastic films and stored at 70°C for 0, 1, 3, and 8 d. These samples were equilibrated at room temperature for 1 h before the evaluation. Differences in the color between the stored cheese samples and non-stored samples were visually evaluated with a five-point scale (1 = imperceptible and 5 = intense) using 10 panel members (20 to 30 y old) according to the method of Ramos et al. (22).

Statistical analysis. The results of visual estimations were applied to statistical analysis. Statistical analyses were performed using Excel 2010 (Microsoft, Redmond, WA) with the add-in software Statcel 4 (OMS, Tokorozawa, Japan). Differences among cheese samples were analyzed by one-way ANOVA, followed by the Tukey-Kramer multiple comparison test. The significance level was set at p < 0.01.

RESULTS AND DISCUSSION

Effects of storage temperature on the browning of Cheddar cheese

First, the effects of storage temperature on the browning of cheese were examined using Cheddar cheese, which is used for processed cheese or other cheese products. Cheddar cheese A packed in a plastic film was stored at various temperatures for 10 d. The L*, a*, and b*-values of cheese samples were measured, before the ΔL*, Δa*, Δb*, and ΔE-values, the color difference, were calculated. Although the cheese samples turned brown gradually, the ΔE-value of samples didn’t correspond to the visual observation, which might be caused by the change to physical properties of cheese at various temperatures. Therefore, the browning or discoloration was evaluated using the L* value. At 70°C, the L*-value decreased linearly with incubation time, but at low temperatures such as 4°C, the L*-value was nearly constant (Fig. 2A). The a* and b*-values changed more greatly and widely at 50, 60 and 70°C than at 4°C (Fig. 2B). The higher the storage temperature, the larger the decrease rate of ΔL*-values. The Arrhenius plot (Fig. 2C), for which the ΔL*-values of cheese samples during storage were used, showed that the browning rate was dependent on the storage temperature and that the apparent activation energy, E_a, was 56 (kJ·mol⁻¹·K⁻¹).

Identification of a precursor of browning of Cheddar cheese A

Then we tried to isolate a precursor of browning of Cheddar cheese using solvent extraction. Here we used Cheddar cheese A, because it turned brown more rapidly than other samples. Cheddar cheese A was extracted with hexane, ethyl acetate, ethanol, and methanol, successively. Each extract and residue was stored at 70°C. As a result, each residue except for the residues after extraction with ethanol and methanol turned brown as much as the non-washed control (Fig. 1A). However, the residue after being washed with methanol hardly turned brown, while Methanol ext. I extract did. These results
suggest that Methanol ext. I contains an important pre-
cursor of the browning. We further analyzed Methanol 
ext. I of Cheddar cheese A before and after storage for 3 d 
at 70˚C with TLC. As a result, a spot (Rf 0.3) of the 
extract detected with the sulfuric acid spray method dis-
appeared after storage for 3 d (Fig. 1B). When the same 
plate was analyzed with the ninhydrin reagent, vari-
ous kinds of dense spots of amino acids appeared, but 
no differences between the spots before and after stor-
age were detected (data not shown). The spot detected 
with the sulfuric acid spray method and disappearing 
with storage was then purified using columns of ODS, 
on exchange resin, and silica gel. The NMR spectrum 
of the isolated spot showed that the isolate seemed to 
be a mixture of \( \text{D-galactose} \) (13C-NMR \( \text{in D}_2\text{O: 61.6 (CH}_2\text{), 61.8 (CH}_2\text{), 65.9 (CH), 69.4 (CH), 69.8 (CH), 69.9 (CH), 71.1 (C), 72.5 (CH), 73.4 (CH), 75.8 (C), 92.9 (CH), 97.1 (CH).}\)

Then we compared an authentic sample of \( \text{D-galactose} \) with the isolate. As a 
result, the Rf value of TLC and the retention time of 
HPLC of the isolate coincided completely with those 
of \( \text{D-galactose} \) (Rf 0.3, retention time 9 min). These 
results indicate that galactose is an important precursor 
of the browning of cheese.

Differences in susceptibility to browning among Cheddar 
cheese samples

We often observe differences in the browning rate 
during storage among cheese samples. Here we compared 
three kinds of Cheddar cheese (A, B, and C), \( \text{D-galactose} \) being isolated from Cheddar cheese A. Appearances of Cheddar cheese A, B, and C stored at 70˚C for 0 to 8 d and their browning scores evaluated visually are shown in Fig. 3. Cheddar cheese A and B turned brown more strongly and faster than Cheddar cheese C.

Then we compared the methanol extracts (Methanol 
ext. II) of Cheddar cheese A, B, and C using TLC. Methanol 
ext. II of Cheddar cheese B showed a different spot 
on TLC from that of Cheddar cheese A. Both spots dis-
appeared after the extracts were stored for 3 d at 70˚C. 
Methanol ext. II of Cheddar cheese C before and after 
storage did not show any spots on TLC, and sugars 
were hardly detected in the extract using HPLC. Next we 
identified the disappearing spot of Methanol ext. II of 
Cheddar cheese B by comparing it with authentic sug-
ars using TLC and HPLC. As a result, the Rf value and
the retention time of the disappearing spot of Cheddar cheese B corresponded completely with those of D-lactose (Rf=0.1, retention time=18 min).

Effects of added sugars on the browning of cheese

The results described above strongly suggest that sugar is a limiting factor for the browning of cheese. Then the effects of the added sugars in cheese on the browning were examined. Here we used Cheddar cheese B containing 6.3 mg/g of lactose, and cheese samples were stored at 40°C to reduce the effect of lactose existing originally on the browning. As shown in Fig. 4, the cheese to which 10 mg/g of D-galactose or D-glucose was added turned brown more intensively during storage than the non-added control. The contribution of galactose to the browning was larger than that of glucose. The addition of 10 mg/g of lactose showed no significant effect on the browning of cheese. Next the dosal effect of galactose on the browning was examined. As shown in Fig. 5, the added amount of galactose correlated strongly with the ΔL*-value (R=-0.985). The strong correlations between the added amount of D-galactose and the Δa*-value (R=0.992), Δb*-value...
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(R=0.981), and ΔE-value (R=0.996) were also apparent. The more galactose was added, the more intensive the browning of the cheese appeared. Bley et al. (23) also reported that the brown color intensity of processed cheese correlated with the galactose concentration in natural cheese, and that most of the galactose was produced by the metabolism of the nongalactose-fermenting strain of \textit{Streptococcus thermophilus}. The utilization of lactose and galactose by various kinds of lactic acid bacteria during cheese making and storage needs to be examined.

As described above, we showed that galactose was the most important factor in the browning of Cheddar cheese during storage. On the other hand, cheese in general contains lactose or glucose in addition to galactose. These sugars seem to be involved in the Maillard reaction and contribute to the browning during storage, although the reaction rate of galactose is faster than those of lactose and glucose. So, we examined the decrease of total sugars in Cheddar cheese containing lactose to which galactose was added was then examined during storage. As shown in Fig. 6A, cheese turned brown more intensively with longer storage times, while total sugars, i.e. galactose and lactose, decreased in parallel longer storage times. The correlation between the amount of decrease in total sugars during storage and the browning was then examined. The decrease in total sugars between cheese samples before and after storage correlated strongly with the L*-value (R=0.967), a*-value (R=0.990, Fig. 6B), and E-value (R=0.988, Fig. 6D). These results strongly suggest that the amount of sugars, especially D-galactose, is an important factor for the browning of cheese.

Relationships between the browning and decrease in sugars of various cheese samples

We showed that the amount of sugars, especially D-galactose, was an important factor for the browning of cheese using Cheddar cheese. Next we examined if this result was the case for other kinds of cheese besides Cheddar. Nine kinds of retail cheese containing different amounts of galactose, glucose, and lactose were stored at 70˚C for 0 and 3 d, before the L*- and a*-values and sugars (galactose, glucose, and lactose) were measured (n=3).

Fig. 7. Decreases in total sugars (galactose, glucose, and lactose) and ΔL*-values of various cheese samples during storage (A), and correlation between decrease in total sugars and Δa*-value (B). Nine kinds of retail cheese (two Camembert cheeses, two Gouda cheeses, two Mozzarella cheeses, and three processed cheeses) were stored at 70˚C for 0 and 3 d, before the L*- and a*-values and sugars (galactose, glucose, and lactose) were measured (n=3).

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