The effect of certain amino acids and browning inhibitors on the 'black spot' phenomenon produced by *Carnimonas nigrificans*

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Abstract: Black spots are sometimes observed in fermented sausages and raw cured meat products, with resultant economic loss. The effect brought about by some amino acids in the development of this browning was evaluated, in aerobiosis at 30 °C and 90–95% RH, in a pork meat mixture with glucose, NaCl and inoculated with *Carnimonas nigrificans* CTCBS1^T at 1.0×10^7 CFU g^{-1}. Amino acids and alternative substances to bisulphite and nitrite as preventative agents were studied for their effect on the browning reaction. The effect of NaCl concentration on browning and on the growth of *Carnimonas nigrificans* CTCBS1^T was also evaluated. Glycine, L-arginine, L-glutamine and L-monosodium glutamate were the amino acids that significantly decreased the *L* values and increased the browning scores in comminuted meat which was mixed with 40 g kg^{-1} NaCl and 20 g kg^{-1} D-(+)-, glucose and inoculated with *Carnimonas nigrificans* CTCBS1^T. *N*-acetyl-L-cysteine, L-cysteine, potassium metabisulphite and propil-3, 4, 5-trihydroxybenzoate prevented browning at 10 and 5 g kg^{-1}. The optimum concentration of NaCl for the growth of *Carnimonas nigrificans* CTCBS1^T was 40 g kg^{-1}.

Keywords: browning; *Carnimonas nigrificans*; amino acids; inhibitors; fermented meat products

INTRODUCTION

The browning of foods is a major problem which can modify the sensorial properties of foods and affect its quality. Browning can follow two different patterns: enzymatic browning occurs as a result of the polyphenoloxidase-catalysed oxidation of phenolic compounds to *o*-quinones which polymerize to form dark pigments[1,2] and among the most important of the possible non-enzymic browning reactions are those involving sugars and amino acids, especially when they are heat treated.

Arnau and Garriga[3] identified a Gram-negative bacteria, which has been accepted as a new genus: *Carnimonas* gen nov sp nov. One species, *Carnimonas nigrificans*,[4] was shown to be responsible for a rust-like colour which becomes dark within 24 h after the inoculation of an overnight culture of the strain *Carnimonas nigrificans* on raw cured meat products (mainly fermented sausages) stored in aerobic conditions, making them unsuitable for marketing. The optimum temperature for browning was 30–35 °C and it did not appear at temperatures above 40 °C. This microorganism induces browning in meat products containing glucose, maltose or dextrin when they are manufactured in an aerobic environment. Potassium bisulphite, sodium nitrite and organic acids prevented black spot formation, as did *Lactobacillus plantarum* CTC 305, which delayed the growth of *Carnimonas nigrificans*.[5]

Other substances such as L-cysteine, *N*-acetyl-L-cysteine and glutathione have been put forward as potential browning inhibitors.[6,7] 4-Hexylresorcinol has been effective in preventing black spot in shrimp caused by polyphenoloxidase activity.[8,9]

The objective of this study was to determine the effect of certain amino acids on the browning produced by *Carnimonas nigrificans* CTCBS1^T and to find additional substances to prevent this problem.

MATERIAL AND METHODS

Bacterial strain and media

Carnimonas nigrificans CTCBS1^T was grown in Tryptone Soya Broth (TSB, Difco Laboratories, Detroit, Michigan USA) at 30 °C and stored frozen at −80 °C in the same medium plus glycerol (200 mg ml^{-1}). The strain was cultured routinely at 30 °C in TSB supplemented with bacto agar (14 g kg^{-1}) (Difco) when solid medium was required.

Effect of NaCl

In order to evaluate the effect of NaCl concentration

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on the growth of the strain and in the production of browning, 10 ml solutions containing NaCl (from 30 to 110 g kg^{-1}, in multiples of 10), D-(+)-glucose (20 g kg^{-1}) and L-monosodium glutamate (20 g kg^{-1}) were inoculated (1%) with an overnight culture of *Carnimonas nigrificans* CTCBS1^T and incubated in a water bath at 30 °C for up to 8 days in closed stirred flasks. The absorbance of the solutions before and after filtration (0.45 μm filter sterile unit, Millex®-HV) was periodically measured at 420 nm[10] with a Shimadzu spectrophotometer (Shimadzu UV-240, Kyoto Japan).

The effect of amino acids and browning inhibitors

The effect of amino acids and browning inhibitors was evaluated using comminuted pork meat containing NaCl (40 g kg^{-1}) and D-(+)-glucose (20 g kg^{-1}). *Gluteus medius* muscles with $pH_{45} > 6.0$ and $pH_{24} < 6.2$ were selected in a commercial slaughterhouse and homogenized at six different times (six batches). In each batch, three controls with no added amino acid, 20 samples with 20 g kg^{-1} of different amino acids and seven potential browning inhibitors at three different levels (1, 5 and 10 g kg^{-1}) were inoculated with 1.0×10^7 CFU g^{-1} of *Carnimonas nigrificans* CTCBS1^T and homogenized in a laboratory cutter (Moulinex type D56). The mixture obtained (100 g) was distributed in Petri dishes without lids and stored at 30 °C and 90–95% relative humidity for 6 days. Analytical grade L-amino acids were purchased from Sigma (Sigma Aldrich Química SA, Madrid, Spain). The amino acids studied were: glycine, monosodium glutamate, glutamine, lysine, histidine, serine, alanine, proline, asparagine, arginine, threonine, methionine, tryptophan, isoleucine, hydroxyproline, leucine, valine, tyrosine, phenylalanine and cystine.

The potential antibrowning substances studied were: *N*-acetylcysteine (Merck, Darmstadt, Germany), 2-*tert*-butyl-4-methoxyphenol (BHA) (Merck, Schuchardt, Germany), L-cysteine, 4-hexylresorcinol (Sigma), potassium metabisulphite, 2,6-Di-*tert*-Butyl-4-methylphenol (BHT) and propyl-3,4,5-trihydroxibenzoate (Panreac SA, Barcelona, Spain). The mean basic composition of the meat for the six batches was (in percentage): moisture, 74.76 ± 0.98; protein, 20.84 ± 0.93; fat, 3.57 ± 1.43 and the mean pH was 5.73 ± 0.11.

The browning effect was evaluated after 6 days by a four-member panel, using an eight-point scale (0 = no browning and 8 = strong browning). All the samples from each batch were evaluated in the same session. Colour was measured in CIELab colour space. Measurements were made using a tri-stimulus colourmeter Minolta Chromameter CR-200 (Minolta Camera Co, Ltd, Osaka, Japan) with an 8 mm diameter measuring area with respect to CIE (1976)[11] reference standard illuminant C and observer angle 2°. Before measuring, the apparatus was calibrated with a white plate reference standard (CR-A43, Minolta Camera Co, Ltd, Osaka, Japan). Lightness (L^*); redness (a^*) and yellowness (b^*), were evaluated on the controls, the samples with amino acids and the samples with antibrowning substances added at 10 g kg^{-1}. The measurements were carried out after the removal of the white slime observed on the surface of the meat mixtures inoculated with *Carnimonas nigrificans* CTCBS1^T.

Effects of potassium metabisulphite and cysteine on the growth of *Carnimonas nigrificans* CTCBS1^T

The effect of potassium metabisulphite and cysteine on the growth of *Carnimonas nigrificans* CTCBS1^T was assessed. The strain was grown in TSB in stirred flasks at 30 °C for 72 h. The medium was supplemented with 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg ml^{-1} of potassium metabisulphite or 0, 1.0, 2.0 and 5.0 mg ml^{-1} of cysteine. Duplicates from each concentration were sampled. *Carnimonas nigrificans* CTCBS1^T was enumerated after serial 10-fold dilutions were plated on Tryptone Soya Agar and incubated aerobically at 30 °C for 72 h.

Statistics

Browning scores different from zero and L values obtained with different amino acids and inhibitors were analysed using variance analysis. The model included: treatment, batch, and their interaction as fixed effects. The differences with respect to the control were tested by the Dunnett test.[12]

RESULTS AND DISCUSSION

The browning reaction observed after the inoculation of *Carnimonas nigrificans* CTCBS1^T in comminuted meat containing NaCl and D-(+)-glucose began with a rust-like colour which became black after several days. During the browning process, L values determined after removing the slime decreased during the storage (Table 1), b values increased during the first day and a values during the second and third day (data not shown). Thereafter, both b and a values tended to decrease or stabilise. For that reason the L values seem

Table 1. Changes of L values in comminuted meat with D-(+)-glucose (20 mg g^{-1}) and NaCl (40 mg g^{-1}) inoculated with *Carnimonas nigrificans* CTCBS1^T and stored at 30 °C and 90–95% RH for 6 days (n = 6)

Day	L means	SD
0	62.2	1.71
1	54.9	1.93
2	45.7	1.90
3	39.3	1.00
6	37.9	1.35

to correspond fairly well with the changes in appearance observed at the end of the browning process.

The *L* values and browning scores in comminuted meat inoculated with *Carnimonas nigrificans* CTCBS1^T with different added amino acids and browning inhibitors after 6 days at 30 °C showed a significant batch effect ($P < 0.05$), but the interaction batch/treatment was not significant. Therefore, the interaction effect was dropped from the model and the batch effect was used as a blocking effect. Using the residual standard deviation of the analyses, the percent coefficient of variation for different treatments (CV) ranged from 1.8 to 4.8 for the *L* values and from 6.0 to 13.0 for the browning scores.

In liquid culture the absorbance of the culture filtrate measured at 420 nm increased during the stationary phase of the growth of *Carnimonas nigrificans* CTCBS1^T (Fig 1), suggesting that the compounds responsible for browning could be secondary metabolites. Both the browning and the growth of the strain were maximum when the NaCl concentration was 40 g kg^{-1} (data not shown). No growth was observed at NaCl concentrations of 90, 100 and 110 g kg^{-1}.

Effect of amino acids

In comminuted meat containing 40 g kg^{-1} of NaCl and 20 g kg^{-1} of D-(+)-glucose, browning was only observed when it was inoculated with *Carnimonas nigrificans* CTCBS1^T. The addition of some amino acids increased the browning both in liquid cultures (data not shown) and in comminuted meat (Table 2).

In comminuted meat, the correlation between *L* values and browning scores for all the treatments containing amino acids was $R = -0.91$. Glycine, L-arginine, L-glutamine and L-monosodium glutamate significantly increased ($P < 0.05$) the browning scores, and decreased *L* values. This was probably due to the higher reactivity of these amino acids rather than meat peptides and proteins in a similar way as in the Maillard reaction[13–15] or an easier metabolisation of these free amino acids than proteins by *Carnimonas nigrificans* CTCBS1^T. L-Phenylalanine, L-hydroxyproline, L-tryptophan, L-asparagine, L-histidine, L-proline, L-alanine, L-isoleucine, L-valine, L-threonine, L-leucine and L-serine did not significantly increase either the browning scores or *L* values ($P > 0.05$) after 6 days. L-Tyrosine and L-cystine significantly increased the *L* values but the scores obtained by panel test were not significantly different to the control, because of the higher variation coefficient of the panel scores. L-Lysine increased the scores ($P < 0.05$) but did not significantly decrease *L* values ($P > 0.05$). L-Cysteine inhibited the browning, probably because of the interaction of the thiol group with the aldehyde to form a thiohemiketal or thioketal, thus blocking the browning.[6] L-Methionine slightly decreased the browning scores and increased *L* values.

Panellists found meat samples tougher when browning took place, which is similar to the toughening

Table 2. *L* values and browning scores in comminuted meat inoculated with *Carnimonas nigrificans* CTCBS1^T with different added amino acids and browning inhibitors after 6 days at 30 °C

Substances	*L mean*	*Scores mean*
N-acetyl-L-cysteine[a]	64.13[b]	0
L-Cysteine[a]	59.30[b]	0
Potassium metabisulphite[a]	57.05[b]	0
Propyl-3,4-5 trihydroxybenzoate[a]	49.40[b]	0
L-Cystine	41.71[b]	4.8
4-Hexylresorcinol[a]	40.96[b]	3.3[b]
L-Methionine	40.63[a]	3.8[b]
L-Tyrosine	39.26[a]	4.8
2-*tert*-butyl-4-methoxyphenol[a]	38.83	4.7
2, 6-Di-*tert*-butyl-4-methylphenol[a]	38.73	4.7
L-Serine	38.31	4.8
L-Leucine	38.27	4.8
L-Threonine	38.05	4.8
Control	37.34	4.8
L-Valine	37.33	4.8
L-Isoleucine	37.32	4.8
L-Alanine	37.16	5.0
L-Proline	37.05	5.0
L-Histidine	36.71	5.1
L-Asparagine	36.72	5.1
L-Tryptophan	36.68	5.1
L-Hydroxyproline	36.10	5.2
L-Phenylalanine	36.02	5.2
L-Lysine	35.50	5.9[b]
L-Monosodium glutamate	35.21[b]	6.2[b]
L-Glutamine	35.11[b]	6.3[b]
L-Arginine	32.80[b]	6.7[b]
Glycine	24.50[b]	7.2[b]
Rsd	1.17	0.43

$n = 6$

[b] *L* values or browning scores significantly different with respect to the control ($P < 0.05$). Scores: 0 = no browning and 8 = strong browning.
All the substances were added at 20 mg g^{-1} except those marked.
[a] which were added at 10 mg g^{-1}.

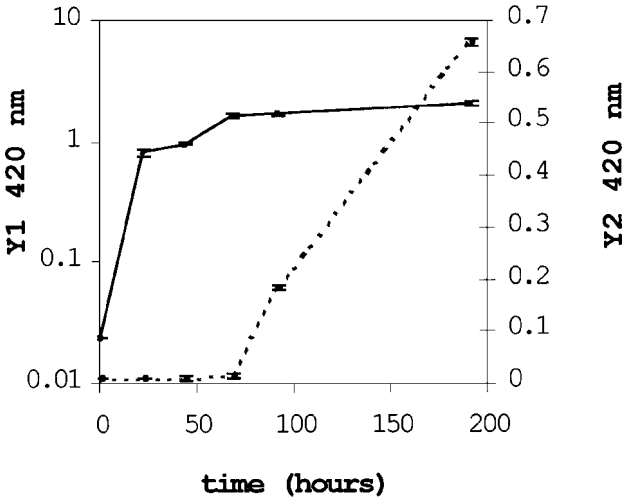

Figure 1. Browning development by *Carnimonas nigrificans* CTCBS1^T in a solution containing 40 mg g^{-1} NaCl, 20 mg g^{-1} D-(+)-glucose and 20 mg g^{-1} L-monosodium glutamate at 30 °C. Y1, Absorbance of the culture at 420 nm (–); Y2, absorbance of the culture filtrate at 420 nm (...). The error bars indicate SD ($n = 3$).

Table 3. Growth of *Carnimonas nigrificans* CTCBS1^T at 30 °C for 72 h in Tryptone Soya Broth supplemented with different concentrations of potassium metabisulphite or L-cysteine

Substances	*mg ml^{-1}*	*CTCBS1 (CFU ml^{-1})*
Potassium metabisulphite	0	7.4×10^8
	0.2	2.6×10^8
	0.4	3.4×10^8
	0.6	1.7×10^8
	0.8	1.1×10^8
	1.0	2.1×10^4
	1.2	1.8×10^4
L-Cysteine	0	7.4×10^8
	1.0	6.2×10^8
	2.0	7.6×10^7
	5.0	8.8×10^5

Results are the means of two replicates.

produced by the Maillard reaction in foods.[16] The browning reaction was arrested when the inoculated samples were vacuum-packed because *Carnimonas nigrificans* CTCBS1^T is obligately aerobic.[4]

Prevention of browning

N-acetyl-L-cysteine, L-cysteine, potassium metabisulphite, and propyl-3, 4, 5-trihydroxybenzoate prevented the browning at 10 and 5 g kg^{-1} (scores = 0). When the concentration was 1 g kg^{-1}, the browning scores ranged from 1 to 3. *N*-acetyl-L-cysteine prevented the browning, probably due to the —SH group[6] in a similar way to L-cysteine. The greater nucleophilic reactivity of the acetylated derivative may explain why it was slightly more effective as a browning inhibitor than cysteine.[7] *N*-acetyl-L-cysteine, L-cysteine, and potassium metabisulphite bleached the rust-like pigment in the first steps of the browning reaction, but were not effective when the colour became black. Moreover, the counts of *Carnimonas nigrificans* in the solutions containing 1 mg ml^{-1} of potassium metabisulphite were 4 $\log_{10}$ lower than the control after 72 h. A 3 $\log_{10}$ decline was observed when the strain was grown in L-cysteine at 5 mg ml^{-1} compared with the control. At 1 mg ml^{-1} of cysteine the counts were similar to the control (Table 3).

Propyl-3,4,5-trihydroxybenzoate was effective in the prevention of browning, 4-hexylresorcinol was only slightly effective, and other phenolic substances such as BHT, ethyl *p*- hydroxy benzoate and propyl-*p*-hydroxy benzoate had no significant effect,[3] maybe because they are specially active against Gram-positive bacteria[16] and *Carnimonas nigrificans* is a Gram-negative bacteria. Moreover, the effect of some of these substances could be affected because of their solubility in water.

The results obtained indicated that the browning produced by *Carnimonas nigrificans* CTCBS1^T with glucose and amino acids, as well as the inhibitory properties of some of the substances studied, shows similarities to the Maillard reaction. However, important differences exist in the temperature pattern, the oxygen effect and sugars involved, which could be related to the metabolism of the *Carnimonas nigrificans*, the strain responsible for the defect.

In conclusion, the browning effect produced by *Carnimonas nigrificans* CTCBS1^T was increased by glycine, L-arginine, L-glutamine and L-monosodium glutamate. *N*-acetyl-L-cysteine, L-cysteine, potassium metabisulphite and propyl-3,4,5-trihydroxybenzoate were useful in the prevention of this defect.

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