Amino Acids (2007) 32: 39–43
DOI 10.1007/s00726-006-0355-5
Printed in The Netherlands

Amino Acids

The influence of manufacture on the free D-amino acid content of Cheddar cheese

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Received November 17, 2005
Accepted February 2, 2006
Published online June 1, 2006;

Summary. The changes in the concentration and those of composition of alanine, aspartic acid and glutamic acid enantiomers were investigated during manufacture of Cheddar cheese. The amount of D-alanine increased continuously during ripening following the liberation of L-alanine originated from the proteolysis of milk proteins. There was slightly more D-aspartic and D-glutamic acid in the dry matter of curd after pressing than before pressurization. The D-amino acid content and the ratio of the D-enantiomers related to the total amount of free amino acids differed significantly among cheeses produced with different single-strain starters. The D-amino acid composition changed during manufacture, but the influence of the strain selection was not significant on the D-amino acid pattern.

Keywords: D-alanine – D-aspartic acid – D-glutamic acid – Cheddar cheese manufacture – Autolysis

Introduction

The D-amino acid content of raw milk with low cell count was reported to be not significant. Neither pasteurization nor UHT heating resulted in significant increase in the amount of D-amino acids (Brückner and Hausch, 1990a; Gandolfi et al., 1992). In contrast to heating, the concentration of D-amino acids in fermented milk products was significantly higher than that of the raw material. Among fermented products ripened cheeses are the richest source of D-amino acids. Mostly D-alanine (D-Ala) was present in the highest quantities and with a few exceptions D-glutamic acid (D-Glu) and D-aspartic acid (D-Asp) were also present (Brückner and Hausch, 1990a, b; Brückner et al., 1992).

The origin of free D-amino acids related with bacterial activity is supposed to be connected with the autolysis of cells and the presence of bacterial racemases (Brückner et al., 1992; Gandolfi et al., 1994; Adams, 1972). The Gram positive bacteria applied in starters during cheese-making possess cell walls with D-amino acid containing peptidoglycan and substituted teichonic acid giving approximately 50% of the dry material of the cell (Schleifer and Kandler, 1972; Friedman, 1999). The retardation and the death phase of the bacterial cell life-span is concerned with cheese ripening (Scott, 1998). During this period the number of non-viable cells increases. These cells in cheese may be more or less intact with complete cell walls or being in *sphaeroplast* form with membranes with varying degree of disintegration depending on the state of lysis (Wilkinson et al., 1995). In the latter stage the D-amino acids may be liberated from the constituents of the cell wall and intracellular izomerases could also release from the cells. Among D-amino acids the amount of D-Ala in fruit juices and milk was reported to be associated with the stage of the bacterial growth. D-Ala content began to increase when the bacteria population was in the retardation phase (Gandolfi et al., 1992, 1994). Because the stage of bacterial growth determines the die-off rates, the increment of D-Ala could also be associated with the number of dead cells. In cheeses this part of the life-span associated with ripening, therefore similar tendencies could be possible during the ripening of cheeses in the D-Ala content. On the other hand, besides the stage of manufacture the intensity of lysis can depend on the manufacturing conditions applied to a given type of cheese varieties. Actually in the case of the same variety of cheese, e.g., starter culture strains with different susceptibility to lysis can be applied (Wilkinson et al., 1995). In our experiment the manufacture of one of the most popular cheeses was investigated and the influence of the processing stages and

the selection of starter culture on the free D-amino acid content were investigated.

Materials and methods

Cheese manufacture

The conventional Cheddar cheese production was applied (Scott, 1998). The raw material of each trial was 100 l of bulkmilk from cows. The casein/fat ratio was adjusted between 0.7 and 1.0 after pasteurization (72 °C, 15 sec). The starter culture used was a 2% (v/v) inoculum of a single-strain *Lc. lactis* ssp. *cremoris* 303 or *Lc. lactis* ssp. *cremoris* AM2. The rennet was added when acidity reached 0.20–0.22%. After cutting the curd/whey the mixture was stirred and cooked (0.2 °C/min to 40 °C). Curds were pitched and after the whey was removed the curds were cheddared until the acidity of whey draining reached 0.68–0.85%. After milling the curd was salted at a rate of 2% (w/w), pressed (75 kPa, 16 h), vacuum wrapped and stored at 8 °C. The first sampling was carried out on the day of the beginning of the processing (0 day) after salting and before pressing. The next sample was taken after pressing (1 day), and the following ones were taken during ripening on the 7th, 28th and 63rd days. Samples were grated and stored at −24 °C after lyophilization. Cheese was produced at the Faculty of Food Science and Technology; strains were obtained from the culture bank of the Department of Microbiology, University College Cork (Ireland).

Chemical analysis

Lyophilized cheese samples were pulverized with a Microculatti grinder. Five grams of sample was weighed into a 100 ml Erlenmeyer flask and 20 ml of 0.1 M HCl was added. The suspensoid was stirred for 3 h with a magnetic stirrer then it was left to steep overnight at 5 °C. The following day the two-phase system was shaken up again, and then centrifuged at 500 *g* for 10 min. Protein was precipitated from the supernatant with equal volume of 25% (w/v) trichloroacetic acid solution with the final concentration of trichlorocetic acid of 12.5%. The suspensoid was centrifuged (500 *g*, 10 min) after 30-min standing. Then 4 ml from the supernatant were placed in a 10 ml volumetric flask and the solution was neutralized with 4 M NaOH solution following dilution with distilled water. The extract was filtered through a 0.45 µm membrane filter before analysis.

During precolumn derivatization with OPA (*o*-phthaldialdehyde) and TATG (1-thio-β-D-glucose tetraacetate) (Sigma, St. Louis, MO, USA) diastereoisomer pairs of the amino acids were produced (Einarsson et al., 1987; Csapó et al., 1995). Derivatization and analysis were carried out with a Merck-Hitachi HPLC comprised of a L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, and AIA data conversion utility for the D-7000 HPLC system manager. The compounds were separated on a 125 mm × 4 mm i.d. column packed with Superspher 60 RP-8e (Merck, Darmstadt, Germany). The initial mobile phase composition was 28% (v/v) methanol and 72% phosphate buffer (50 mM, pH 7). After ten-minute isocratic elution the ratio of acetonitrile was increased from 0 to 17% and the volume of phosphate buffer decreased from 72% to 55%. From the 40th minute the volume of acetonitrile was increased from 17 to 40% while the ratio of the phosphate buffer changed from 55 to 36% and that of methanol from 28 to 24%. The flow rate was 1 ml/min, and the oven temperature was 40 °C. Solvents (acetonitrile and methanol) were HPLC gradient grade (Merck, Darmstadt, Germany). The derivatives were detected with a fluorescence detector (λ_{ex} 325 nm, λ_{em} 420 nm).

Statistical analysis

Cheese manufacture was repeated three times with both strains (*Lc. lactis* ssp. *cremoris* 303 and *Lc. lactis* ssp. *cremoris* AM2). The influence of processing steps and the starter culture selection on the D-amino acid content of the products was evaluated with multiple analysis of variance. Besides the concentration of free D-amino acids, the D-amino acid composition [(D-amino acid/ΣD-amino acid) · 100] and the percentage of the D-enantiomer [(D/D + L) · 100] were calculated and regarded as variables.

The equation of the used linear model was the following:

$$Y_{ijk} = \mu + B_i + C_j + BC_{ij} + e_{ijk}$$

where

Y_{ijk} = the kth observation in the ijth treatment combination,
μ = the least squares mean,
B_i = the effect of the ith class of factor B (manufacturing step) expressed as a deviation from μ,
C_j = the effect of the jth class of factor C (strain) expressed as a deviation from μ,
BC_{ij} = the interaction effect of the ith class of factor B and the jth class of factor C expressed as a deviation from $\mu + B_i + C_j$ and
e_{ijk} = the random error associated with the kth observation in the ijth treatment combination.

In order to compare the influence of the cheesemaking steps on the of D-amino acid content within one processing protocol the manufactures with two different strains were separately evaluated with one way analysis of variance. In these particular cases the equation of the linear model was:

$$Y_{ij} = \mu + B_i + e_{ij}$$

where

Y_{ij} = the jth observation in the ith treatment,
μ = the least squares mean,
B_i = the effect of the ith class of factor B (manufacturing step) expressed as a deviation from μ,
e_{ij} = the random error associated with the jth observation in the ith treatment.

In case of significant difference among treatment means ($P < 0.05$) the comparison of that was accomplished with the Student-Newman-Keuls test. Data analysis was carried out with the use of SPSS for Windows 10.0 (1999) statistical program.

Results

Firstly the D-amino acid content of the semi-finished Cheddar cheeses was evaluated separately depending on the type of the starter (Table 1, part A and B). The D-Ala values at the beginning of the manufacture (day 0 and 1) were very close to the concentration that was determined in yoghurt and fresh cheese (Brückner and Hausch, 1990a). The amount of D-Ala showed a continuous increase during processing, especially in the ripening period. Though, the extent of this increment was not outstanding. By the time of the last sampling (9th week of ripening) it did not reach the values that were detected in ripened cheeses (Brückner and Hausch, 1990b). Probably the length of the ripening period was not enough for the accelerated release of this D-amino acid.

The D-Asp and the D-Glu content of curd slightly increased during pressing. As values were counted on the basis of dry material this change cannot be attributed to the

Table 1. The D-alanine, D-aspartic acid and D-glutamic acid content of semi-finished Cheddar cheese in the function of the stage of manufacture and the used starter strain (mg/100 g dry matter) (n = 3)

Examined amino acid	Elapsed time from the beginning of the manufacture (days)				
	0	1	7	28	63
	Stage of manufacture				
	Before pressing	After pressing	Ripening	Ripening	Ripening
A *Lactococcus lactis* subsp. *cremoris* 303					
D-Ala	$1.8^{a} \pm 0.8$	$2.4^{ab} \pm 0.5$	$3.1^{abc} \pm 0.9$	$3.8^{bc} \pm 0.8$	$4.5^{c} \pm 0.6$
D-Asp	$0.98^{a} \pm 0.33$	$1.9^{b} \pm 0.4$	$2.2^{b} \pm 0.6$	$2.2^{b} \pm 0.5$	$2.4^{b} \pm 0.4$
D-Glu	$4.1^{a} \pm 0.5$	$6.0^{b} \pm 0.4$	$6.6^{b} \pm 1.1$	$6.4^{b} \pm 0.8$	$6.4^{b} \pm 0.5$
B *Lactococcus lactis* subsp. *cremoris* AM2					
D-Ala	$1.0^{a} \pm 0.7$	$1.2^{a} \pm 0.6$	$1.7^{b} \pm 0.7$	$2.0^{b} \pm 1.0$	$2.3^{c} \pm 0.9$
D-Asp	$0.59^{a} \pm 0.31$	$1.1^{b} \pm 0.7$	$1.4^{b} \pm 0.7$	$1.5^{b} \pm 0.9$	$1.5^{b} \pm 0.8$
D-Glu	$2.7^{a} \pm 1.4$	$3.6^{a} \pm 2.4$	$4.3^{a} \pm 2.3$	$4.2^{a} \pm 3.1$	$4.1^{a} \pm 2.3$

^{abc} Averages in one row with common superscript do not differ ($P \geq 0.05$)

decrease of the water content. During pressing there is an acceleration of growth of the cell count (Scott, 1998) and the number of dead starter cells is theoretically low. The value of the pressure applied to form the curd into a shape was four orders of magnitude lower than pressure values used in order to reduce the cell count (Koncz et al., 2003). However, it cannot be excluded that more traits together such as pressing and the increase of salt content and that of osmotic pressure may induce the destruction of certain cells, or exert some stress on microorganisms which resulted in the production of more D-Asp and D-Glu.

Though these two amino acids are present in the cell wall (Schleifer and Kandler, 1972; Tipper and Wright, 1979), their concentration did not change significantly during the maturation of Cheddar until the 9th week. It is in agreement with the findings of others (Gandolfi et al., 1992, 1994), who stated that D-Ala is the first D-amino acid whose amount began to increase due to bacterial activity while the amount of D-Asp and D-Glu remained unchanged.

The six manufacturing processes with the two different strains were also evaluated together with multiple analyses of variance. In this case, with the exception of D-Ala, the influence of the processing steps has less effect on the D-amino acid content than the selection of the starter culture. The choice of strains exerted a significant effect on the D-Ala, D-Asp and D-Glu content ($P < 0.01$) of the semi-finished products (Table 2). Cheeses inoculated with *Lc. lactis* ssp. *cremoris* 303 contained more free D-amino acids than the ones acidified with *Lc. lactis* ssp. *cremoris* AM2. This result can be connected with the difference between strains in the D-amino acid formation capacity. Supposedly their susceptibility to autolysis is different, but this hypothesis could be accepted or rejected with the knowledge of the activity of intracellular enzymes.

The D-amino acid composition continuously changed during cheese manufacture. The ratio of D-Asp was slightly higher after pressing than prior to pressurization. During ripening the ratio of D-Ala increased, and that of D-Glu

Table 2. The influence of the stage of manufacture and the selection of starter culture strain on the D-alanine, D-aspartic acid and D-glutamic acid content and the D-amino acid composition of semi-finished Cheddar cheese

Factors	D-amino acid concentration (mg/100 g dry matter)				D-amino acid composition (D-amino acid/Σ D-amino acid) (%)		
	D-Ala	D-Asp	D-Glu	D-Asp + D-Glu + D-Ala	D-Ala	D-Asp	D-Glu
Manufacturing steps (B)	**	*	NS	NS	**	***	***
Strain (C)	***	**	***	**	NS	NS	NS
Interaction (B × C)	NS	NS	NS	NS	NS	NS	NS

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3. The free D-amino acid composition and the percentage ratio of the D-enantiomer of free amino acids in Cheddar cheese during manufacture (mg/100 g dry matter) (n = 6)

	Elapsed time from the beginning of the manufacture (days)				
	0	1	7	28	63
	Stage of manufacture				
	Before pressing	After pressing	Ripening	Ripening	Ripening
D-amino acid composition (%)					
D-Ala/ΣD-amino acid	$24^{ab} \pm 5$	$23^{a} \pm 3$	$25^{ab} \pm 3$	$29^{bc} \pm 3$	$32^{c} \pm 3$
D-Asp/ΣD-amino acid	$14^{a} \pm 1$	$18^{b} \pm 1$	$18^{b} \pm 1$	$19^{b} \pm 1$	$18^{b} \pm 1$
D-Glu/ΣD-amino acid	$62^{c} \pm 5$	$59^{c} \pm 3$	$57^{bc} \pm 3$	$53^{ab} \pm 3$	$50^{a} \pm 3$
D-enantiomer ratio $(D/D+L) \cdot 100$ (%)					
Ala	$54^{b} \pm 15$	$47^{a} \pm 13$	$48^{a} \pm 13$	$45^{a} \pm 13$	$44^{a} \pm 13$
Asp	$28^{a} \pm 8$	$36^{b} \pm 7$	$39^{b} \pm 7$	$38^{b} \pm 6$	$36^{b} \pm 7$
Glu	$25^{a} \pm 14$	$26^{a} \pm 7$	$52^{b} \pm 15$	$82^{c} \pm 4$	$81^{c} \pm 2$

[abc] Averages in one row with common superscript do not differ ($P \geq 0.05$)

decreased and the proportion of D-Asp remained unchanged (Table 3). Despite of the processing steps, the choice of the starter strain did not exert an effect on the D-amino acid pattern of the products (Table 2).

The percentage ratio of the D-enantiomer within a given free amino acid content was also calculated (Table 3). The ratio of D-Ala practically did not change during ripening because the concentration of both enantiomers increased at a similar rate and the ratio of the enantiomers was approximately 50–50%. While the release of D-Ala can be associated with the lysis of bacteria the appearance of free L-Ala is connected with the enzymatic proteolysis of paracasein owning to starter proteinases and peptidases hydrolyzing peptides to lower molecular weight peptides and free amino acids. The increase of the concentration of D-Ala followed the liberation of L-Ala originated from milk proteins. Due to this tendency the question of the origin of free D-Ala may arise. Some part of it could be originated from the cell wall, but besides this it may be formed from free L-Ala if the bacterial alanine racemase could operate outside the bacteria. If possible the L-D conversion could accelerate during ripening. With the greater extent of bacterial lysis the activity of the released racemase may increase, on the other hand the amount of its substrate (free L-Ala) also increases due to hydrolysis of milk proteins.

The ratio of the D-enantiomers in the free amino acid pool was higher ($P < 0.01$) in Cheddar cheeses inoculated with *Lc. lactis* ssp. *cremoris* 303 than in cheeses acidified with *Lc. lactis* ssp. *cremoris* AM2 because the D-amino acid content was higher in the first case, but the amount of the released L-enantiomer did not differ significantly among strains.

Acknowledgements

The cheese samples were produced at the Faculty of Food Science and Technology, University College Cork. The professional help of Prof. Dr. Patrick F. Fox, Dr. Paul McSweeney and Baukje Folkertsma is gratefully acknowledged. We are grateful to the Sapientia Foundation, Institute of Research Programs for the financial support.

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