Short communication: Reducing agents attenuate methylglyoxal-based browning in Parmesan cheese

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ABSTRACT

The microbial production of methylglyoxal in cheese has been linked to the formation of brown pigmentation and distinctive volatiles. This study investigated methods for preventing methylglyoxal-induced browning in Parmesan cheese through the addition of reducing agents. Cheeses were treated with the reducing agents sodium bisulfite, glutathione, and erythorbate at 2:1 and 4:1 molar ratios with added methylglyoxal, and then incubated at 10°C. Colorimetric methods were used to determine degree of browning at 0, 3, and 6 d. Sodium bisulfite and glutathione inhibited the browning reactions of methylglyoxal compared with the control. Erythorbate was much less effective than the other compounds at inhibiting browning, yet was significantly less browned than the control. These reducing agents are thought to act as strong nucleophiles that can form thiohemiketals and thioketals at the carbonyl carbons of methylglyoxal.

Key words: reducing agent, low temperature browning, methylglyoxal, Parmesan cheese

Short Communication

The development of brown pigmentation and concomitant flavors during cheese ripening has been attributed to the formation of methylglyoxal, an α -dicarbonyl resulting from microbial metabolism (McDonald, 1992; Divine et al., 2012). Such browning has significant detrimental effects on the flavor and appearance of cheeses so affected, potentially reducing their economic value. Because low-temperature, methylglyoxal-based browning is different from typical Maillard reactions that proceed slowly below 80°C, it presents a unique issue for cheese manufacturers seeking to control such activity (Divine et al., 2012).

Methylglyoxal is most commonly formed as an intermediate in the methylglyoxal pathway, a bypass of more

common glycolytic pathways (Figure 1; Booth et al., 2003). Other routes for methylglyoxal formation may involve pathways for acetone metabolism and amino acid breakdown (Kalapos, 1994). The methylglyoxal pathway can occur in prokaryotes as an alternative to the Embden-Meyerhof-Parnas (EMP) pathway. Unlike the EMP pathway, however, the methylglyoxal pathway does not produce any energy in the form of ATP and is thought to be a mode of recycling phosphate (Russell, 1998; Booth et al., 2003). In glycolysis, fructose-1,6-diphosphate is broken down by an aldolase to form dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). In the EMP pathway, triose phosphate isomerase will convert the DHAP into GAP, and the 2 newly formed GAP equivalents will continue to be catabolized to form pyruvate. In contrast, the methylglyoxal pathway employs methylglyoxal synthase to catalyze the formation of methylglyoxal from DHAP. From there, methylglyoxal is metabolized to other intermediates via the glyoxalase system and is eventually converted to pyruvate. The glyoxalase system is thought to exist in many organisms as a means of protection against methylglyoxal's ability to interact with and degrade critical functional proteins in the cell (Booth et al., 2003). Starter cultures typically used in cheese production (e.g., *Lactobacillus helveticus*) were shown to produce 0.2 μ g of methylglyoxal per mL of de Man, Rogosa, and Sharpe medium modified to simulate cheese during early ripening (McDonald, 1992).

Excess methylglyoxal intermediates may react with amine groups, forming advanced glycation end-products and a brown pigmentation similar to that produced by the Maillard reaction (Figure 1; Divine et al., 2012). Methylglyoxal has specifically been shown to react with proteins (e.g., casein) and free amino acids (Kalapos, 1994). This type of protein-diketone reaction will create brown pigmentation and characteristic volatiles, such as alkylpyrazines (McDonald, 1992; Divine et al., 2012). In Parmesan cheese, researchers found that the concentration of methylglyoxal was reduced as cheese browned during aging and thus hypothesized that methylglyoxal had reacted with free amino acids in the cheese to form brown pigmentation (McDonald, 1992).

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Figure 1. Methylglyoxal formation and metabolism (Russell, 1998; Silva et al., 2005).

The research also suggested that a concentration of 2 to 3.5 μg of methylglyoxal/g of cheese (after pressing) was necessary for the formation of brown pigmentation during aging (McDonald, 1992).

Proposed methods to prevent methylglyoxal-induced browning of Parmesan cheese potentially include choosing proper starter cultures and reducing contamination from bacteria capable of contributing to methylglyoxal generation (McDonald, 1992). Although many commonly used starter cultures (e.g., *Lb. helveticus*) may produce methylglyoxal, such production would most likely not yield levels sufficient to cause browning. It is instead thought that contamination of cheese during processing with wild-type lactic acid bacteria is responsible for the higher concentrations of methylglyoxal. This is consistent with the observation that less browning occurred in brine-salted cheese than direct-salted cheese and that more diacetyl (another α -dicarbonyl similar to methylglyoxal) is produced during aerobic conditions (McDonald, 1992). Because consistent color and flavor is critical to the successful manufacture of cheese varieties, there is a need to establish a means of controlling methylglyoxal-based browning reactions.

One such approach for the prevention of methylglyoxal-mediated browning would involve the addition of chemical reducing agents to directly prevent methylglyoxal from interacting with amine groups. Sulfites and sulfur-containing amino acids have been shown to prevent nonenzymatic browning in model sugar-protein systems (Molnar-Perl and Friedman, 1990; Friedman, 1996). Additionally, sulfhydryl groups are effective as antibrowning agents because of their reducing and free radical scavenging capacities as strong nucleophiles (Friedman, 1996). Many sulfur-containing compounds are Food and Drug Administration-approved and generally regarded as safe (GRAS), such as the salt sodium bisulfite and the tripeptide glutathione. Other common antioxidants, such as erythorbic acid, show functionality in preventing other types of browning reactions. Figure 2 shows potential chemical reactions of methylglyoxal with either protein or the antibrowning compounds. These mechanisms are modeled after similar pathways in the literature but substitute the aldehyde group of a reducing sugar with that of methylglyoxal (Friedman and Molnar-Perl, 1990; Isaac et al., 2006). In typical Maillard browning, the amine functional group of protein will condense with the aldehyde of reducing sugars or other substrates, forming N-substituted glycosylamines that may eventually form advanced glycation end-products (Isaac et al., 2006). The antibrowning agents used in the current study can prevent this reaction by modifying the aldehyde of methylglyoxal before it can react with protein. Due to the high reducing potential of sulfur, we expect that the sulfur-containing compounds will act as nucleophiles and donate electrons to methylglyoxal's aldehyde carbon (Friedman, 1996). The resulting sp^3 -hybridized carbon can no longer act as a strong electrophile and thus would not condense with the amino groups of protein. The mechanism of erythorbic acid, which will be in the form of erythorbate due to its low acid dissociation constant (pK_a) of 2.1, is expected to be similar to that of its stereoisomer, ascorbic acid. Because the reaction is reversible, however, it may not be as effective as the sulfur-containing compounds. In addition, its stereoisomer, ascorbic acid, has been shown to brown itself in similar nonenzymatic pathways; it is therefore possible that erythorbate will also brown (Jackson et al., 1960).

Given the complexities of these browning reactions and the various chemical mechanisms potentially affected or altered by such reducing agents, we hypothesize that these reducing agents will act to prevent or inhibit methylglyoxal-mediated browning in cheese.

Three Parmesan cheeses were acquired from various suppliers to represent standard cheeses commonly available at the supermarket. Cheese samples were ground in a blender (Vita-Mix, Cleveland, OH) on high for 20 s.

Figure 2. Proposed pathways of methylglyoxal deactivation for sulfite ion, glutathione, and erythorbic acid (Friedman and Molnar-Perl, 1990; Isaac et al., 2006).

The ground cheeses (2.0-g samples) were divided among treatment conditions into 8-mL glass vials with Teflon septa and plastic screw caps (Supelco, Bellefonte, PA). Each vial was treated with 8.6 μL of a 40% aqueous methylglyoxal solution (Sigma-Aldrich, St. Louis, MO) equivalent to 2.0 mg of methylglyoxal/g of cheese. Higher concentrations of methylglyoxal than typical biological levels were used to accelerate the reaction. Based on the potential stoichiometry of the proposed reactions with methylglyoxal (2 carbonyl groups), antibrowning compounds were added at a molar ratio of either 2:1 or 4:1 (treatment compound to methylglyoxal); this corresponds to approximately 11.5 and 23 mg of NaHSO_3 (Fisher Scientific, Fair Lawn, NJ), 34.1 and 68.2 mg of glutathione (Sigma-Aldrich), or 19.6 and 39.2 mg of D-(–)-isoascorbic acid (Sigma-Aldrich), respectively. One negative control treatment had no methylglyoxal or antibrowning compounds added (hereafter, the control treatment) and another positive control treatment had methylglyoxal but lacked any anti-browning agent (hereafter, the methylglyoxal treatment). Vials were sealed and incubated at 10°C (aging temperature) for 6 d. Preliminary studies demonstrated that this time-frame was sufficient to capture the onset and inhibition of browning under these conditions (data not shown).

Degree of browning was determined through colorimetry (Colorflex 45°/0°, Hunterlab, Reston, VA). Samples were read in a 64-mm glass sample cup at times of 0, 3, and 6 d. All measurements were performed in triplicate. Statistical analysis was performed to determine significant mean differences and factor effects at $\alpha < 0.05$ (JMP version 7.0.1; SAS Institute Inc., Cary, NC).

Results from colorimetry trials are reported in Table 1 with ANOVA results in Table 2. Darker cheeses that have undergone browning are described by low L^* (lightness) and high a^* (red) and b^* (yellow) values, whereas white cheeses have higher L^* and lower a^* and b^* values. A single value of total color change, referred to as ΔE , was calculated as $\Delta E = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$; ΔE increases positively as the degree of browning advances. Figure 3 shows the change in ΔE values over time for each condition.

Degree of browning inhibition can be calculated based on a formula devised by Molnar-Perl and Friedman (1990), with ΔL replaced by ΔE . We calculated the percentage inhibition of browning as follows:

$$\text{Inhibition of browning (\%)} = (\Delta E_{\text{methylglyoxal}} - \Delta E_{\text{sample}}) \times 100 / \Delta E_{\text{methylglyoxal}}$$

Table 1. Lightness (L*), redness (a*), yellowness (b*), color change (ΔE), and inhibition of browning (%) mean values (SD in parentheses) for different treatments at 0, 3, and 6 d¹

Time and treatment ²	L*	a*	b*	ΔE	% Inhibition
0 d					
Control	73.0 (1.72)	−0.20 (0.44)	16.7 (1.36)	—	—
Methylglyoxal	71.4 (1.26)	−0.55 (0.65)	19.2 (3.11)	—	—
2:1 NaHSO ₃	71.6 (2.91)	−0.85 (0.51)	19.3 (1.22)	—	—
4:1 NaHSO ₃	69.9 (2.03)	−1.04 (0.37)	16.0 (1.01)	—	—
2:1 Glutathione	71.2 (1.99)	−0.24 (0.59)	18.1 (1.36)	—	—
4:1 Glutathione	70.0 (2.32)	−0.31 (0.38)	17.7 (0.91)	—	—
2:1 Erythorbate	72.0 (2.68)	−0.46 (0.68)	20.7 (2.75)	—	—
4:1 Erythorbate	71.7 (2.45)	−0.47 (0.48)	20.6 (3.54)	—	—
3 d					
Control	73.3 (2.16)	−0.36 (0.52)	16.9 (1.89)	0.69 (0.51)	—
Methylglyoxal	65.6 (2.69)	3.22 (1.75)	36.5 (2.69)	18.6 (1.51)	—
2:1 NaHSO ₃	69.5 (3.62)	0.78 (0.61)	28.8 (3.01)	9.87 (4.29)	47.0 (23.1)
4:1 NaHSO ₃	72.6 (1.86)	−0.92 (0.48)	20.9 (2.94)	5.70 (2.68)	69.9 (12.7)
2:1 Glutathione	71.1 (1.95)	−0.07 (0.94)	21.8 (2.24)	3.73 (2.46)	80.7 (12.1)
4:1 Glutathione	70.6 (1.82)	−0.36 (0.63)	20.0 (0.82)	2.89 (0.82)	84.6 (4.13)
2:1 Erythorbate	66.2 (4.70)	4.10 (2.91)	32.8 (2.08)	14.4 (3.71)	22.0 (25.7)
4:1 Erythorbate	65.9 (3.85)	3.86 (2.24)	31.3 (4.76)	13.0 (1.76)	30.1 (11.1)
6 d					
Control	74.3 (1.62)	−0.39 (0.47)	17.5 (1.49)	1.60 (0.24)	—
Methylglyoxal	61.2 (3.39)	5.54 (1.79)	38.7 (2.77)	23.0 (3.23)	—
2:1 NaHSO ₃	67.1 (3.87)	1.90 (1.10)	32.6 (3.89)	15.1 (5.42)	35.7 (15.0)
4:1 NaHSO ₃	71.5 (2.02)	−1.27 (0.93)	21.3 (2.63)	5.63 (1.39)	75.4 (5.71)
2:1 Glutathione	69.9 (2.26)	0.12 (1.23)	24.1 (1.04)	6.26 (1.13)	72.9 (1.19)
4:1 Glutathione	69.7 (2.21)	−0.21 (0.67)	22.7 (1.64)	5.20 (1.56)	77.6 (4.37)
2:1 Erythorbate	60.4 (5.64)	7.62 (3.32)	35.8 (1.40)	21.0 (2.96)	6.19 (27.0)
4:1 Erythorbate	61.4 (4.97)	6.99 (2.25)	35.0 (2.22)	19.3 (2.47)	14.1 (22.9)

¹Increased browning was correlated with low L* (lightness), low percentage inhibition, and high a* (redness), b* (yellowness), and ΔE values.

²Control = no methylglyoxal or antibrowning compounds added; methylglyoxal = methylglyoxal but no anti-browning agent added; NaHSO₃, glutathione, and erythorbate = addition of treatment compound at a 2:1 or 4:1 molar ratio to methylglyoxal.

Table 2. Full-factorial ANOVA for dependent variables and the factors treatment (T) and time in days (D)

Dependent variable and factor ¹	df	Sum of squares	F-ratio	P-value
L*				
T	7	479	7.80	<0.0001
D	2	231	13.2	<0.0001
T × D	14	334	2.72	0.005
a*				
T	7	228	17.6	<0.0001
D	2	111	30.0	<0.0001
T × D	14	137	5.27	<0.0001
b*				
T	7	1,910	46.6	<0.0001
D	2	1,290	110	<0.0001
T × D	14	536	6.53	<0.0001
ΔE				
T	7	1,510	46.3	<0.0001
D	2	1,870	201	<0.0001
T × D	14	822	12.6	<0.0001
% I				
T	7	33,100	35.8	<0.0001
D	2	24,300	91.7	<0.0001
T × D	14	17,200	9.30	<0.0001

¹L*, a*, b* = lightness, redness, and yellowness, respectively; ΔE = color change; %I = percentage inhibition of browning.

Figure 3. Color change (ΔE) of different cheese trials over time (0, 3, and 6 d). High ΔE values are consistent with increased browning. Control = no methylglyoxal or antibrowning compounds added; methylglyoxal = methylglyoxal but no antibrowning agent added; NaHSO₃, glutathione, and erythorbate = addition of treatment compound at a 2:1 or 4:1 molar ratio to methylglyoxal.

where ΔE describes the color change values, and $\Delta E_{\text{methylglyoxal}}$ refers to the color change of the positive browning condition (cheese and methylglyoxal without antibrowning agent).

Sulfite used at a 2:1 concentration had 47.0% (3 d) and 35.7% (6 d) inhibition; sulfite used at a 4:1 concentration was much more effective, inhibiting color change by 69.9% (3 d) and 75.4% (6 d; Table 1). Because sulfite inhibits browning much more effectively at a 4:1 ratio than it does at a 2:1 ratio, it seems likely that the irreversible formation of the thioketal molecule is necessary to inhibit methylglyoxal-based browning (Figure 2). Formation of the thiohemiketal intermediate will not fully prevent methylglyoxal-protein interactions; the thiohemiketal may either react with protein at its ketone group or dissociate back into its methylglyoxal and sulfite ion constituents due to the reversibility of its production (Friedman and Molnar-Perl, 1990). A higher concentration of substrate will favor the formation of the thiohemiketal intermediate and thus increase thioketal production. Furthermore, the higher sulfite concentration may allow the second carbonyl group to form thiohemiketals and thioketals.

Unlike sodium bisulfite, glutathione was effective at inhibiting color change at both 2:1 and 4:1 (Table 1). Slightly higher levels of inhibition were achieved using glutathione at a 4:1 molar ratio (84.6% at 3 d and 77.6% at 6 d), and a slightly less, although still high, level of inhibition (80.7% at 3 d and 72.9% at 6 d) at a 2:1 molar ratio. The greater inhibition at 4:1 levels again suggests that thioketal formation may be necessary for complete inhibition, although it appears to

be less necessary for glutathione-based inhibition. The large size of the glutathione molecule might cause steric hindrances when bonded to methylglyoxal, thus reducing browning to a greater extent than the smaller sulfite ion, and reducing the need for thioketal formation or for reactions at the ketone carbon. Because glutathione is an intermediate in methylglyoxal metabolism *in vivo* (Figure 1; Silva et al., 2005), its availability may further prevent browning by decreasing the concentration of methylglyoxal produced by starter cultures.

Erythorbate was the least effective antibrowning agent based on its inhibition values. Although erythorbate had some slight inhibition at molar concentrations of both 2:1 (22.0% at 3 d and 6.19% at 6 d) and 4:1 (30.1% at 3 d and 14.1% at 6 d), there was a very large variance in the results. This was likely due to biological differences in the samples; we observed that erythorbate was least effective on the raw milk Parmesan cheese. Several factors explain why erythorbate may be a less effective antibrowning compound. First, erythorbate may undergo nonenzymatic browning in a similar mechanism as its stereoisomer, ascorbic acid (Jackson et al., 1960). Also, its mechanism for methylglyoxal inactivation is reversible, thus potentially preventing the formation of brown pigmentation for only a short time. Furthermore, erythorbate is unable to form the strong thiohemiketal and thioketal bonds that prevent browning in the sulfite and glutathione mechanisms (Figure 2).

This study used 2 mg/g of methylglyoxal to accelerate the rate of browning; however, 1/1,000 of that concentration has been shown to typically cause browning

in aging cheese over 1 mo (2 to 3.5 $\mu\text{g/g}$ methylglyoxal; McDonald, 1992). Therefore, antibrowning agents may be applied to cheeses; for example, to cheese shreds as part of the anticaking or flow agent application, at much lower concentrations than those used in these studies. For both sulfite and glutathione, an approximate molar ratio of 4:1 (antibrowning agent to methylglyoxal) was determined to be most effective at inhibiting the formation of brown pigmentation in cheese. This corresponds to approximately 20 $\mu\text{g/g}$ of sodium bisulfite and around 60 $\mu\text{g/g}$ of glutathione. Both of these compounds are generally regarded as safe (GRAS) according to the US Food and Drug Administration. Sulfites are regulated more specifically and may not be used at levels >200 ppm ($\mu\text{g/g}$) in dairy products (Foulk, 1993). Erythorbate was least effective in these studies, although future research may be able to better use this common antioxidant, perhaps by combining it with other compounds capable of complementing its activity in this application. It may also be of interest to investigate other means to invoke reducing potential in the cheese systems through either chemical means or the selection of starter or adjunct bacteria capable of influencing methylglyoxal metabolism.

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