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Purification and Characterization of Cystathionine β-Lyase from *Lactococcus lactis* subsp. *cremoris* B78 and Its Possible Role in Flavor Development in Cheese

ARNO C. ALTING,* WIM J. M. ENGELS, SASKIA VAN SCHALKWIJK, AND FRED A. EXTERKATE

Department of Biophysical Chemistry, Netherlands Institute for Dairy Research (NIZO), 6710 BA Ede, The Netherlands

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An enzyme that degrades sulfur-containing amino acids was purified from *Lactococcus lactis* subsp. *cremoris* B78; this strain was isolated from a mixed-strain, mesophilic starter culture used for the production of Gouda cheese. The enzyme has features of a cystathionine β-lyase (EC 4.4.1.8), a pyridoxal-5′-phosphate-dependent enzyme involved in the biosynthesis of methionine and catalyzing an α,β-elimination reaction. It is able to catalyze an α,γ-elimination reaction as well, which in the case of methionine, results in the production of methanethiol, a putative precursor of important flavor compounds in cheese. The native enzyme has a molecular mass of approximately 130 to 165 kDa and consists of four identical subunits of 35 to 40 kDa. The enzyme is relatively thermostable and has a pH optimum for activity around 8.0; it is still active under cheese-ripening conditions, viz., pH 5.2 to 5.4 and 4% (wt/vol) NaCl. A possible essential role of the enzyme in flavor development in cheese is suggested.

Proteolysis in Gouda cheese is essential for the ultimate generation of the typical cheese flavor. Chymosin, the main constituent of rennet, is primarily responsible for initial cleavage of caseins in cheese (43), while the action of the lactococcal cell envelope proteinase is essential for an efficient secondary proteolysis, leading to the formation of small peptides and amino acids (17, 18) and for normal flavor development (37).

The typical flavor of Gouda cheese and of other cheese types is recovered in the water-soluble fraction of the cheeses (12). The results of fractionation experiments have shown that low-molecular-mass components (<500 Da) are responsible for these typical cheese flavors. The water-soluble fraction contains small peptides, amino acids, free fatty acids, and possibly degradation products thereof. Although it is known that peptides can taste bitter (28) or delicious (44) and that amino acids can taste sweet, bitter, or broth-like (33), the direct contribution of peptides and amino acids to flavor is probably limited to a basic taste. The components responsible for the typical cheese flavor are assumed to be part of the volatile fraction, and they may be associated with proteolysis via the production of specific amino acids, which are the precursors of these flavor components. In fact, a Gouda cheese-like flavor can be generated by incubation of methionine with cell extract of *Lactococcus lactis* subsp. *cremoris* B78 (13).

The degradation of amino acids may take place via enzymic pathways involving different types of enzymes, which either act as a deaminase, decarboxylase, or transaminase or convert amino acid side chains (24, 26). Since such enzymes are likely to be located intracellularly, lysis or permeabilization of starter bacteria is a prerequisite for the accessibility of these enzymes to external substrates. In fact, the appearance in a young Gouda cheese of specific degradation products of the action of an intracellular endopeptidase indicates lysis of starter bacteria at an early stage of the cheese-ripening process (2, 17).

As part of our work on the relationship between proteolysis and flavor development in Gouda cheese (16–18), we are studying the enzymic degradation of amino acids by lactococci, which is supposed to be an important step in the production of flavor components. In this paper, the isolation, purification, and characterization of an enzyme with features of cystathionine β-lyase from *L. lactis* subsp. *cremoris* are described. The enzyme is able to degrade various sulfur-containing amino acids and is active under cheese-ripening conditions.

MATERIALS AND METHODS

Chemicals. All amino acids and amino acid derivatives, pyruvic acid, iodoacetic acid, iodoacetamide, hydroxylamine, DL-penicillamine, phenylhydrazine, DL-cycloserine, and semicarbazide were obtained from Sigma Chemicals (St. Louis, Mo.); EDTA was obtained from BDH Limited (Poole, United Kingdom); carboxymethoxylamine, 3-methyl-2-benzothiazolinonhydrazone hydrochloride hydrate, and 4-methylthio-2-oxobutyric acid (KMBA) were obtained from Aldrich-Chemie GmbH & Co. (Steinheim, Germany); 5,5′-dithiobis-(2-nitrobenzoic acid) was obtained from Janssen Chimica (Beerse, Belgium); pyridoxal-5′-phosphate (PLP) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany); *N*-ethylmaleimide was obtained from Merck (Darmstadt, Germany); and nisin A was purified from Nisaplin (nisin A content, 2.5% [wt/wt]; Aplin & Barrett, Trowbridge, United Kingdom) (36).

Organism and growth. Strain B78 was isolated from the mixed-strain, mesophilic starter culture Bos (used in The Netherlands for the production of Gouda-type cheese). This DL-type starter consists of mainly *L. lactis* subsp. *cremoris* strains, *Lactococcus lactis* subsp. *lactis*, and the citric acid-fermenting *L. lactis* subsp. *lactis* bv. diacetylactis (D) and *Leuconostoc* (L) strains. Strain B78 was identified as *L. lactis* subsp. *cremoris*. The organism was grown (overnight at 30°C; final pH, 4.6 to 4.8) in milk and was subsequently harvested as described by Exterkate (15). The organism requires methionine for its growth in the methionine assay medium of Difco (Detroit, Mich.).

Purification. Washed cells from a 7.5-liter overnight culture, suspended in 150 ml of a 20 mM potassium phosphate (KP_i) buffer (pH 7.5) containing 1 mM EDTA and 20 μM PLP, were frozen in liquid nitrogen, and after thawing, the cells were disrupted by sonication of the suspension for 16 periods of 15 s (on ice, thereby keeping the temperature below 10°C) with the XL2020 sonicator of Heat Systems Ultrasonics (New York, N.Y.). The disrupted cells were centrifuged for 30 min at 30,000 × *g* in a J2-21M centrifuge (Beckman Instruments, Palo Alto, Calif.) to remove intact cells and cell particles from the crude cell extract. Solid ammonium sulfate was added to the cell extract to a final concentration of 35% (wt/vol). Insoluble material was removed by centrifugation (30,000 × *g*), and additional ammonium sulfate was added to a final concentration of 55% (wt/vol) to precipitate the enzyme-containing material. After a subsequent centrifugation step, the precipitate was collected and dissolved in the KP_i-EDTA-PLP buffer

* Corresponding author. Mailing address: NIZO, P.O. Box 20, 6710 BA Ede, The Netherlands. Phone: 31-318-659534. Fax: 31-318-650400.

described above. This solution was extensively washed and concentrated in the same buffer with a 200-ml ultrafiltration cell (Amicon Corporation, Danvers, Mass.) equipped with a 30-kDa cutoff Omega membrane (Filtron, Northborough, Mass.). The retentate was filtered through a 0.22-μm-pore-size filter (Millipore Corporation, Bedford, Mass.) and injected (2-ml fractions) onto a MonoQ HR 5/5 (Pharmacia, Uppsala, Sweden) anion-exchange column equilibrated with the KP_i-EDTA-PLP buffer. Proteins were eluted at 1 ml min^{-1} with a 0.1 to 0.45 mM NaCl gradient in the same buffer. Fractions containing the enzyme activity were pooled, concentrated, and filtered as described above and subsequently injected (500-μl fractions) onto a Superose 12 HR 10/30 (Pharmacia) gel filtration column equilibrated with KP_i-EDTA-PLP buffer. After elution at 0.4 ml min^{-1}, the fractions containing the enzyme activity were pooled. The column was calibrated with the following marker proteins: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa [plus dimer, 136 kDa]), ovalbumin (45 kDa), β-lactoglobulin (36-kDa dimer), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). All were obtained from Pharmacia (except for β-lactoglobulin, which was purified from whey). All steps were carried out at 0 to 4°C, and enzyme fractions were stored at −20°C.

Enzyme assays. In order to trace the enzyme activity during the different purification steps and to characterize the enzyme, we routinely used the method described by Esaki and Soda (14) for the determination of keto acid production (for characterization, a protein concentration of 7 μg ml^{-1} in a final volume of 500 μl was used) and the method described by Uren (41) for the determination of thiol formation (for characterization, a protein concentration of 3.5 μg ml^{-1} in a final volume of 1 ml was used). In both cases, cystathionine (5 mM, final concentration) was used as a substrate and the incubation mixtures in 0.1 M KP_i–20 μM PLP (pH 8.0) were held at 30°C for 15 min. For the specificity studies with the purified enzyme, other amino acids and amino acid derivatives were used as the substrate under similar conditions. The influences of inhibitors were established after a 10-min preincubation of the purified enzyme fraction at room temperature with different inhibitors at the indicated concentrations at pH 8.0. To investigate the pH dependence, two buffer systems were used: KP_i-Tris-glycine (pH 6.0 to 9.5) and sodium acetate-KP_i-Tris (pH 4.5 to 8.0), at a concentration of 0.05 M for each constituent. The temperature sensitivity (stability and optimum) was determined at the pH optimum (pH 8.0). The thermal stability of the enzyme was examined by heating of samples in buffer (0.1 M KP_i–20 μM PLP [pH 7.0]) for 10 min at various temperatures (20 to 70°C) prior to the assay. Ammonia production was measured by the ammonia determination test of Boehringer. A Cary 1E UV-VIS spectrophotometer (Varian, Victoria, Australia) was used in all cases.

Identification of reaction products. Substrate degradation was monitored and product identification was performed with an LKB type 451 amino acid analyzer (Pharmacia). Dynamic headspace gas chromatographic (GC) analyses were performed for identification of the volatile sulfur-containing compounds formed during the degradation of methionine by the action of the purified enzyme. For that purpose, a 10-ml sample was purged with helium (150 ml/min, 30 min) at room temperature, and the volatile compounds were collected on an adsorption trap packed with Carbotrap (80 mg) and Carbosieve SIII (10 mg). After the sampling procedure, the different compounds were desorbed (10 min, 250°C) and cryofocused (−100°C) onto the head of the capillary column of the GC with a thermal-desorption-cold trap injection device (Chrompack, Middelburg, The Netherlands) connected to a Carlo Erba MEGA 5360 GC equipped with a flame-photometric detector (SSD 250) in the sulfur mode (Carlo Erba, Milan, Italy). GC separations were performed with a fused silica capillary column (60 m by 0.32 mm) coated with DB1 (df, 1.0 μm) in stationary phase (J&W Scientific, Folsom, Calif.). During analysis, the oven temperature was held at 40°C for 1 min and then raised (10°C min^{-1}) to 50°C and kept at this temperature for 1 min, raised (2°C min^{-1}) to 60°C, and then programmed at a rate of 10°C min^{-1} to 245°C; this temperature was maintained for 25 min. Identification of sulfur-containing compounds was achieved with retention times of standard compounds. Thin-layer chromatography (TLC) was used to study the possibility of KMBA formation from methionine by the action of the purified enzyme. Samples were withdrawn from an incubation mixture and applied to a cellulose TLC plate (Merck) and developed in butanol-acetone-dimethylamine (40% aqueous solution)-water (40:40:8:20 [vol/vol]). Methionine and KMBA were applied as standards. Both could be located by first spraying the TLC plate with 0.1 M potassium bichromate solution in water-acetic acid (1:1 [vol/vol]) and then spraying it with 0.1 M silver nitrate solution in water. Sulfur-containing compounds appeared as yellow derivatives.

Protein quantitation. Protein concentrations were estimated by the micromethod of Bradford (4) with the Coomassie protein assay reagent and the instructions of the Pierce Chemical Company (Rockford, Ill.) and with crystalline serum albumin (fraction V; BDH) as the standard.

PAGE. Polyacrylamide gel electrophoresis (PAGE) was performed with a Phast system, a Midget cell (Pharmacia), or a Mini Protean II cell (Bio-Rad Laboratories, Hercules, Calif.), all according to the respective instruction manuals. Sodium dodecyl sulfate (SDS)-PAGE was carried out with 12.5% polyacrylamide gels with either the Phast system or the Midget cell. Native PAGE was performed with the Mini Protean II cell in combination with a 4 to 15% gradient Mini Protean II ready gel (Bio-Rad). The proteins were visualized by Coomassie brilliant blue staining or silver staining with Phastgel blue R and the Phastgel silver kit (Pharmacia), respectively. Low- and high-molecular-mass marker proteins (Pharmacia) were used as references.

TABLE 1. Purification scheme for the cystathionine β-lyase activity of *L. lactis* subsp. *cremoris* B78

Step	Total protein (mg)	Total activity (U[a])	Sp act (U/mg)	Purification (fold)	Recovery (%)
Cell extract	980	13.2	0.014	1.0	100
Ammonium sulfate precipitation	185	12.2	0.066	4.7	92
Anion-exchange chromatography	8.4	9.3	1.1	76	71
Gel filtration chromatography	1.7	3.5	2.1	150	21

[a] One unit (U) is defined as 1 μmol of thiol generated per min.

RESULTS

Purification. Table 1 shows the purification scheme of cystathionine lyase. Most of the methionine-converting activity in the crude cell extract appeared to be copurified with this lyase (described below). The enzyme was purified 150-fold in a three-step procedure with 21% recovery. After desalting of the various ammonium sulfate fractions, most of the thiol-generating activity appeared to be present in the 35 to 55% ammonium sulfate (wt/vol) fraction. With anion-exchange chromatography for the next purification step, all of the activity was eluted between 0.35 and 0.38 M NaCl. In the final gel filtration step, most activity was eluted at 165 kDa; the purest fraction was used for characterization.

The protein was judged to be homogeneous by SDS-PAGE (12.5% polyacrylamide) stained with Coomassie brilliant blue (Fig. 1A). Two additional bands, corresponding to low-molecular-mass proteins, could be detected with the more sensitive silver staining (Fig. 1B). After separation of the gel filtration fraction in a native 4 to 15% gradient polyacrylamide gel, the single band running at an apparent molecular mass of 130 kDa was cut out of the gel, and the protein was then extracted with 20 mM KP_i (pH 7.5)–20 μM PLP as extraction buffer. This fraction contained both the cystathioninase and methioninase activities and appeared as a single band with a size of 35 to 40

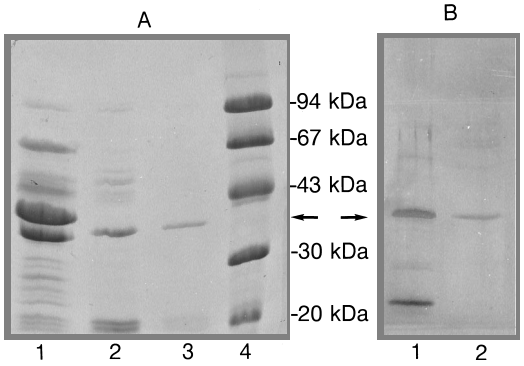

FIG. 1. SDS-PAGE (12.5% polyacrylamide) in combination with Coomassie brilliant blue staining (A) or silver staining (B) showing the different purification steps of the cystathionine β-lyase activity. (A) Lanes 1 to 4, the 35 to 55% AS fraction, the MonoQ fraction, the Superose 12 fraction, and the Pharmacia low-molecular-mass markers, respectively. (B) Lanes 1 to 2, the Superose 12 fraction and the fraction extracted from the 130-kDa band (4 to 15% gradient PAGE), respectively. The position of cystathionine β-lyase is indicated by the arrows.

TABLE 2. Substrate specificity of the purified cystathionine β-lyase

Substrate[a]	Substrate concn (mM)	Relative activity (%)[b]
Lanthionine[c]	5	169
L-Cystathionine	5	100
L-Homoserine	10	96
L-Cystine	5	64
L-Djenkolic acid	5	58
L-Cysteine	10	12
L-Homocysteine	5	4
L-Methionine	10	1

[a] No activity could be measured with the following substrates: L-arginine (5 mM), L-asparagine (5 mM), L-aspartic acid (5 mM), L-glutamic acid (5 mM), L-glutamine (5 mM), L-histidine (5 mM), L-lysine (5 mM), 4-methylthio-2-oxobutyric acid (10 mM), and nisin A (1 mg/ml). The last two substrates were measured by thiol determination for enzyme activity.

[b] Keto acid determination.

[c] A mixture of D,L- and *meso*-lanthionine.

kDa by SDS-PAGE (12.5% polyacrylamide) followed by silver staining (Fig. 1B).

Enzyme characterization. The molecular mass of the native enzyme estimated by gel filtration is approximately 165 kDa, which is somewhat higher than that estimated by gradient PAGE (described above). The activity of the purified enzyme was optimal between pH 7.5 and 8.5 with cystathionine as the substrate. The activity in the pH range 5.0 to 5.5 was about 10 to 15% of the activity at the optimal pH. The enzyme activity decreased rapidly above pH 8.5. The enzyme was stable up to 60°C over a period of 10 min. The activity declined rapidly above 60°C, and the enzyme was completely inactivated by being heated at temperatures above 70°C. There was no significant loss of activity after storage for 20 h at 4 or 20°C or after lyophilizing of samples containing the enzymic activity; repeated freezing (−20°C) and thawing or storage of the preparations for months at −20°C also had no dramatic effect on enzyme activity.

In order to demonstrate the cofactor requirement, the purified enzyme, with or without a hydroxylamine treatment (10 mM), was dialyzed against a large volume of 20 mM KP_i (pH 7.5) in a Slide-a-lyzer (Pierce Chemical Company). Treatment with hydroxylamine or a 20-h dialysis of the untreated enzyme resulted in complete inactivation of the enzyme. The activities of the dialyzed treated and untreated enzyme fractions could be restored to 56 and 63%, respectively, by addition of PLP. Taken together, these results indicate that PLP is required for enzyme activity.

Table 2 shows that the enzyme has a relatively broad specificity towards sulfur-containing amino acids as measured by keto acid formation. Degradation of lanthionine, cystathionine, homoserine, cystine, djenkolic acid, cysteine, homocysteine, and methionine (in that order of susceptibility) was observed. With the thiol determination method, similar results were obtained with the substrates lanthionine, cystathionine, cystine, djenkolic acid, and methionine. In all cases, a keto component, ammonia, and a thiol group could be detected. Both cysteine and homocysteine could be identified as degradation products of cystathionine by amino acid analysis. Therefore, it seems that the enzyme is capable of catalyzing both the α,β-elimination and α,γ-elimination reactions. No KMBA was detected with the purified enzyme when methionine was used as a substrate, and no thiol formation was detected with KMBA as a substrate. These results suggest a simultaneous deamination and C-S-lyase action (α,γ-elimination) catalyzed by the purified enzyme rather than a coupled reaction mechanism involving two enzymes (and the formation of the intermediate KMBA), which apparently are also present in the crude cell extract (11). Deaminase activities involving arginine, glutamine, or asparagine, but no other amino acids, were detected in the crude cell extract as well (1), but none of these activities were found with the purified enzyme. The deaminase activities found in the cell extract are probably due to the action of a specific arginine deiminase (30) and glutaminase-asparaginase (31), respectively, which are enzymes found in many bacteria.

The volatile (degradation) products of methionine conversion were identified by GC as methanethiol, dimethyldisulfide, and dimethyltrisulfide (Fig. 2). Mainly dimethyldisulfide (but also dimethyltrisulfide) was detected, probably because under aerobic conditions these components are formed from methanethiol. Although the C-S bond in lanthionine is easily split by the purified enzyme, no cleavage of the C-S bonds in nisin A, a peptide containing five lanthionine rings, was observed (25). Apparently a free amino group is required for α,β-elimination.

The effects of some inhibitors upon the activity of the cystathionine lyase activity are summarized in Table 3. The enzyme was strongly inhibited by carbonyl reagents such as hydroxylamine, DL-penicillamine, phenylhydrazine, semicarbazide, and 3-methyl-2-benzothiazolinone hydrazone, which are known inhibitors of PLP-dependent enzymes. Other inhibitors of PLP-dependent enzymes, like DL-cyclo-serine, carboxymethoxylamine (irreversible inhibitor, substrate analog), DL-propargylglycine (irreversible inhibitor), and β-cyano-L-alanine (competitive inhibitor) also strongly inhibited the enzyme. The sulfhydryl-reactive agents iodoacetic acid, iodoacetamide, and

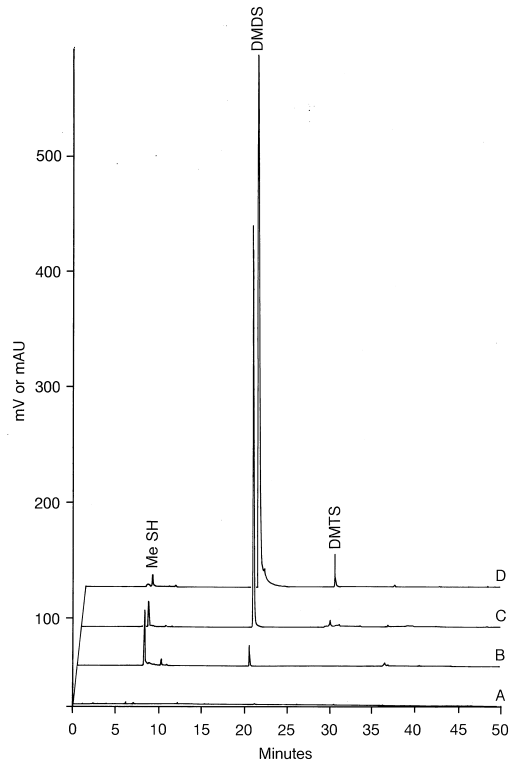

FIG. 2. Identification of the volatile (degradation) products of methionine conversion as identified by thermal desorption cold trap GC after incubation for 120 min (C) or 360 min (D) under the conditions described in Materials and Methods or after an incubation for 360 min without enzyme addition (A) or without substrate addition (B). Me SH, methanethiol; DMDS, dimethyldisulfide; DMTS, dimethyltrisulfide.

TABLE 3. Effects of inhibitors on cystathionine β-lyase activity

Inhibitor	Inhibitor concn	Relative activity (%)	
		Thiol determination	Keto acid determination
Hydroxylamine	1 mM	1	4
	10 mM	2	0
DL-Penicillamine	1 mM	ND[a]	97
	10 mM	ND	57
Phenylhydrazine	1 mM	19	ND
Semicarbazide	1 mM	91	87
	10 mM	7	48
3-Methyl-2-benzothiazolinone hydrazone	1 mM	ND	91
	10 mM	ND	53
N-Ethylmaleimide	1 mM	ND	162
	10 mM	ND	151
Iodoacetamide	1 mM	ND	107
	10 mM	ND	136
Iodoacetic acid	1 mM	ND	105
	10 mM	ND	113
Glycine	1 mM	ND	102
	10 mM	ND	94
DL-Propargylglycine	1 mM	1	1
	10 mM	0	0
β-Cyano-L-alanine	1 mM	37	67
	10 mM	6	13
Carboxymethoxylamine	1 mM	67	9
	10 mM	6	0
DL-Cycloserine	1 mM	89	93
	10 mM	51	61
EDTA	1 mM	106	102
	10 mM	94	93
Tris	1 mM	101	105
	10 mM	101	96
NaCl	0.4% (wt/vol)	100	ND
	4% (wt/vol)	69	ND

[a] ND, not determined.

N-ethylmaleimide caused no marked inhibition of the enzyme. The slight apparent activation is due to the reaction of the sulfhydryl agents with 3-methyl-2-benzothiazolinone used in the keto acid determination. There was also no effect of the chelating agent EDTA. The activity of the enzyme was only slightly reduced by 4% NaCl.

DISCUSSION

The results in this paper indicate that the enzyme purified from *L. lactis* subsp. *cremoris* B78 is cystathionine β-lyase (EC 4.4.1.8), a PLP-dependent enzyme which is involved in the biosynthesis of methionine. It is responsible for the degradation of cystathionine into homocysteine, pyruvate, and ammonia. Subsequently, homocysteine is methylated to form methionine (21).

Although this microorganism, like most *L. lactis* strains, is auxotrophic for the amino acid methionine, there are indications that a biosynthetic route for methionine exists in *L. lactis*, but it is probably interrupted (6). The existence of gene defects in *L. lactis* related to the biosynthesis of amino acids has been established by comparison of the nucleotide sequences of some of these genes of a nondairy strain that is prototrophic for the amino acids leucine, isoleucine, valine, and histidine with the homologous sequences from auxotrophic dairy strains. The auxotrophy seems to be the result of accumulated mutations and deletions within these genes (6, 9, 20). There are also indications that some *L. lactis* subsp. *lactis* strains are able to grow in a methionine-deficient medium at a very low growth rate, suggesting that these strains possess all of the genes needed for the synthesis of this amino acid (6). With a single-step mutagenesis, Deguchi and Morishita (8) were able to restore the ability to synthesize certain amino acids in *L. lactis* auxotrophic for these amino acids.

To our knowledge, a cystathionine β-lyase from lactococci has not been described before. The enzyme has been purified from the gram-negative bacteria *Escherichia coli* (10, 38), *Salmonella typhimurium* (23, 35), and *Bordetella avium* (19) and has been partly purified from *Paracoccus denitrificans* (5). Analogous to the cystathionine β-lyases occurring in these bacteria, the lactococcal enzyme is a tetrameric protein with identical subunits approximately 40 kDa in size. The amino-terminal sequence determination of the protein extracted from the 40-kDa band (SDS-PAGE [12.5% polyacrylamide]) unequivocally showed a single amino-terminal sequence, indicating that the 40-kDa band is free of other proteins (data not shown). No sequence similarity could be found between this amino-terminal sequence of the lactococcal enzyme and the amino-terminal sequences of cystathionine β-lyase from *E. coli* (3), *B. avium* (19), and *S. typhimurium* (35). However, it should be mentioned that the amino-terminal region of the enzyme in these gram-negative bacteria is not very conserved. At the moment, the amino-terminal sequence is being used to pick up the gene encoding this lactococcal cystathionine β-lyase.

The enzyme has an alkaline pH optimum, and like many other enzymes of biosynthetic pathways, it is not endowed with an absolute specificity. The relative activity exhibited by the lactococcal enzyme towards different substrates was found to decrease in the order lanthionine > cystathionine > homoserine > cystine > djenkolic acid > (homo)cysteine > methionine. This order of preference for the different substrates and the identification of cystathionine degradation products show that the enzyme prefers to catalyze the α,β-elimination reaction, but it is capable of catalyzing an α,γ-elimination reaction as well. The preference for lanthionine, cystathionine, cystine, and djenkolic acid is in accordance with the results found for other known bacterial cystathionine β-lyases (5, 10, 19, 23, 35, 41). It is known that cystathionine γ-lyase isolated from *Streptomyces phaeochromogenes* also catalyzes the α,β-elimination reaction at about one-seventh the rate of the α,γ-elimination reaction (34). The *Paracoccus* enzyme differs from the lactococcal enzyme by not degrading cysteine, homoserine, and methionine (5).

The lactococcal enzyme is very sensitive to propargylglycine and β-cyanoalanine, which are also strong inhibitors of mammalian cystathionine γ-lyase (42). In contrast to the lactococcal enzyme, the cystathionine β-lyases of *E. coli* and *B. avium* are not inhibited by propargylglycine. Carboxymethoxylamine, a strong inhibitor of PLP-dependent *S*-alkyl-L-cysteine lyase of *Acacia farnesiana* (32), also strongly inhibits the lactococcal enzyme. Like most of the PLP-dependent enzymes, the enzyme is very sensitive to carbonyl reagents. The chelating agent EDTA and sulfhydryl reagents have no effect upon the activity of the lactococcal enzyme, showing that metal ions and thiol groups are not essential for its activity. In this respect, the *L. lactis* enzyme differs from the *P. denitrificans* and *B. avium* enzymes, which are both sensitive to sulfydryl reagents. Moreover, under the same experimental conditions, the lactococcal enzyme is heat stable within the range 20 to 60°C, whereas the *Bordetella* enzyme is heat stable only up to 40°C (19).

The impact of sulfur-containing compounds on the development of flavor in cheese has been discussed and is widely accepted (29). The importance of methanethiol and dimethyldisulfide has been emphasized, but several other sulfur compounds have also been identified (7). Green and Manning (22)

have investigated the volatile sulfur compounds in cheddar cheese and have concluded that methanethiol is important in the development of distinctive cheddar-like flavors. However, the nature of its flavor-conferring properties has not yet been elucidated. Methionine is accepted as the most important precursor for methanethiol in cheese. It was possible to achieve an improvement of the cheddar flavor by incorporation of a free or fat-encapsulated methanethiol-generating methionine γ-lyase–methionine system into cheddar cheeses. However it was concluded that methanethiol alone did not cause the true cheddar flavor (29). Recently, Engels and Visser (13) showed that the cell extract of *L. lactis* subsp. *cremoris* B78 is capable of generating a Gouda cheese-like flavor from methionine. Contrary to other authors (27), we associate methanethiol production in cheese with cystathionine β-lyase activity and other enzymes of the lactic starter bacteria in the first place and not with enzyme activities derived from nonstarter bacteria or with nonenzymic reactions.

The cystathionine β-lyase is still active at the pH of a normal Gouda cheese, and even the presence of a high salt concentration did not strongly inhibit its activity. The activity of the present enzyme in intact but energetically exhausted (39) cells involving internal (generated) substrate or substrate that enters the cell by passive diffusion is probably limited. Lysis of cells is therefore a prerequisite to the full realization of the enzyme activity with respect to cheese ripening. In a 5-month-old cheese, some of the cells showed a locally degraded cell wall and a seemingly intact membrane (40). These cells probably did not burst because of an osmotically and structurally determined stabilization by the cheese environment (38). Even in the case of a clear membrane rupture, the cell content remained largely within the cell envelope structure (40) because of the semisolid gel structure in which the cell is embedded. Therefore, it is not expected that the activity of the present enzyme decreases after lysis of cells in cheese as a result of dilution of the cofactor. In view of this and its relatively high stability, and considering the fact that sulfur-containing flavor components have very low threshold values for perception, the enzyme could play an important role in the cheese-ripening process, despite the fact that the conditions in cheese are far from optimal for its activity.

In conclusion, it has been shown that cystathionine β-lyase, of which the function within the growing cell is not primarily related to amino acid catabolism, can be significantly involved in amino acid conversion. In cheese, this relatively low level of activity might still be important if viewed in the light of the often extremely low concentrations at which volatile flavor products of these actions or subsequent reactions can contribute to flavor.

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