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Lactobacillus reuteri DSM 20016: purification and characterization of a cystathionine γ-lyase and use as adjunct starter in cheesemaking

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Summary. A homo-tetrameric ~160-kDa cystathionine γ-lyase was purified to homogeneity from *Lactobacillus reuteri* DSM 20016 by four chromatographic steps. The activity was pyridoxal-5′-phosphate dependent and the enzyme catalyzed the α,γ-elimination reaction of L-cystathionine, producing L-cysteine, ammonia and α-ketobutyrate. The enzyme was active towards a range of amino acids and amino acid derivatives, including methionine. The pH and temperature optima were found to be 8·0 and 35 °C, respectively. Isoelectric pH (pI) was ~5·0 as determined by two-dimensional electrophoresis. Sensitivity to chemical inhibitors was typical of lactococcal cystathionine γ- and β-lyases, except it was inhibited by sulphydryl reagents. The N-terminal sequence was MKFNTQLIHGGNSED, which had 100% homology with cystathionine β-lyase of *Lb. reuteri* 104R (Accession Number CAC05298). *Lb. reuteri* DSM 20016, together with 10 other strains of non-starter lactic acid bacteria, was used as adjunct starter in the production of miniature Canestrato Pugliese-like cheeses. After 40 d ripening, the water-soluble extract of the cheeses with added *Lactobacillus fermentum* DT41 and *Lb. reuteri* DSM 20016 contained the highest enzyme activities on cystathionine and methionine substrates. Determinations of methanethiol, dimethyl sulphide, dimethyl disulphide and dimethyl trisulphide in the miniature cheeses confirmed the findings of enzyme activities.

Keywords: *Lactobacillus reuteri* DSM 20016, cystathionine γ-lyase, cheese.

Flavour development during cheese maturation is a very complex process, and is not entirely understood. After primary and secondary proteolysis, it is now widely believed that microbial enzymes involved in amino acid catabolism may play a key role in flavour development (Hemme *et al.* 1982; Weimer *et al.* 1999). These enzymes produce flavour compounds as a result of their catalytic breakdown of amino acids (McSweeney & Sousa, 2000).

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In particular, the catabolism of sulphur amino acids is considered important, as sulphur compounds are believed to be major actors in the development of a typical cheese flavour (Dias & Weimer, 1999). Together with transaminases and methionine γ-lyase, cystathionine lyases are key enzymes in sulphur metabolism. They essentially catabolise cystathionine by one of two mechanisms: α- and β-elimination resulting in the production of ammonia, pyruvate and homocysteine, or α- and γ-elimination which produces cysteine, α-ketobutyrate and ammonia. Both cystathionine-β-lyase (CβL; EC 4.4.1.8) and cystathionine-γ-lyase (CγL; EC 4.4.1.1) are pyridoxal-5′-phosphate-dependent enzymes (Alting *et al.* 1995; Bruinenberg *et al.* 1997; Smacchi & Gobbetti, 1998). Although the main physiological role of these enzymes is the production of cysteine and homocysteine from cystathionine, they can also produce methanethiol from methionine (Alting *et al.* 1995; Bruinenberg *et al.* 1997). Methanethiol and *S*-methylthioesters, resulting from the reaction between methanethiol and acyl CoAs, were recently judged as important in the aroma and flavour of both surface-ripened and internal bacterially ripened cheeses (Lamberet *et al.* 1997; Helinck *et al.* 2000).

Dias & Weimer (1998) showed cystathionine degrading ability in several strains of lactococci and lactobacilli. Cystathionine lyases have been isolated from a number of microorganisms including *Saccharomyces cerevisae* (Yamagata *et al.* 1993) and *Escherichia coli* (Laber *et al.* 1996). Alting *et al.* (1995) purified CβL from *Lactococcus lactis* subsp. *cremoris* B78, a starter strain used in the manufacture of Gouda-type cheeses. Purification and characterisation of CγL from *Lc. lactis* subsp. *cremoris* SK11 was reported by Bruinenberg *et al.* (1997). *Lactobacillus fermentum*, a strain associated with Italian and Swiss type cheese, produces CγL with high activity still detectable under cheese ripening conditions (Smacchi & Gobbetti, 1998). Dobric *et al.* (2000) reported on the cystathionine lyase of *Lc. lactis* subsp. *cremoris* MG1363 and its unique dual catalytic ability. This enzyme can carry out both α-, β- and α-, γ-elimination reactions on the same substrate.

We previously studied the amino acid catabolism in cheese-related bacteria such as non-starter lactic acid bacteria (NSLAB) and smear bacteria, which received lesser attention (Curtin *et al.* 2001). In particular, we found the greater cystathionine lyase activity in *Lb. reuteri* DSM 20016, *Brevibacterium linens* 10 and *Corynebacterium ammoniagenes* 8. A quadratic response surface methodology was applied to study the interactive effect of temperature, pH and NaCl on enzyme activity and we found that the cystathionine lyase activity of *Lb. reuteri* DSM 20016 well adapted to cheese-ripening conditions. *Lb. reuteri* is an obligatory heterofermentative bacterium (Hammes & Vogel, 1995) widely distributed in sourdoughs and cheeses, in the latter case as a component of the indigenous or adventitious microflora of milk. *Lb. reuteri* is also used as a probiotic strain in the manufacture of *Symbalance* yoghurt produced by the Swiss company, Tonilait.

This paper describes the purification and characterisation of a cystathionine γ-lyase enzyme from *Lb. reuteri* DSM 20016, and the use of *Lb. reuteri* DSM 20016 and other NSLAB strains in the manufacture of miniature cheeses to evaluate the cystathionine lyase activity during ripening.

Materials and methods

Chemicals

All amino acids and amino acid derivatives, inhibitors, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), EDTA, pyridoxal-5′-phospate (PLP) and markers for

two-dimensional-electrophoresis were from the Sigma Chemical Co. (St Louis, MO-63178, USA). Pre-packed fast protein liquid chromatography (FPLC) columns of DEAE-cellulose, Superose 12 HR 10/30, Phenyl-Superose 5/5, Mono-Q HR 5/5, protein mass standards for SDS-PAGE, reagents and immobiline strips for IPG-phore were from Amersham Pharmacia Biotech (S-75182, Upssala, Sweden). The SPB1-Sulphur column (30 m, 0·32 ID, 100% poly-dimethylpolysiloxane coated, 4 μm film thickness, fused silica capillary column) was from Supelco (Bellefonte, PA, USA). Unless otherwise stated, all others chemicals were obtained from Farmitalia Carlo Erba (I-20159, Milano, Italy).

Enzyme purification

Cells were harvested from 12 l of an overnight culture of *Lb. reuteri* DSM 20016 grown at 30 °C in MRS broth (De Man *et al.* 1960). Cytoplasmic extract was obtained by the method of Coolbear *et al.* (1992) with the following modifications: the extraction buffer 50 mM-Tris-HCl, pH 7·5 contained 1 mM-EDTA and 0·01 mM-PLP, and once resuspended in isotonic buffer, the spheroplasts were ultrasonically treated for 10 cycles of 30 s by using an Ultrasonic A180 G Instrument (PBI International, I-20153, Milano, Italy).

After freeze-drying (Edwards Mod IPTB; Edwards, I-20090, Trezzano, Italy), cytoplasmic extract was resuspended in 50 mM-potassium phosphate (KPi) buffer, pH 7·5, containing 1 mM-EDTA and 0·01 mM-PLP and applied to a DEAE-cellulose anion exchange column (55 × 1·6 cm inside diameter). Proteins were eluted by a linear gradient of 0–0·5 M-NaCl, at a flow rate of 90 ml/h. Fractions with enzyme activity were pooled, dialysed (Dialysis Tubing, cut-off 12000 Da) and concentrated by freeze-drying. After resuspension in 50 mM-KPi–1 mM-EDTA–0·01 mM-PLP buffer containing 0·15 M-NaCl, the sample was applied to an FPLC Superose 12 HR 10/30 gel filtration column. The same buffer with a flow rate of 0·3 ml/min was used to elute the proteins. Active fractions from gel filtration were further purified on an FPLC phenyl Superose 5/5 hydrophobic interaction column and a Mono Q HR 5/5 column as described by Smacchi & Gobbetti (1998).

Protein concentration during purification steps was determined by the method of Bradford (1976), using bovine serum albumin as standard.

Enzyme assays

CγL activity was assayed by measuring the amount of keto acids (Esaki & Soda, 1987), ammonia (DHFF CHAMB, I-20093, Milan, Italy) and free thiols (Ferchichi *et al.* 1985) formed from cystathionine. This latter method was used for further enzyme characterization. Under standard conditions, the reaction mixture consisted of 2 μl 5 mM-DTNB in ethanol, 25 μl 15 mM-L-cystathionine or other amino acid derivatives, 3·6 μl sodium azide (final concentration of 0·5 g/l), 300 μl enzyme preparation and 15·6 μl reaction buffer (50 mM-KPi–1 mM-EDTA–0·01 mM-PLP, pH 8·0). Controls without substrate and without enzyme sample were included and incubation was carried out at 35 °C. After 60 min of incubation the reaction mixture was centrifuged at 12000 ***g*** for 2 min, and absorbance of the supernatant was read at 412 nm. Increase of absorbance was calculated as difference between $O.D._{412}$ at 60 min and T_0. One unit of activity (U) was defined as the concentration (μM) of thiols produced in 10 min. Specific activity was defined as U per mg protein. The concentration of thiols was determined by a standard curve obtained with solutions of known concentrations of cysteine.

The method described by Ferchichi *et al.* (1985) was used to determine the enzyme

activity contained in the water-soluble extract (WSE) of the various miniature cheeses. The only modification in the reaction mixture was the use of 500 μl WSE instead of the enzyme preparation. Controls without substrate and without WSE were included. Specific activity was expressed as μM of thiols produced in 10 min per g of total cheese.

The presence of CβL and/or CγL activities was indirectly investigated by the ninhydrin method (Gaitonde, 1967; Yamagata *et al.* 1993) and subsequently confirmed by HPLC determination of cysteine and homocysteine as enzymatic end products. Separation was carried out with a Waters Corporation HPLC (Milford, Massachusetts, 01757, USA) equipped with a 600E pump, Diode Array detector and Millenium 32 software. A Nova-Pak C18 (3·9 × 300 mm, Waters Corporation) column was used and isocratic elution was performed with 0·1 g trifluoroacetic acid/l at a flow rate of 0·2 ml/min.

Effect of temperature on activity was determined by incubating the enzyme in 50 mM-KPi–1 mM–EDTA-0·01 mM-PLP buffer, pH 8·0 at temperatures between 10 and 45 °C. Heat stability and the pH optimum were assayed as described by Smacchi & Gobbetti (1998). Dependence on PLP for activity was established by dialysing the sample against 150 times volume of buffer (50 mM-KPi, pH 8·0) at 4 °C for 20 h. Activity was then assayed with and without the addition of PLP.

For substrate specificity studies, selected amino acids were substituted for cystathionine and activity was assayed under the standard conditions. The effect of inhibitors was determined by preincubating the enzyme for 10 mins at room temperature with the 2·5-and 5-mM concentrations of inhibitor and subsequent assay under standard conditions.

Molecular mass determination

Relative molecular mass of the purified enzyme was estimated both by FPLC gel filtration and by SDS-PAGE as described by Smacchi & Gobbetti (1998). Non-denaturating SDS-PAGE was performed with 4–15% acrylamide gradient. Proteins were stained by Comassie brilliant blue (Sigma). Low and high molecular mass marker proteins (Pharmacia) were used.

Isoelectric point (pI) determination

The isoelectric point (pI) was determined using two-dimensional (2D)-electrophoresis by using amyloglucosidase, pI 3·8; ovalbumin, pI 5·1; carbonic anhydrase, pI 7·0; and myoglobin, pI 7·6 as markers.

The purified fraction was directly resuspended in denaturating buffer composed of 8 M-urea, 40 g/l CHAPS (3-3 cholamidopropyl dimethylammonium-1-propane sulphonate), 40 mM-Tris base and 65 mM-dithioerythritol (DTE). Two-dimensional gel electrophoresis was performed using the immobiline/polyacrylamide system, essentially as described by Görg *et al.* (1988) and Hochstrasser *et al.* (1988). Isoelectric focusing was carried out on immobiline strips providing a non-linear 3–10 pH gradient by IPG-phore, at 15 °C. Voltage was increased from 300 to 5000 V during the 1st 5 h, then stabilized at 8000 V for 8 h. After electrophoresis, IPG strips were equilibrated for 12 min against 6 M-urea, 300 g glycerol/l, 20 g SDS/l, 0·05 M-Tris-HCl, pH 6·8, and 20 g DTE/l, and for 5 min, against 6 M-urea, 300 g glycerol/l, 20 g SDS/l, 0·05 M-Tris-HCl, pH 6·8, 25 g iodioacetamide/l, and 0·5% bromophenol blue. The second dimension was carried out with 12% polyacrylamide gels (18 cm × 20 cm × 1·5 mm), at 40 mA/gel constant current and at 10 °C for approximately 5 h until the dye front reached the bottom of the gel. Gels were silver stained as

described by Hochstrasser *et al.* (1988) and Oakley *et al.* (1980). Protein maps were scanned with a laser densitometer (Molecular Dynamics 300s) and analysed with the Image master 2D elite computer software (Pharmacia).

N-terminal amino acid sequence

Electroblotting was performed using a BioRad Mini Trans Blot system, and blotted onto polyvinylidene difluoride membrane (PVDF) according to Matsudaira (1987); the PVDF membrane was stained with Amido Black.

Membranes were air dried at 37 °C and stored at −20 °C for further analysis. Protein bands were cut out and their amino acid sequence determined by Edman degradation using an automatic Protein/Peptide Sequencer (mod 470A; Applied Biosystem Inc., Foster City, CA 94404, USA) connected on-line with a phenylthiohydantoin-amino acid analyser model 120A and a control/Data Module model 900A (Applied Biosystems Inc.).

The sequence comparison was performed by using EMBL/GenBank/DDBJ databases.

Manufacture of miniature cheeses

Miniature cheeses were manufactured according to the modified protocol of Rehman *et al.* (1998). Modifications concerned the use of ewes' milk, the amount of liquid rennet (0·3 ml/l of milk and the use of commercial *Lb. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* as starters ($\sim 10^7$ cfu/g of cheese milk). Curd was held at 42 °C for 2–3 h until pH 4·5–5·0 was reached and the cheeses were ripened for 40 d at 10–15 °C and environmental humidity 95 %. These modifications were in agreement with the large scale protocol used for the manufacture of Canestrato Pugliese, one of the most typical ewes' cheese of Southern Italy.

Lb. reuteri DSM 20016, *Lb. brevis* AM8, *Lb. alimentarius* O8, *Lb. confusus* 10XF1, *Lb. hilgardii* 51B, *Lb. casei* subsp. *pseudoplantarum* 2742, *Lb. curvatus* 2770, *Lb. pentosus* ATCC 8041, *Lb. fermentum* DT41, *Lb. paracasei* 3970 and *Lb. casei* subsp. *casei* 2764 were used as adjunct starters in miniature cheeses. Adjunct starters were cultivated in MRS broth at 30 or 37 °C for 24 h. Cells were harvested by centrifugation, washed twice with distilled water and resuspended in a small amount of pasteurised milk. Adjunct starters were co-inoculated with starter LAB to have an initial concentration of $\sim 10^8$ cfu/g of cheese milk. A control without adjunct starters was also manufactured. *Str. thermophilus* and lactobacilli (under anaerobiosis) were enumerated by incubating in M17 and MRS agar media at 42 °C for 48 h and at 30 and 42 °C, respectively. For lactobacilli, the results for plate counts were confirmed by microscopic observation and by physiological and biochemical assay (Hammes & Vogel, 1995) and API CHL System (bioMerieux, Marcy-l'Etoile, France). The APILAB Plus version 4.0 program (bioMerieux) was used to analyze the fermentation profiles obtained with the identification strips. Some isolated were also submitted to SDS-PAGE analysis of the cell wall protein profiles (De Angelis *et al.* 2001).

The WSE of each cheese was prepared according to the modified method of Kuchroo & Fox (1982). Modifications concerned the dialysis (dialysis tubing, cut-off 12000 Da) of the WSE for 24 h at 4 °C against 50 mM-KPi–1 mM-EDTA–0·01 mM-PLP buffer, pH 8·0, to eliminate interference of compounds contained in the extracts (e.g. free thiol groups) and sterile filtration (0·22 μm pore size, Syrfil Filter, Nucleopore, Costar Corporation, Cambridge, MA 02140, USA) in order to avoid interference due to cellular activity (Gobbetti *et al.* 1997). CγL activity of the pure cultures of

adjunct starters was also determined. Twenty-four-hour old cells of adjunct starters were cultivated in MRS broth (Difco), harvested by centrifugation, washed twice in 0·05 M-Tris-HCl, pH 7·5 and resuspended in the same buffer at an A_{620} of 1 (measured on a 1 : 10 diluted cell suspension). These cells suspensions (10^8 cfu/ml) were used to determine the CγL activities as descibed above.

Determination of volatile sulphur compounds

Methanethiol, dimethyl sulphide (DMS), dimethyl disulphide (DMDS) and dimethyl trisulphide (DMTS) in the miniature cheeses were determined as described by Purge and Trap Injector (PTI) (Chromapack, Middelburg, The Netherlands) High resolution (5890 series II-HP) Gas Chromatograph Mass selective A HP detector (MSD 5971) (Hewlett Packard, Palo Alto, California, USA) (PTI-HRGC-MS) integrated system. After ripening, a cheese slurry was produced by adding 10 ml distilled water and 3 g ice to 5 g cheese. Volatile sulphur compounds from the samples contained in a vessel were removed by the purging gas, passed through a condenser set to −10 °C and then focused in a cryogenic trap (wide bore Chromapack CP-SIL 5 CB column) pre-cooled to −120 °C. The trap was quickly heated to 200 °C and the collected volatiles were pushed into the top of a SPBI-Sulphur column (Supelco). Helium was used as the carrier gas at a flow rate of 1 ml/min. Initial column temperature of 30 °C was held for 1 min, increased to 200 °C at the rate of 10 deg C/min and maintained for 10 min. Peak area of the eluting compounds was determined using electronic integration software and the data were analysed on a personal computer. Compounds were identified by comparing retention times with authentic compounds. Volatile sulphur compounds were quantified by preparing standard curves with authenitc compounds.

Statistical analysis

Enzymatic measurement from three independent replicates were subjected to one way analysis of variance at the Computer Centre of the University of Perugia using SAS (1985); for multiple comparison the Tukey test was used and the alpha value for all experiments was set at 0·05.

RESULTS

Enzyme characterization

The enzyme was purified 19·5 fold in a four-step procedure with ∼ 5% recovery. After the final anion-exchange on Mono Q column, SDS-PAGE showed a single protein band stained at the same position as the marker protein of 40 kDa (Fig. 1). A single peak at a position corresponding to ∼ 160 kDa was eluted by gel filtration on Superose 12; non-denaturating SDS-PAGE also yielded a single protein with a molecular mass of approximately 157 kDa (data not shown), thus leading to the presumption that the enzyme consisted of four identical subunits of ∼ 40 kDa. Most of the biochemical characteristics of the enzyme were virtually identical to that found for the purified CγL from *Lb. fermentum* DT41 (Smacchi & Gobbetti, 1998): molecular mass, pH (8·0) and temperature 35 °C optima, heat stability (complete inactivation after heating at 60 °C for 5 min) and PLP-dependent activity. At pH 6·0 and 5·0, the enzyme had a relative activity of 40 and 20%, respectively, while at pH 9·0 it maintained ∼ 90% of the maximum activity. Approximately 50% of the maximum activity was maintained at 15 °C.

CγL of *Lb. reuteri* DSM 20016 was active towards all the amino acids and amino acid derivatives used. Activity towards the following substrates decreased in the order: L-cystathionine > DL-cystathionine > L-djenkolic acid > L-cystine > L-cys-

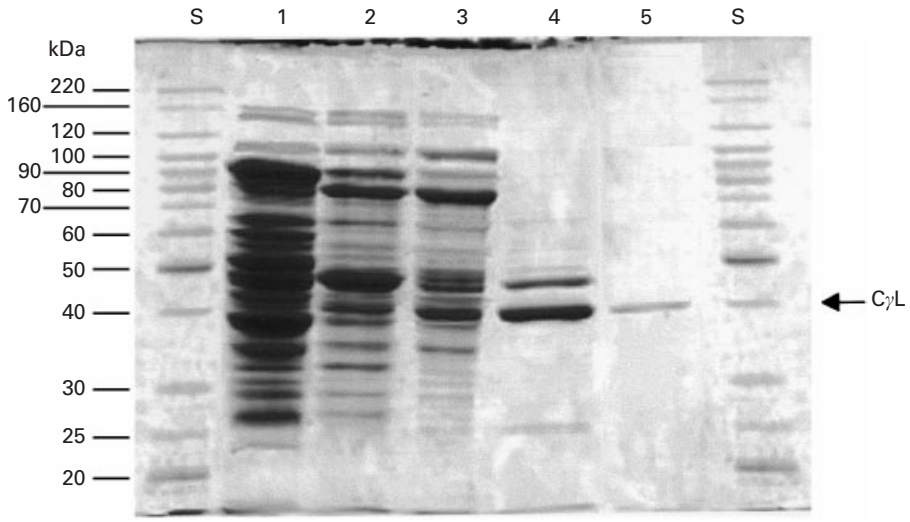

Fig. 1. SDS-PAGE (12% polyacrylamide) with Coomassie Blue staining showing the different purification steps of the cystathionine lyase from *Lactobacillus reuteri* DSM 20016. Lane S: Standard. Lanes 1–5, cytoplasmic extract, fractions obtained after chromatography on DEAE Cellulose, Superose 12, Phenyl Superose and Mono Q. The position of cystathionine γ-lyase is indicated by an arrow.

Table 1. *Substrate specificity of the cystathionine γ-lyase of* Lactobacillus reuteri *DSM 20016*

(Values are means for $n = 3$)

Substrate	Relative activity[1], %
L-Cystathionine	100[a]
DL-Cystathionine	95[b]
L-Djenkolic acid	77·9[c]
L-Cystine	70·1[d]
L-Cysteine	39·6[e]
L-Methionine	32·3[f]
L-Serine	21·3[g]

[1] The relative activity on amino acids and amino acids derivatives was calculated assuming the activity on L-cystathionine to be 100% which was equivalent to 100 U/mg protein. One unit of activity (U) is defined as μM of thiols formed in 10 min.

[a,b,c,d,e,f,g] Values in the same column without a common superscript were significantly different: $P < 0{\cdot}05$.

teine > L-methionine > L-serine (Table 1). Enzymatic degradation of these substrates always resulted in the formation of a free thiol group, a keto acid component and ammonia as reaction products. L-Cysteine, as determined by HPLC analysis, and not L-homocysteine was produced by degradation of L-cystathionine (Fig. 2*a*, *b*). These results indicated that the bacterium possesses CγL activity. As shown in Table 1, L-cysteine is in turn actively degraded by the CγL activity of *Lb. reuteri* DSM 20016.

Carbonyl reagents such as MBHT, semicarbazide, DL-penicillamine and phenylhydrazine markedly decreased the enzyme activity, especially at the highest concentration (5 mM) assayed (Table 2). Other known inhibitors of PLP-dependent enzymes such as DL-propargylglycine caused inhibition at both 2·5 and 5 mM concentrations. The sulphydryl reagents, NEM and iodoacetamide, were also inhibitory at both the concentrations assayed. The chelating agent EDTA was not inhibitory. At 50 g NaCl/l the enzyme retained 58·7% activity.

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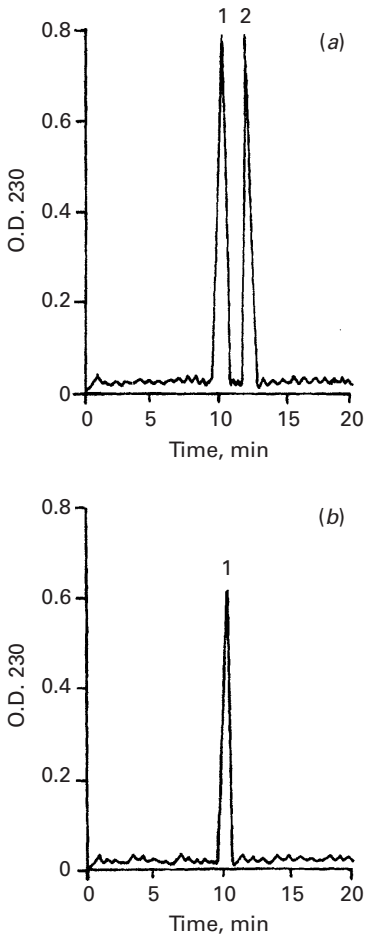

Fig. 2. HPLC separation of DTNB-derivatized products: (*a*) L-cysteine (1) and L-homo-cysteine (2) reacted with DTNB, (*b*) purified enzyme reaction using cystathionine as a substrate, with DTNB, for 60 min.

As determined by 2D-electrophoresis, the isoelectric point (pI) of the CγL of *Lb. reuteri* DSM 20016 was found to be $\sim$ 5·0.

The N-terminal sequence of the CγL of *Lb. reuteri* DSM 20016 was MKFNTQLIHGGNSED. Sequence comparison performed on the EMBL/GenBank/DDBJ databases revealed 100 % homology with CβL of *Lb. reuteri* 104R (Accession Number CAC05298).

Manufacture of miniature cheeses

Cystathionine lyase activities of the pure cultures of adjunct starters (10^8 cfu/ml), used in the production of miniature cheeses, on cystathionine and methionine substrates varied. It was greatest in *Lb. fermentum* DT41 and *Lb. reuteri* DSM 20016 (6·84 and 5·3 U/g and 2·8 and 1·6 U/g, respectively) and ranged between 0 and 1·78 for all the other strains. After 40 d ripening, all the cheeses had 28–30 % moisture, 32–34 % fat, 25–26 % protein and 20–25 % pH 4·6-soluble nitrogen/total nitrogen. These values were in agreement with the average values commonly found in industrial manufacture of Canestrato Pugliese cheese (Albenzio *et al.* 2001). The concentration of starter cultures decreased from 10^8 cfu/g after 1 d manufacture

Table 2. *Effect of inhibitors on the activity of the cystathionine γ-lyase from* Lactobacillus reuteri *DSM 20016*

(Values are means for $n = 3$)

	Relative activity[2]	
Inhibitors[1]	2·5 mM[3]	5 mM[3]
NEM	24[f]	19[d]
Iodacetamide	22·6[g]	n.d.
MBTH	76·5[b]	31[c]
Semicarbazide	36·2[d]	10·7[e]
EDTA	102[a]	94·5[a]
DL-Penicillamine	23·9[f]	3·8[f]
Phenylhydrazine	66[c]	2·8[g]
DL-Propargylglycine	28·3[e]	1·9[h]
NaCl[4]	100[a]	58·7[b]

[1] NEM, N-ethylmaleimide; MBTH, 3-methyl-2-benzothiazolinone; EDTA, ethylenediaminetetra acetic acid.

[2] Relative activity on amino acid and amino acid derivatives was calculated assuming the activity on L-cystathionine to be 100% which was equivalent to 100 U/mg of protein. One unit of activity (U) is defined as μM of thiols formed in 10 min.

[3] Concentration of inhibitor.

[a,b,c,d,e,f,g,h] Values in the same column without a common superscript were significantly different: $P < 0·05$.

[4] Assayed at 25 and 50 g/l.

n.d., not detected.

Table 3. *Cystathionine lyase activity contained in the water soluble extracts of miniature cheeses produced with several lactobacilli as adjunct starters*

(Values are means for $n = 3$)

Adjunct starters	Activity on L-Cystathionine, U/g[1]	Activity on L-Methionine, U/g
Lb. fermentum DT41	5·6[a]	1·84[a]
Lb. reuteri DSM 20016	4·33[b]	0·79[b]
Lb. hilgardii 51B	1·43[c]	0·1[d]
Lb. confusus 10XF1	0·92[d]	0·36[c]
Lb. alimentarius 08	0·7[e]	n.d.
Lb. pentosus ATCC 8041	0·19[f]	0·1[d]
Lb. casei subsp. *casei* 2764	0·11[g]	0·02[e]
Lb. casei subsp. *pseudoplantarum* 2742	0·09[g]	n.d.
Lb. paracasei 3970	n.d.	n.d.
Lb. brevis AM8	n.d.	n.d.
Lb. curvatus 2770	n.d.	n.d.
Control cheese[2]	n.d.	n.d.

[1] One unit of activity (U) is define as μM of thiols formed in 10 min.

[2] Control cheese without adjunct starters.

n.d., not detected.

[a,b,c,d,e,f,g] Values in the same column without a common superscript were significantly different: $P < 0·05$.

to $\sim 5 \times 10^6$ cfu/g at the end of ripening, while all the adjunct starters had cell numbers of $\sim 5 \times 10^8$ cfu/g throughout ripening (data not shown). The control cheese without adjunct starters reached a NSLAB level of 10^6 cfu/g, only after 40 d ripening. At this stage of ripening, WSE from cheeses were produced and assayed for cystathionine lyase activity on cystathionine and methionine substrates (Table 3). The enzyme activity on both substrates was greater in cheeses containing *Lb. fermentum* DT41 and *Lb. reuteri* DSM 20016 (5·6 and 4·33 U/g and 1·84 and 0·79 U/g, respectively) and lower in cheeses with all the other adjunct starters. Miniature

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Table 4. *Methanethiol, dimethyl sulfide (DMS), dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) concentrations (pg/g) in the miniature cheeses produced with several lactobacilli as adjunct starters*

(Values are means for $n = 3$)

Adjunct starters	Methanethiol, pg/g	DMS, pg/g	DMDS, pg/g	DMTS, pg/g
Lb. fermentum DT41	2·0[a]	3·0[a]	32·2[a]	0·6[a]
Lb. reuteri DSM 20016	1·5[b]	2·2[b]	24·7[b]	0·4[b]
Lb. confusus 10XF1	0·8[c]	0·3[d]	5·2[d]	n.d.
Lb. hilgardii 51B	0·8[c]	0·4[c]	8·5[c]	0·1
Lb. alimentarius 08	0·5[d]	0·3[d]	5·2[d]	n.d.
Lb. brevis AM8	0·2[e]	n.d.	5·2[d]	n.d.
Lb. casei subsp. *pseudoplantarum* 2742	0·1[f]	0·2[e]	3·0[e]	n.d.
Lb. curvatus 2770	0·1[f]	n.d.	2·4[f]	n.d.
Lb. pentosus ATCC 8041	0·1[f]	n.d.	2·4[f]	n.d.
Lb. casei subsp. *casei* 2764	0·1[f]	n.d.	2·0[g]	n.d.
Lb. paracasei 3970	n.d.	n.d.	2·0[g]	n.d.
Control cheese	n.d.	n.d.	2·5[f]	n.d.

n.d., not detected.
[a,b,c,d,e,f,g] Values in the same column without a common superscript were significantly different: $P < 0·05$.

cheese produced without adjunct starters did not contain detectable cystathione lyase activity. These results agreed with the determination of volatile sulphur compounds in miniature cheeses (Table 4). Methanetiol, DMS, DMDS and DMTS were always present at the highest concentrations when *Lb. reuteri* DSM 20016 and *Lb. fermentum* DT41 were used as adjunct starters. In comparison, the other cheeses contained very low levels of these volatile sulphur compounds which only slightly differed from those of the control cheese. Other unknown volatile sulphur compounds were present in the elution profile of miniature cheeses produced *Lb. reuteri* DSM 20016 and *Lb. fermentum* DT41 as adjunct starters.

Discussion

After four chromatographic steps a ~ 160 kDa homo-tetrameric CγL of *Lb. reuteri* DSM 20016 was purified to homogeneity. Its N-terminal sequence shares 100 % homology with the CβL of *Lb. reuteri* 104R. The sequence related to *Lb. reuteri* 104R was submitted by Satoh *et al.* (2000) on the EMBL database but no reports on the biochemical characterization of the enzyme were published. Our study showed that α-ketobutyrate, ammonia and L-cysteine and not homocysteine were produced from the hydrolysis of cystathionine, thus defining as a CγL and not CβL the enzyme of *Lb. reuteri* DSM 20016 (Weimer *et al.* 1999).

To our knowledge, CγL enzymes have only been isolated from *Sac. cerevisae* (Yamagata *et al.* 1993), *Lc. lactis* subsp. *cremoris* SK11 (Bruinenberg *et al.* 1997) and *Lb. fermentum* DT41 (Smacchi & Gobbetti, 1998). In a recent paper, Dobric *et al.* (2000) reported on a cystathionine lyase from *Lc. lactis* subsp. *cremoris* MG1363 which, uniquely, was capable of both α, γ and α, β elimination reactions on the same substrate, thus possessing both CγL and CβL activities.

CγL of *Lb. reuteri* DSM 20016 had several biochemical features (e.g. pH and temperature optima, inhibition by carbonyl reagents and DL-propargylglycine and tetrameric form consisting of 4 subunits with molecular mass of approximately 35–48 kDa) in common with other CγL and CβL enzymes (Alting *et al.* 1995; Laber *et al.* 1996) and in particular with the CγL of *Lb. fermentum* DT41 (Smacchi &

Gobbetti, 1998). The operon for cysteine catabolism of *Lb. fermentum* strain BR11 has been sequenced (Turner *et al.* 1997). It is organised like that of *Lb. reuteri* 104R (Satoh *et al.* 2000) and the putative protein sequences of the CγL, transmembrane protein, ATP-binding protein and surface protein mapA are virtually identical. In particular, the comparison between the available CγL DNA sequences revealed 88% of homology thus justifying the elevated biochemical similarity. Nevertheless, some features seemed to distinguish CγL of *Lb. reuteri* DSM 20016 from the other enzymes studied. It retained ~ 40% of the maximum activity in the presence of 5% NaCl, while the enzyme of *Lc. lactis* subsp. *cremoris* SK11 was more sensitive (two-fold reduction in activity by 5% NaCl; Bruinenberg *et al.* 1997). The CβL of *Lc. lactis* subsp. *cremoris* B78 (Alting *et al.* 1995) was reported to experience a slight reduction in activity in the presence of 4% NaCl. CγL of *Lb. reuteri* DSM 20016 had 77% residual activity on cystine whereas this amino acid was the preferred substrate for the CγL of *Lb. fermentum* DT41 and *Lc. lactis* subp. *cremoris* MG1363 (Dobric *et al.* 2000; Smacchi & Gobbetti, 1998). Differently from other CγL enzymes (Alting *et al.* 1995; Bruinenberg *et al.* 1997; Smacchi & Gobbetti, 1998; Dobric *et al.* 2000), sulphydryl reagents did affect the activity of CγL of *Lb. reuteri* DSM 20016 suggesting that thiol groups may be significant for activity. The isoelectric point (pI) was determined only on the CγL from *Sac. cerevisae* (Yamagata *et al.* 1993) which agreed with those reported for the enzyme of this study (pI of ~ 5·0).

Metabolism of sulphur in bacteria associated with cheese is a topic of interest. Volatile sulphur compounds, especially methanethiol, are well correlated with the desirable flavour of several cheeses. In spite of the cited studies on purification and characterization of enzymes responsible for the amino acid catabolism, contradictory results were found when these enzyme activities were determined in the cheese matrix (Weimer *et al.* 1999). In particular, the importance and especially the release of CγL and CβL enzymes in cheese is only partially known. Since these are enzymes used in the biosynthetic pathways, it is reasonable to suspect that they may be regulated by extracellular cysteine and/or methionine concentration and be expressed at low levels in the cells. Compared to the optimum activity on cystathionine, the CγL of *Lb. reuteri* DSM 20016 showed markedly higher hydrolysis of methionine than enzymes of *Lc. lactis* subsp. *cremoris* SK11 and B78 (32·3 vs. 1·0%; Alting *et al.* 1995; Bruinenberg *et al.* 1997). In this study, we also investigated the cystathionine lyase activities and related volatile sulphur compounds contained in the water-soluble extracts of Canestrato Pugliese-like miniature cheese with several lactobacilli used as adjunct starters. Within the 11 strains of adjunct starters screened, the highest activities on both cystathionine and methionine substrates were found in the cheeses with added *Lb. fermentum* DT41 and *Lb. reuteri* DSM 20016. These results totally agreed with the determination of methanethiol, DMS, DMDS and DMTS in the cheese matrix.

Lb. reuteri is distributed in cheeses as a component of the NSLAB microflora and is well known for its antimicrobial and probiotic activity (Ganzle *et al.* 2000; Saavedra, 2000; O'Sullivan *et al.* 1992).

As shown in this study, its use in addition to starter cultures may have practical implications for cheese ripening to boost the amount of CγL enzymes and related sulphur compounds in the cheese matrix during ageing.

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