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Effect of added α-ketoglutaric acid, pyruvic acid or pyridoxal phosphate on proteolyis and quality of Cheddar cheese

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Abstract

Cheddar cheese curds were supplemented with 1, 5 or 20 g of α-ketoglutarate or pyruvic acid or 1.2 g pyridoxal-5[1]-phosphate/kg cheese curd. The higher levels of keto-acids (5 or 20 g/kg curd) caused undesirable changes in the physico-chemical properties of resultant cheese. All levels of α-ketoglutarate reduced the pH of the cheese and promoted syneresis during pressing, while pyruvic acid increased the pH of the cheese. The numbers of starter and non-starter lactic acid bacteria were not affected by the addition of keto-acids or pyridoxal-5[1]-phosphate. α-Ketoglutarate or pyruvic acid, at 1 g/kg, or pyridoxal-5[1]-phosphatase, at 1.2 g/kg cheese curd, did not influence primary proteolysis in the cheese. The highest and lowest concentrations of total and individual free amino acids were found in the cheeses treated with pyruvic acid or α-ketoglutarate, respectively. The concentrations of most amino acids were lower in the cheeses treated with pyridoxal-5[1]-phosphate than in the control. The results of this study suggest that α-ketoglutarate and pyridoxal-5[1]-phosphate enhanced the degradation of most amino acids in Cheddar cheese while pyruvic acid promoted the formation of amino acids. The cheeses treated with α-ketoglutarate were more mature than the control cheese of the same age while pyruvic acid-treated cheese had a better flavour than the control.

Keywords: Cheddar cheese; Keto acids; Amino acids

1. Introduction

The biochemistry of cheese ripening is a complex process which involves three primary events, i.e. proteolysis, lipolysis and glycolysis, which are catalyzed by enzymes indigenous to milk, from the coagulant, starter bacteria, and the adventitious non-starter microflora (see Fox, 1998; Fox, Connor, Guinee, & O' Brien, 1996; Fox, Law, McSweeney, & Wallace, 1993). Proteolysis is a major biochemical event during the ripening of Cheddar cheese (see Fox & McSweeney, 1996; Fox, Singh, & McSweeney, 1994). Although the end products of proteolysis are amino acids, the further breakdown of these by decarboxylation, deamination, desulphurylation and demethiolation is considered to be an integral part of the overall process of flavour development in cheese (Gripon, Monnet, Lamberet, & Desmazeaud, 1991; Law, 1987). Several sapid and aromatic compounds are produced *via* the catabolism of amino acids, which has been reviewed by Fox (1998) and Fox and Wallace (1997).

Lactococci possess enzyme activities by which amino acids are transformed into aroma compounds. Engels and Visser (1996) reported the formation of the typical flavour of Gouda by incubating lactococcal cellular extracts with Met. Roudout-Algaron and Yvon (1998) reported that, in vitro, lactococci can degrade aromatic and branched chain amino acids to aroma compounds, such as hydroxy and branched carboxylic acids. The first step in the degradation for these amino acids is transamination which requires the presence of an α-keto acid acceptor for the amino group. Yvon, Berthelot and Grippon (1999) reported that α-ketoglutarate is the main α-keto acid acceptor for amino acid transamination and that the addition of α-ketoglutarate to St Paulin-type cheese strongly enhanced the degradation of aromatic and branched chain amino acids and Met.

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Banks et al. (2000) used α-ketoglutarate to enhance amino acid catabolism in Cheddar cheese and reported that adding α-ketoglutarate to Cheddar curd at 5 g/kg curd enhanced the catabolism of Leu and Ile by approximately 20%, while the production of Glu was enhanced by approximately 30%. Substantial increases in the concentrations of several volatile components, potentially arising from amino acid catabolism, were observed by Banks et al. (2000).

During the biosynthesis of amino acids, all the carbons of Ala and Val and four of the six carbons of Leu are obtained from pyruvate (Burgess, 1983). Pyruvate also provides, on average, 2.5 of the six carbons of Lys.

Yvon, Thirouin, Rijnen, Frometier, and Gripon (1997) purified and characterized a pyridoxal-5[1]-phosphate-dependent transaminase from *Lactococcus lactis* subsp. *cremoris*. Pyridoxal-5[1]-phosphatase, the coenzyme form of vitamin B6, is involved in a variety of reactions in the metabolism of α-amino acids, including transamination, α-decarboxylation, racemizations, aldolizations, and the β-decarboxylation of Asp (Frey, 1983).

The objectives of this study were to investigate the effect of added α-ketoglutarate, pyruvic acid or pyridoxal-5[1]-phosphate on the metabolism of amino acids in Cheddar cheese and the subsequent effect on the quality of the cheese.

2. Materials and methods

2.1. Cheese manufacture

Lactococcus lactis ssp. *cremoris* 227 was obtained from the Microbiology Department, University College, Cork. The culture was grown in sterilized (110 °C×10 min) reconstituted (10%, w/w) low-heat skim milk powder and was used as starter at a level 2% (v/v)

Cheddar cheese was manufactured from two 100 l lots of milk in 100 l vats on two occasions (trials 1 and 2) by a standard protocol. The curds obtained, after milling, were mixed together and then divided into eight 2-kg lots. One lot of curd was kept as control (C); three lots were treated with α-ketoglutarate at 1, 5 or 20 g/kg (KG1, KG5 and KG20), another three lots with pyruvic acid at 1, 5 or 20 g/kg (PA1, PA5 and PA20) and the 8th lot with 1.2 g pyridoxal-5[1]-phosphate/kg curd (PP). These chemicals, which were obtained from Sigma Chemical Co., St. Louis, MO, USA, were mixed with salt and added to the curds at salting. Thus, on each day of cheese manufacture, eight cheeses were obtained, i.e. C, KG1, KG5, KG20, PA1, PA5, PA20 and PP. The levels of α-ketoglutarate were similar to those used by Yvon et al. (1999) in St. Paulin-type cheese and similar levels of pyruvic acid were used. The level of pyridoxal-5′-phosphate was chosen randomly.

2.2. Compositional analysis

The 1-day-old cheeses were analyzed in duplicate for fat (IIRS, 1995), protein (%N×6.38; AOAC, 1990), moisture (AOAC, 1990) and salt (Fox, 1963). The pH of the cheese was determined by mixing cheese and distilled water in the ratio of 1:1 and then placing the electrodes of a pH meter directly into the slurry.

2.3. Bacteriological analysis

Starter bacteria in the cheeses were enumerated on LM17 agar (Oxoid, Basingstoke, UK), incubated at 30 °C for 3 days (Terzaghi & Sandine, 1975). Non-starter lactic acid bacteria were enumerated in the cheeses during ripening on Rogosa agar (E-Merck, Darmstadt, Germany), incubated aerobically for 5 days at 30 °C.

2.4. Assessment of proteolysis

Samples (200 g) were taken periodically during ripening for the assessment of proteolysis and frozen at −20 °C until analyzed. Water-soluble fractions (WSF) of the cheeses were prepared according to the method of Kuchroo and Fox (1982). The N content of the WSF was determined in duplicate by the macro-Kjeldahl method. The water-insoluble fraction (WISF) and WSF of the cheeses were analyzed by urea-polyacrylamide gel electrophoresis (PAGE) using the stacking gel system of Andrews (1983); the gels were stained by using a modification of the method of Blakesley and Boezi (1977) with PAGE Blue G-90. Part of the WSF was fractionated by ethanol (70%); the ethanol-soluble and -insoluble fractions were analyzed by reversed-phase high performance liquid chromatography (RP–HPLC), as described by Lynch, McSweeney, Fox, Cogan, and Drinan (1997).

Total free amino acids in the cheeses were determined in triplicate by the Cd-ninhydrin method (Folkertsma & Fox, 1992). Individual free amino acids were analyzed in the WSF fraction of the cheeses; 1 ml of WSF was deproteinised by mixing with 1 ml of 2% (w/v) TCA and allowed to stand for 10 min before centrifugation (Microcentaur, MSE, UK) at 14 400 ***g*** for 10 min. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2. Fifty microlitres of the supernatant in buffer were taken and analyzed on a 120×4 mm cation-exchange column (Na^+ form) using a Beckman 6300 amino acid analyzer (Beckman Instruments Ltd, High Wycombe, UK).

2.5. Sensory analysis

Cheeses were graded after 2, 3, 5 and 6 months of ripening by two trained graders from the Irish Department of Agriculture, Food and Forestry, on the basis of

flavour and texture on a scale of 0 to 10. The graders also noted the perceived maturity of the cheeses.

3. Results and discussion

3.1. General

In general, results presented are the means of both cheesemaking trials. The addition of 5 or 20 g α-ketoglutarate or pyruvic acid caused undesirable changes to the texture and composition of the cheese and the graders found them unacceptable even after 2 months: therefore, the cheeses treated with 5 or 20 g of α-ketoglutarate/kg curd were not studied for the full period of ripening. The cheese with 20 g α-ketoglutarate/kg curd did not mat properly and was crumbly even on day 1. The addition of pyridoxal-5-phosphate changed the colour of the curd to yellowish, but the natural colour of the cheese was restored during the first month of ripening. The cheese treated with 1 g of α-ketoglutarate or pyruvic acid or 1.2 g pyridoxal-5′-phosphate/kg curd were studied for 6 months.

3.2. Composition

The levels of moisture and salt varied (Table 1). The cheese treated with 20 g α-ketoglutarate/kg curd had the lowest content of moisture (34.3%) and salt (1.10%), suggesting that the addition of α-ketoglutarate promoted syneresis during pressing of the curd. Yvon et al. (1999) reported no significant changes in the moisture content of St Paulin cheese on the addition of α-ketoglutarate (20 g/kg cheese). The addition of pyruvic acid at 20 g/kg curd had no marked affect on the moisture of the cheese but reduced the retention of salt compared with the control. The protein content increased with the increasing level of α-ketoglutarate but the level of fat was not affected markedly by the addition of α-ketoglutarate, probably due to loss of fat in the whey expressed from these cheeses. The levels of fat and protein were not affected markedly by the addition of pyruvic acid or pyridoxal-5[1]-phosphate.

α-Ketoglutarate resulted in a marked decrease in the pH of the cheeses, pro rata the level of α-ketoglutarate added. The decrease in the pH of the curd explains the extensive syneresis of the curd; Yvon et al. (1999) reported that the addition of α-ketoglutarate to St Paulin cheese caused an increase in the pH. The addition of pyruvic acid increased the pH of the cheese. The addition of 1 g α-ketoglutarate or 1 g pyruvic acid or 1.2 g pyridoxal-5[1]-phosphate/kg cheese caused only small changes to the composition or pH of the resultant cheeses and were acceptable according to commercial graders; hence only these cheeses were evaluated for proteolysis.

3.3. Bacteriological

All the cheeses had a similar number of starter bacteria on day 1 ($\sim 10^9$ cfu g^{-1}; Table 2). These results suggest that the addition of α-ketoglutarate, pyruvic acid or pyridoxal-5[1]-phosphate did not promote the death of starter bacteria during pressing. In Cheddar cheese, the maximum number of starter bacteria is attained before the addition of salt (McSweeney et al., 1995). A reduction of two log cycles of starter bacteria was observed in the control cheese and in the cheeses treated with 1 or 5 g α-ketoglutarate or pyruvic acid/kg cheese curd during 60 days of ripening. Twenty grams of α-ketoglutarate/kg curd caused a 4 log cycle reduction in the number of starter bacteria, probably due to the low moisture and pH in the cheese. The starter bacteria did not die off during the first 60 days of ripening in the cheeses treated with 20 g pyruvic acid or 1.2 g pyridoxal-5′-phosphate/kg cheese curd. Perhaps the significant increase in the pH of the cheeses caused by 20 g pyruvic acid prolonged the viability of the starter

Table 1
Composition and pH of 1 day-old control and experimental cheeses treated with α-ketoglutaric (1, 5 or 10 g/kg, KG1, KG5, KG10) or pyruvic acid (1, 5 or 10 g/kg, PA5, PA1, PA10) or pyridoxal-5[1]-phosphate (1.2 g PP per kg)[a]

Cheese	Moisture (%)	Fat (%)	Protein (%)	Salt (%)	pH
Control	38.1	30.0	26.6	1.60	5.13
KG1	38.1	29.5	26.0	1.57	5.10
KG5	35.7	30.5	28.0	1.45	4.99
KG20	34.3	29.5	29.2	1.10	4.81
PA1	38.4	30.5	27.3	1.61	5.15
PA5	38.0	30.0	27.8	1.76	5.29
PA20	38.5	30.0	27.0	1.35	5.58
PP	38.7	30.0	27.2	1.60	5.11

[a] Means of cheeses in two trials analyzed in duplicate.

Table 2
Numbers of starter bacteria (cfu g^{-1} cheese) in control and experimental cheeses treated with α-ketoglutaric (1, 5 or 10 g; KG1, KG5, KG10 or pyruvic acid (1, 5 or 10 g; PA5, PA1, PA10) or pyridoxal-5[1]-phosphate (1.2 PP) g per kg of cheese curd during ripening[a]

Cheese	1 day cfu$\times 10^9$	7 days cfu$\times 10^9$	30 days cfu$\times 10^8$	60 days cfu$\times 10^8$
Control	1.87	1.86	5.09	2.28
KG1	1.62	1.69	3.51	3.51
KG5	1.54	1.56	2.32	5.07
KG20	1.73	1.56	7.4	2.31
PA1	1.92	1.89	4.91	4.95
PA5	1.93	1.82	5.3	6.40
PA20	1.85	1.87	13.3	14.0
PP	1.90	1.88	20.5	28.9

[a] Means of cheeses of two trials analyzed in duplicate.

bacteria as did the vitamin pyridoxal-5[1]-phosphate. No counts of starter bacteria were taken at 90 days of ripening as the non-starter lactic acid bacteria (NSLAB) started growing.

No growth of NSLAB was observed in any of the cheeses up to 2 months of ripening but all 6-month-old cheeses had a similar number of NSLAB (10^7 cfu g^{-1} cheese; Table 3), suggesting that the addition of α-ketoglutarate, pyruvic acid or pyridoxal-5′-phosphate did not interfere in the growth of NSLAB in cheese during ripening.

3.4. Proteolysis

Addition of 1 g α-ketoglutarate or 1 g pyruvic acid or 1.2 g pyridoxal-5′-phosphate/kg cheese curd caused minor changes to the level of WSN in the cheeses during ripening (Table 4). No marked differences were observed in the urea-PAGE of WISF of cheeses during ripening (not shown), suggesting that α-ketoglutarate, pyruvic acid or pyridoxal-5[1]-phosphate did not influence the activity of residual chymosin or indigenous plasmin in cheese during ripening, since chymosin and plasmin are principally responsible for the production of WSN and the level of proteolyis detected by urea-PAGE of WISF of cheeses (Fox et al., 1993; Visser, 1977).

Urea-PAGE of WSF of cheeses also showed no marked differences between the cheeses treated with α-ketoglutarate or pyruvic acid or pyridoxal-5[1]-phosphate and the controls and RP–HPLC chromatograms showed only minor differences between the cheeses (not shown).

The level of total amino acids was highest in the cheese treated with pyruvic acid and lowest in the cheese treated with α-ketoglutarate (Table 5), suggesting that α-ketoglutarate enhanced the catabolism of some amino acids while pyruvic acid enhanced their formation. Yvon et al. (1999) and Banks et al. (2000) reported that α-ketoglutarate promoted the degradation of amino acids in St Paulin and Cheddar cheeses, respectively. The 6-month-old cheese supplemented with pyridoxal-5[1]-phosphate also had an increased concentration of total amino acids (as mg Leu/g cheese).

The concentrations of Tyr and Arg were highest in the cheese treated with α-ketoglutarate, suggesting that α-ketoglutarate enhanced the production of these amino acids (Table 6) but the concentration of most of other amino acids was lowest in this cheese. Trp was not

Table 3
Numbers of non-starter lactic acid bacteria[a] during ripening (cfu g^{-1} cheese) in control and experimental cheeses which were treated with α-ketoglutaric (1g; KG1) or pyruvic (1; PA1) acid or pyridoxal-5[1]-phosphate (1.2 g, PP) per kg of cheese curd

Cheese	90 days cfu×10^6	150 days cfu×10^6	180 days cfu×10^7
Control	3.90	4.00	4.20
KG1	1.95	3.71	2.81
PA1	1.13	6.10	4.61
PP	26.0	9.96	2.32

[a] Means of cheeses of two trials analyzed in duplicate.

Table 4
Water-soluble nitrogen[a], expressed as % of total N, in control and experimental cheeses treated with α-ketoglutaric (1 g; KG1) or pyruvic (1; PA1) acid or pyridoxal-5[1]- phosphate (1.2 g; PP) per kg of cheese curd during ripening

Cheese	1 day	60 days	120 days	180 days
Control	7.70	17.6	24.6	23.5
KG1	8.31	18.5	26.2	24.7
PA1	8.12	17.3	24.1	23.2
PP	8.34	18.7	25.7	24.7

[a] Means of cheeses of two trials analyzed in duplicate.

Table 5
Changes in the concentrations[a] of total amino acids (mg Leu/g) in control and experimental cheeses treated with α-ketoglutaric (1 g; KG1) or pyruvic (1 g; PA1) acid or pyridoxal-5[1]- phosphate (1.2 g; PP) per kg of cheese curd

Cheese	1 day	60 days	120 days	180 days
Control	1. 2	2.3	4.4	8.5
KG1	1.9	2.1	4.2	7.0
PA1	2.0	2.3	4.4	9.3
PP	2.0	2.3	4.4	9.2

[a] Means of cheeses of two trials analyzed in triplicate.

Table 6
Concentration of amino acids[a] (μg g^{-1} cheese) in 6 month-old control and experimental cheeses that were treated with 1 g of α-ketoglutaric (KG1) or pyruvic (PA1) acid or 1.2 g pyridoxal-5[1]- phosphate (PP) per kg of cheese curd

Amino acid	Control	KG1	PA1	PP
Asp	170	135	172	143
Thr	99	76	103	85
Ser	343	323	350	322
Glu	636	379	590	583
Gln	46	41	45	45
Ala	139	119	146	161
Val	330	283	337	309
Met	190	150	195	185
Ile	71	58	75	73
Leu	957	782	992	976
Tyr	155	195	189	153
Phe	518	452	557	546
His	113	98	74	83
Lys	205	182	200	199
Trp	1175	00	1161	1182
Arg	27	64	61	13
Total	5491	4254	5528	5321
NH_3	141	134	170	122

[a] Means of cheeses of two trials analyzed in duplicate.

Table 7
Sensory scores (means of two trials) awarded by two graders from the Irish Department of Agriculture, Food and Forestry on a 0–10 scale[a] for control and experimental cheeses that were treated with α-ketoglutaric (1, KG1; 5, KG5 or 10 g, KG10) or pyruvic (1, PA1; 5, PA5 or 10 g, PA10) acid or pyridoxal-5[1]- phosphate (PP, 1.2 g) per kg of cheese curd during ripening

Cheese	60 days		90 days		150 days		180 days	
	Flavour	Body	Flavour	Body	Flavour	Body	Flavour	Body
Control	6.0	7.0	6.0	7.0	7.0	7.0	5.0	6.0
KG1	7.0	7.0	6.0	7.0	7.0	7.0	4.0	3.0
PA1	6.0	7.0	7.0	6.0	9.0	7.0	7.0	6.0
PP	5.0	7.0	7.0	7.0	8.0	6.0	4.0	3.0

[a] 0 = minimum score, 10 = maximum score.

found in the cheese treated with α-ketoglutarate, perhaps because it is catabolised completely in the presence of α-ketoglutarate. Yvon et al. (1999) reported that Trp is catabolised rapidly to indole-3-acetate in St Paulin cheese in presence of α-ketoglutarate. The concentrations of Glu, Gly and Lys were markedly lower in the cheese containing α-ketoglutarate than all other cheeses. Yvon et al. (1999) also reported significant degradation of Glu in presence of α-ketoglutarate while, Banks et al. (2000) reported enhanced (by about 30%) production of Glu in Cheddar cheese supplemented with 20 g α-ketoglutarate/kg curd. Degradation of Met was also observed in the presence of α-ketoglutarate, in agreement with the findings of Yvon et al. (1999).

The concentration of most amino acids in this study was highest in the cheese treated with pyruvic acid, while the cheese containing pyridoxal-5[1]-phosphate had a lower concentration of the majority of the amino acids than the control. These results suggest that pyruvic acid, which provides carbons in the formation of amino acids, enhanced the formation of amino acids, while pyridoxal-5[1]-phosphatase promoted their catabolism.

3.5. Sensory analysis

The commercial graders awarded the highest flavour scores to 60 day-old cheese supplemented with 1 g α-ketoglutarate/kg curd (Table 7) and considered it to be as mature as 90 day-old commercial Cheddar cheese. The cheese treated with pyruvic acid was consistently awarded the best scores for flavour and after 90, 150 and 180 days of ripening it had a strong pleasant flavour. The 3 month-old cheese containing α-ketoglutarate or pyruvic acid was perceived to be as mature as 5 and 4 month-old commercial Cheddar cheese, respectively. The 3 month-old cheese supplemented with pyridoxal-5[1]-phosphate was perceived to be as mature as 4 month-old commercial cheeses. Banks et al. (2000) reported that the aroma of Cheddar cheese containing 20 g α-ketoglutarate/kg curd, was enhanced, with values at 6 weeks equivalent to those seen in an 18 week control sample. The 6 month-old cheeses treated with 1 g α-ketoglutarate or 1.2 g/kg pyridoxal-5[1]-phosphate received poor scores for flavour and body. The texture of cheese treated with 1 g α-ketoglutarate or 1.2 g/kg pyridoxal-5[1]-phosphate was crumbly.

4. Conclusions

α-Ketoglutarate, pyruvic acid and pyridoxal-5[1]-phosphate influenced the catabolism of amino acids, especially Glu, Gly, Lys Met and Trp, in Cheddar cheese. Pyruvic acid promoted the production of amino acids in Cheddar cheese. α-Ketoglutarate improved the maturity of Cheddar cheese. Pyruvic acid substantially improved the flavour of Cheddar cheese. Further experiments, using smaller concentration increments of α-ketoglutarate, pyruvic acid and pyridoxal-5[1]-phosphate should be undertaken to confirm the preliminary results reported here.

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