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Purification and Characterization of Cystathionine γ-Lyase from *Lactococcus lactis* subsp. *cremoris* SK11: Possible Role in Flavor Compound Formation during Cheese Maturation

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A cystathionine γ-lyase (EC 4.4.1.1) (γ-CTL) was purified to homogeneity from a crude cell extract of *Lactococcus lactis* subsp. *cremoris* SK11 by a procedure including anion-exchange chromatography, hydrophobic interaction chromatography, and gel filtration chromatography. The activity of SK11 γ-CTL is pyridoxal-5′-phosphate dependent, and the enzyme catalyzes the α,γ-elimination reaction of L-cystathionine to produce L-cysteine, α-ketobutyrate, and ammonia. The native enzyme has a molecular mass of approximately 120 to 200 kDa and apparently consists of at least six identical subunits of 20 kDa. In this respect, the SK11 enzyme clearly differs from other bacterial cystathionine lyases, which are all tetrameric proteins with identical subunits of approximately 40 kDa. In addition, the specific activity of purified SK11 γ-CTL toward L-cystathionine is relatively low compared with those reported for other bacterial cystathionine lyases. The SK11 enzyme shows a broad substrate specificity. In the case of L-methionine, the action of SK11 γ-CTL results in the formation of methanethiol, a volatile sulfur compound known to be required in flavor development in cheddar cheese. The α,β-elimination reaction of L-cysteine is also efficiently catalyzed by the enzyme, resulting in the formation of hydrogen sulfide. Although the conditions are far from optimal, cystathionine γ-lyase is still active under cheddar cheese-ripening conditions, namely, pH 5.0 to 5.4 and 5% (wt/vol) NaCl. The possible role of the enzyme in cheese flavor development is discussed.

The maturation of cheese is a complex process, and the major biochemical reactions contributing to cheese flavor development are carbohydrate metabolism, lipolysis, and proteolysis. Proteolysis is due to the combined action of a variety of enzymes: chymosin (rennet), indigenous plasmin, and the microbial proteinases and peptidases originating from the starter and nonstarter lactic acid bacteria. It is now well established that a well-balanced process of breakdown of milk proteins into small peptides and amino acids is essential for the development of a good cheese flavor (for a review, see reference 9).

The water-soluble fraction of various types of cheese makes a major contribution to the intensity of cheese flavor (5, 7, 22). Low-molecular-weight (<500-Da) compounds are responsible for the cheese flavor of the water-soluble fractions. These low-molecular-weight compounds may include small peptides, amino acids, short-chain free fatty acids, and breakdown products of such components (7). Although small peptides and free amino acids appear to make a significant contribution to a savory, brothy type of flavor (5), volatile products of cheese ripening are considered to be responsible for the actual cheese flavor (27). The development of volatile cheese flavor compounds may be associated with the enzymatic conversion of specific amino acids. For instance, a Gouda cheese-like flavor was formed by incubation of L-methionine with a cell extract of the lactococcal strain *Lactococcus lactis* subsp. *cremoris* B78 (8). In addition, incorporation of L-methionine and the enzyme methioninase from *Pseudomonas putida* into cheddar cheese resulted in accelerated cheese flavor development (18).

It is now believed that amino acid catabolism, mediated by enzymes originating from lactic acid bacteria, may play a major role in the formation of flavor components during cheese ripening (27, 30). The different types of enzymes involved in these reactions have decarboxylase, deaminase, or transaminase activity, or they modify amino acid side chains. The primary reaction products are amines, other amino acids, α-keto acids, and sulfur compounds (14).

As a first step to elucidate the nature of cheese flavor development and the contribution of amino acid catabolism in lactic acid bacteria, the breakdown of sulfur-containing compounds by lactic acid bacteria was studied. In this paper, we report the purification and biochemical characterization of cystathionine γ-lyase (γ-CTL) from *Lactococcus lactis* subsp. *cremoris* SK11. This enzyme can utilize various sulfur-containing substrates including L-methionine, from which it produces methanethiol, a volatile sulfur compound that appears to play an essential role in the development of cheddar cheese flavor (18, 27).

MATERIALS AND METHODS

Chemicals. All enzyme substrates and inhibitors were obtained from Sigma Chemical Co. (St. Louis, Mo.); 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 3-methyl-2-benzothiazolinone hydrazone, pyridoxal-5′-phosphate (PLP), 2,2-dihydroxy-1,3-indanedione (nynhydrin), 2-(*N*-morpholino)ethanesulfonic acid (MES), 1,3-bis[tris(hydroxymethyl)methylamino]propane (bis-Tris-propane), and α-ketobutyrate were obtained from Sigma Chemical Co. The amino acid derivatization reagent kit, involving 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), was obtained from Waters Associates (Milford, Mass.). Tris and EDTA were obtained from Bio-Rad (Hercules, Calif.). Unless mentioned otherwise, all other reagents used in this study were analytical grade and were obtained from BDH (Poole, United Kingdom).

Enzyme purification. *L. lactis* subsp. *cremoris* SK11 was grown overnight at 30°C in 10 liters of lactose-M17 broth (Merck AG, Darmstadt, Germany) supplemented with 0.025% (wt/vol) L-methionine. Cells were harvested and washed twice in 20 mM potassium phosphate (KP_i) buffer (pH 7.2) containing 1 mM EDTA, 0.01 mM PLP, and 1 mM phenylmethylsulfonyl fluoride. The cells were resuspended in 40 ml of KP_i-EDTA-PLP-phenylmethylsulfonyl fluoride and disrupted with ultrasonic waves (ultrasonic processor XL; Heat Systems, Farmingdale, N.Y.) for 10 cycles of 30 s of sonication and 30 s of cooling on ice. The suspension was centrifuged (12,000 × *g* for 30 min at 4°C) to remove cell debris,

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TABLE 1. Purification of γ-CTL from *L. lactis* subsp. *cremoris* SK11

Purification step	Total amt of protein (mg)	Total activity (U)[a]	Sp act (U/mg of protein)	Purification (fold)	Yield (%)
Cell extract	1,040	1,352	1.3	1.0	100
Anion-exchange chromatography (first)	37	226	6.1	4.8	17
Hydrophobic interaction chromatography	1.60	28.5	17.8	14.1	2.1
Anion-exchange chromatography (second)	0.46	13.9	30.2	24.0	1.0
Gel filtration chromatography	0.05	3.1	64.7	51.3	0.23

[a] One unit (U) is defined as 1 nmol of thiol produced per min at 30°C with L-cystathionine as the substrate.

and the supernatant (cell extract) was incubated at 30°C for 30 min with DNase and RNase (Boehringer, Mannheim, Germany) at a final concentration of 10 μg/ml to remove residual DNA and RNA.

The cell extract was applied to a MemSep 1010 DEAE anion-exchange chromatography cartridge (Millipore, Bedford, Mass.) connected to a ConSep LC100 system (Millipore). The cartridge was equilibrated with KP_i-EDTA-PLP buffer containing 40 mM potassium chloride (KCl), and proteins were eluted at 6 ml/min in a 40 to 600 mM KCl gradient in the same buffer. The enzyme-active fractions were pooled, concentrated, and washed in KP_i-EDTA-PLP buffer by ultrafiltration in a Centricon-30 microconcentrator with a 30-kDa cutoff membrane (Amicon, Beverly, Mass.). Ammonium sulfate was added to the pooled fractions to a concentration of 1 M, and they were applied to a HiTrap phenyl-Sepharose HP hydrophobic interaction column (Pharmacia Biotech, Uppsala, Sweden). The bound protein was eluted at 1 ml/min by lowering the ammonium sulfate concentration in KP_i-EDTA-PLP buffer, with the enzyme activity eluting from the column between 630 and 400 mM ammonium sulfate. The enzyme-active fractions were collected and concentrated by ultrafiltration and subjected a second time to DEAE anion-exchange chromatography as described above, except that in this step a flow rate of 1.2 ml/min was used. Final purification of the concentrated enzyme solution was achieved by gel filtration high-pressure liquid chromatography (HPLC) with a TSK-Gel G3000 SW column (7.5 mm by 60 cm) equipped with a TSK guard column SW (7.5 mm by 7.5 cm) (Tosoh Corp., Tokyo, Japan). Chromatography was carried out with 100 mM KP_i-EDTA-PLP buffer (pH 7.2) in a Waters HPLC system consisting of a 626 gradient pump (flow rate, 1 ml/min), 600S pump controller, 717plus autosampler, 996 photodiode array detector, and Millennium 2010 V2.10 chromatography manager software (Waters Associates, Milford, Mass.). For determination of the molecular mass of the native enzyme, the column was calibrated with an HPLC marker protein kit (USB, Cleveland, Ohio) containing glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), myokinase (32 kDa) and cytochrome *c* (12.4 kDa). The fractions that contained high enzyme activities were pooled and concentrated by ultrafiltration with 20 mM KP_i-EDTA-PLP buffer (see above). Throughout the purification, enzyme activity was monitored by determination of thiol formation (see below) with L-cystathionine as a substrate. The purified enzyme was stored at −40°C.

Enzyme assays. Enzyme activity toward various substrates was measured by determination of thiol formation with DTNB as described by Uren (28) and determination of keto acid production with 3-methyl-2-benzothiazolinone hydrazone as described by Soda (25), using α-ketobutyrate as a standard. The standard reaction mixture contained 0.1 M KP_i buffer (pH 8.0), 0.01 mM PLP, and 1 mM substrate. The reaction mixtures were kept at 30°C for 30 min, and in all assays, the reaction velocity was linearly proportional to the amount of extract present. All assays were performed with a THERMOmax microplate reader, using the SOFTmax Software package V2.31 (Molecular Devices, Menlo Park, Calif.).

Effect of temperature, pH, and inhibitors on the enzyme activity. The effects of inhibitors were determined by preincubating the purified enzyme fraction with different inhibitors at the indicated concentrations in the assay buffer (pH 8.0) for 10 min at 20°C. The effect of pH on enzyme activity was measured in 0.1 M MES buffer (pH 4.5 to 7.5) and 0.1 M bis-Tris-propane buffer (pH 6.5 to 9.5). The effect of temperature on enzyme activity was determined by preheating the enzyme in the assay buffer (pH 8.0) for 10 min at temperatures from 25 to 70°C. Enzyme activities were determined as described above.

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Mini-Protean II cell system and a 12% (wt/vol) polyacrylamide Mini-Protean II Tris-glycine Ready Gel as specified by the manufacturer (Bio-Rad, Hercules, Calif.). Wide-range molecular mass marker proteins (Mark12; Novex) containing myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6 kDa), insulin B chain (3.5 kDa), and insulin A chain (2.5 kDa) were used as a reference. Native PAGE was performed on a 4 to 15% (wt/vol) polyacrylamide gradient Mini-Protean II Ready Gel (Bio-Rad) with high-molecular-mass marker proteins (Pharmacia) consisting of thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa) as a reference. Proteins were visualized with Coomassie brilliant blue or by silver staining with Silver Stain Plus (Bio-Rad).

Protein quantification. The protein concentration was determined by a protein assay (Bio-Rad) based on the method of Bradford (3), with bovine serum albumin as the standard.

Identification of reaction products. Headspace gas chromatography (GC) analysis was used to identify volatile sulfur-containing products from degradation of L-cysteine and L-methionine by the purified enzyme at 30°C. For this purpose, 350 μl of reaction mixture (protein concentration, 5 μg/ml) was flushed with helium in the GC headspace vial to obtain anaerobic conditions and sealed with PTFE-butyl seals (Perkin-Elmer, Überlingen, Germany). Headspace samples of 5 ml were analyzed in a Tracor 550 gas chromatograph (Tracor, Austin, Tex.) fitted with a glass column (1.8 m by 0.32 cm) packed with Carbopack B-HT-100 (Supelco Inc., Bellefonte, Pa.) and a flame photometric detector in the sulfur mode. The injector was kept at ambient temperature, and nitrogen (flow rate, 60 ml/min) was used as a carrier gas for GC separation at a constant oven temperature of 60°C. The headspace components were identified by comparison of their retention times with authentic standards.

Amino acid products of L-cystathionine degradation by the purified enzyme were identified by (i) HPLC analysis of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatives as described by Liu (19) and (ii) HPLC analysis (at 300 nm) of reaction mixtures containing DTNB derivatives of free thiol group-containing amino acids (28). In the latter method, reaction mixtures were separated by HPLC on a Hi-Pore RP-318 analytical reversed-phase column (250 by 4.6 mm; Bio-Rad) equipped with a reversed-phase Delta-Pak C_{18} guard column (Waters Associates). HPLC solvent A consisted of water and 0.1% (vol/vol) trifluoroacetic acid; solvent B consisted of water, acetonitrile (50%, vol/vol), and trifluoroacetic acid (0.1%, vol/vol). The gradient program used 100% solvent A for 5 min from the time of sample injection, a linear gradient from 0 to 100% solvent B over a period of 30 min, and an isocratic step at 100% solvent B for 10 min. The flow rate throughout the gradient program was 1 ml/min. Amino acid products were identified by using retention times of AQC and DTNB derivatives of authentic amino acid standards.

L-Cysteine production was measured by the acid ninhydrin procedure (10), and ammonia formation was measured with the ammonia determination kit (Boehringer) as specified by the manufacturer.

RESULTS

Purification of γ-CTL. A summary of the purification of γ-CTL from *L. lactis* subsp. *cremoris* SK11 is shown in Table 1. With anion-exchange chromatography, most of the enzyme activity eluted from the column between 140 and 250 mM KCl. Overall, the enzyme was purified 51-fold, with an activity yield of 0.23%. Enzyme purification was monitored by analyzing aliquots of protein fractions from each chromatographic step by SDS-PAGE. A single polypeptide band (20 kDa) was seen in the final enzyme preparation involving Coomassie brilliant blue and silver staining (Fig. 1, lane 6). The final enzyme preparation eluted from the gel filtration column as a single peak at a position corresponding to about 200 kDa. However, nondenaturing PAGE of the enzyme preparation yielded a single protein band with a molecular mass of approximately 120 kDa (Fig. 2A, lane 2). The 120-kDa protein band was cut out of the gel, and the enzyme was eluted in 0.1 M Tris-HCl buffer (pH 8.0) supplemented with 0.01 mM PLP. The eluate contained γ-CTL activity toward L-cystathionine, and subsequent SDS-PAGE analysis with silver staining revealed one protein band of about 20 kDa (Fig. 2B, lane 3). These results show that the enzyme was purified to homogeneity. This enzyme preparation was used for further biochemical characterization.

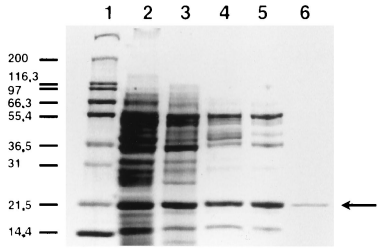

FIG. 1. SDS-PAGE analysis of peak fractions of L-cystathionine-degrading activity after the different chromatographic steps. Lanes: 1, molecular mass marker proteins; 2, crude cell extract; 3, first anion-exchange column; 4, hydrophobic interaction column; 5, second anion-exchange column; 6, gel filtration column. Molecular mass markers (in kilodaltons) are indicated on the left. The arrow indicates the position of γ-CTL.

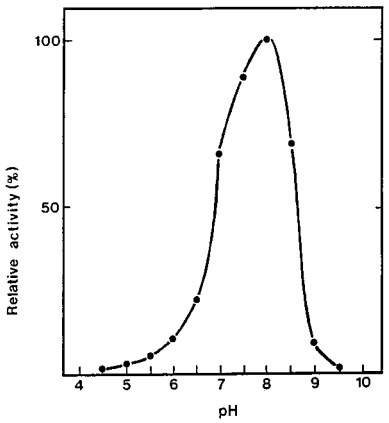

FIG. 3. Effect of pH on the L-cystathionine-degrading activity of γ-CTL.

Enzyme characterization. The activity of the purified enzyme was highest at pH 8.0 (Fig. 3). At values below pH 5.0 and above pH 9.0, no enzyme activity could be detected. The effect of temperature on enzyme activity was determined by using L-cystathionine as a substrate. The enzyme was stable up to 40°C for 10 min, whereas only 25% of the activity remained at 50°C for 10 min. The enzyme was completely inactivated at temperatures above 55°C. No loss of enzyme activity was observed after repeated freezing (−40°C) and thawing or upon storage of the enzyme at −40°C for 4 months.

To establish the cofactor dependence, the purified enzyme fraction was thoroughly washed with 20 mM sodium phosphate buffer (pH 7.2) by ultrafiltration, resulting in a complete loss of enzyme activity toward L-cystathionine. The enzyme could be reactivated to 61% of the original activity upon addition of 0.01 mM PLP to the assay buffer. Alternatively, the rate of hydrolysis of various substrates by the purified enzyme was reduced twofold when PLP was omitted from the assay buffer.

The specificity of the purified enzyme toward various substrates is shown in Table 2. The relative activity exhibited by the enzyme decreased in the following order: lanthionine, L-cystathionine, L-cysteine, L-cystine, L-djenkolic acid, *S*-methyl-L-cysteine, L-homocysteine, and L-methionine. Enzymatic degradation of these substrates invariably resulted in the formation of free thiol group component, a keto acid component, and ammonia as reaction products. L-Serine and L-homoserine were not degraded by the enzyme. The degradation of some substrates (lanthionine, L-cystathionine, L-cysteine, L-djenkolic acid, and L-methionine) can be determined by either the thiol assay or keto acid assay. In these cases, both assays yielded similar results for the specific enzyme activities.

It has been reported (2, 11, 23) that L-cystathionine degradation by cystathionine lyases can proceed via two reactions: either an α,β-elimination reaction resulting in the formation of L-homocysteine, pyruvate, and ammonia, or an α,γ-elimination reaction resulting in the production of L-cysteine, α-ketobutyrate, and ammonia. L-Cysteine was identified as the sole sulfur-containing amino acid product of L-cystathionine degradation by the purified enzyme via (i) amino acid determination by AQC derivatization of reaction products and (ii) analysis of DTNB derivatives of reaction products. L-Homocysteine production from L-cystathionine was not detected by either of the above methods. In addition, the enzymatic release of L-cysteine from L-cystathionine was confirmed by determination of L-cysteine production by using the acid ninhydrin procedure (data not shown). Altogether, these results strongly suggest that the enzyme purified from *L. lactis* subsp. *cremoris* SK11 is a γ-CTL, capable of only the α,γ-elimination reaction of L-cystathionine.

The volatile sulfur-containing products formed during anaerobic incubation at 30°C of L-cysteine and L-methionine with purified γ-CTL were identified by GC. Hydrogen sulfide and methanethiol were the sole volatile sulfur compounds found during degradation of L-cysteine and L-methionine, respectively (Table 3). These results show that the enzyme is capable of the α,β-elimination reaction when L-cysteine is a substrate,

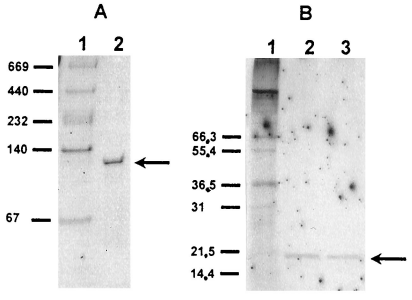

FIG. 2. Nondenaturing PAGE (A) and SDS-PAGE (B) analysis of purified SK11 γ-CTL with Coomassie brilliant blue staining and silver staining, respectively. (A) Lanes: 1, marker proteins; 2, purified SK11 γ-CTL. (B) Lanes: 1, marker proteins; 2, purified SK11 γ-CTL; 3, protein eluted from the 120-kDa band cut out from the native PAGE gel. Molecular mass markers (in kilodaltons) are indicated on the left. The position of γ-CTL is indicated by the arrows.

TABLE 2. Substrate specificity of γ-CTL from *L. lactis* subsp. *cremoris* SK11

Substrate	Relative activity (%)[a]
Lanthionine[b]	150
L-Cystathionine	100
L-Cysteine[c]	73
L-Cystine	45
L-Djenkolic acid	31
S-Methyl-L-cysteine	16
L-Homocysteine[c]	12
L-Methionine	1
L-Serine[c]	0
L-Homoserine[c]	0

[a] Values are the mean of two determinations (standard deviation is less than 5%).
[b] A mixture of DL- and *meso*-lanthionine.
[c] Keto acid determination.

TABLE 3. Relative amounts of volatile sulfur compounds formed during anaerobic incubation at 30°C of L-methionine and L-cysteine with and without purified γ-CTL from *L. lactis* subsp. *cremoris* SK11 and during incubation of γ-CTL without substrates

Compound and conditions	Relative peak area			
	Hydrogen sulfide production after:		Methanethiol production after:	
	6 h	24 h	6 h	24 h
L-Cysteine, no γ-CTL added	37	61	—[a]	—
L-Cysteine + γ-CTL	23,814	76,290	—	—
L-Methionine, no γ-CTL added	—	—	—	—
L-Methionine + γ-CTL	—	—	150	701
γ-CTL, no substrates added	—	—	—	—

[a] —, no detectable peak.

whereas L-methionine is a substrate for the α,γ-elimination reaction. The ratio of hydrogen sulfide and methanethiol production from L-cysteine and L-methionine is similar to the ratio of specific enzyme activities toward these substrates as determined by keto acid and thiol group formation (Table 2). In comparison with production levels after 6 h of incubation, the amount of hydrogen sulfide and methanethiol produced after 24 h of incubation increased 3.2- and 4.7-fold, respectively, indicating that γ-CTL remains active at 30°C for more than 6 h.

The effects of various chemical reagents on γ-CTL activity are shown in Table 4. The enzyme activity was strongly inhibited by the carbonyl reagents hydroxylamine and 3-methyl-2-benzothiazolinone hydrazone. Carbonyl reagents such as DL-penicillamine and semicarbazide also had an inhibitory effect on enzyme activity, but only at higher concentrations. The enzyme was also strongly inhibited by other known inhibitors of PLP-dependent enzymes (15) such as DL-cycloserine, DL-propargylglycine, and carboxymethoxylamine. The sulfhydryl reagents iodoacetic acid, *N*-ethylmaleimide, and iodoacetamide had no inhibitory effect on the enzyme activity. The chelating reagent EDTA also had no inhibitory effect on enzyme activity. The activity of the enzyme was reduced twofold by addition of 5% (wt/vol) NaCl to the assay buffer.

DISCUSSION

In this report, we describe the purification and characterization of γ-CTL (EC 4.4.1.1) from *L. lactis* subsp. *cremoris* SK11. The enzyme was purified to homogeneity (Fig. 1, lane 6) from the crude cell extract by a four-step procedure. Based on the specific activity, the enzyme was purified 51-fold with a recovery of 0.23%. The enzyme catalyzed the conversion of L-cystathionine into α-ketobutyrate, ammonia, and L-cysteine, with the last compound being further degraded into hydrogen sulfide, pyruvate, and ammonia.

Recently, the purification and characterization of a CTL from *L. lactis* subsp. *cremoris* B78 has been reported (2). Although the two lactococcal enzymes share many properties, they clearly differ with respect to their catalytic activity, subunit molecular mass, and number of subunits (see below). A CTL has been isolated from the gram-positive actinomycete *Streptomyces phaeochromogenes* (23), and the purification of CTLs from the gram-negative bacteria *Escherichia coli* (6), *Salmonella typhimurium* (13), *Bordetella avium* (11), and *Paracoccus denitrificans* (4) has been reported. The molecular mass of the native form of SK11 γ-CTL, as estimated by native PAGE (Fig. 2A) and gel filtration chromatography, was approximately 120 and 200 kDa, respectively. The reason for this variance is not known. Using similar methods, Alting et al. (2) also reported different values (130 and 165 kDa) for the molecular mass of native CTL from strain B78. Nevertheless, given an apparent subunit size of 20 kDa (Fig. 1 and 2B), these results imply that native SK11 γ-CTL consists of at least six identical subunits. In this respect, SK11 γ-CTL clearly differs from CTLs from strain B78 and from *S. phaeochromogenes*, as well as the other bacterial CTLs, which are all tetrameric proteins with identical subunits of approximately 40 kDa (2, 4, 6, 11, 13, 23).

Amino-terminal sequence determination of SK11 γ-CTL revealed a single amino-terminal sequence (data not shown), providing additional evidence that the final enzyme preparation is homogeneous and free from other polypeptides. No significant sequence similarity was found between the amino-terminal sequence of SK11 γ-CTL and the amino-terminal sequences of other bacterial CTLs from strain B78 (1), *S. phaeochromogenes* (23), *E. coli* (6), *S. typhimurium* (13), and *B. avium* (11). In addition, no significant sequence similarity was observed between the amino-terminal sequence of SK11 γ-CTL and protein sequences present in the SwissProt Protein Sequence Database (15 October 1996 release).

TABLE 4. Effect of inhibitors on γ-CTL activity

Inhibitor	Inhibitor concn	Relative activity (%)[a]	
		Thiol determination[b]	Keto acid determination[b]
Hydroxylamine	1 mM	2	7
	10 mM	3	0
DL-Penicillamine	1 mM	ND[c]	105
	10 mM	ND	74
Semicarbazide	1 mM	109	50
	10 mM	9	5
3-Methyl-2-benzothiazolinone hydrazone	1 mM	59	45
	10 mM	11	9
Iodoacetamide	1 mM	ND	116
	10 mM	ND	93
Iodoacetic acid	1 mM	ND	112
	10 mM	ND	98
N-Ethylmaleimide	1 mM	ND	105
	10 mM	ND	ND
DL-Propargylglycine	1 mM	4	0
	10 mM	3	0
Carboxymethoxylamine	1 mM	14	31
	10 mM	0	0
DL-Cycloserine	1 mM	65	69
	10 mM	27	15
EDTA	1 mM	98	105
	10 mM	93	85
NaCl	0.5% (wt/vol)	91	102
	5% (wt/vol)	45	51

[a] 100% activity is the enzyme activity toward L-cystathionine (1 mM) with no inhibitor added.

[b] Values are the mean of two determinations; the standard deviation is less than 3% for the thiol determination and 5% for the keto acid determination.

[c] ND, not determined.

The specific activity of purified SK11 γ-CTL toward L-cystathionine is low compared with that reported for other bacterial CTLs: approximately 30-fold lower in comparison with the enzymes from strain B78 (2) and *S. phaeochromogenes* (23) and up to 1,000-fold lower compared with the *B. avium* enzyme (11). However, the available evidence indicates that with respect to protein structure, SK11 γ-CTL is clearly different from the other bacterial CTLs (see above). Comparison of the complete amino acid sequence of SK11 γ-CTL with that of other CTLs may shed light on the catalytic mechanisms of these enzymes and define their evolutionary relationships.

Removal of PLP from the SK11 γ-CTL by ultrafiltration completely inactivated the enzyme, and enzyme activity could be partly restored by addition of PLP. Furthermore, the activity of the enzyme is abolished by hydroxylamine (see below), a carbonyl reagent known to inhibit most PLP-dependent enzymes (15). These results strongly suggest that SK11 γ-CTL contains PLP as a cofactor. The enzyme activity of CTL from strain B78 was also PLP dependent (2).

Although SK11 γ-CTL is heat stable up to 40°C, it quickly loses catalytic activity at higher temperatures, while the B78 enzyme is heat stable up to 60°C (2). Both lactococcal enzymes are stable upon repeated freezing and thawing or storage for months at −20 and −40°C for the B78 and SK11 enzymes, respectively.

The SK11 γ-CTL has optimum activity at an alkaline pH (Fig. 3) and demonstrates a relatively broad substrate specificity (Table 2), as is the case with strain B78 CTL (2) and most other enzymes involved in metabolism of sulfur and sulfur amino acids (15). The substrate specificity of SK11 γ-CTL resembles that of the strain B78 (2) enzyme in being most active with lanthionine and L-cystathionine and far less active with L-cystine, L-djenkolic acid, L-homocysteine, and L-methionine. However, in contrast to the B78 enzyme, SK11 γ-CTL is unable to degrade L-homoserine and shows relatively high activity toward L-cysteine (Table 2). With regard to the latter substrate, the specificity of SK11 γ-CTL also differs from the other bacterial cystathionine β-lyases (4, 6, 11, 13). Both L-homoserine and L-cysteine are effective substrates for γ-CTL from *S. phaeochromogenes* (23) and rat liver (16). The relative activity of SK11 γ-CTL toward lanthionine, L-cystathionine, L-cystine, and L-djenkolic acid is similar to that of the other bacterial CTLs (2, 4, 6, 11, 13).

The SK11 γ-CTL appears to catalyze only the α,γ-elimination reaction of L-cystathionine. In this respect, the enzyme differs from the CTL of strain B78 (2) and γ-CTL from *S. phaeochromogenes* (23), which are both able to catalyze the α,β-elimination and α,γ-elimination reaction of L-cystathionine. However, L-cysteine is efficiently converted by SK11 γ-CTL, indicating that this enzyme can catalyze the α,β-elimination reaction as well as the α,γ-elimination reaction.

The effects of inhibitors on SK11 γ-CTL activity were comparable to the results found for the strain B78 enzyme (2); both enzymes are sensitive to carbonyl reagents but are not inhibited by EDTA and sulfhydryl reagents, indicating that metal ions and thiol groups are not required for their activity. With respect to sensitivity to sulfhydryl reagents, the lactococcal enzymes differ from CTL of *P. denitrificans* (4) and *B. avium* (11), which both showed marked inhibition by *N*-ethylmaleimide and iodoacetamide. In contrast to the CTL of *E. coli* (6) and *B. avium* (11), the SK11 γ-CTL is strongly inhibited by DL-propargylglycine, which is also known to inhibit γ-CTL from *S. phaeochromogenes* (23) and rat liver (29). Although the conditions are far from optimal, SK11 γ-CTL is still active at the pH and salt concentration observed in first-grade cheddar cheese (17, 24) and Gouda cheese (31).

Importantly, SK11 γ-CTL was found to produce the volatile sulfur-containing compounds methanethiol and hydrogen sulfide from the amino acids L-methionine and L-cysteine, respectively (Table 3). Alting et al. (2) reported the formation of methanethiol, dimethyldisulfide, and dimethyltrisulfide (the last two compounds were probably oxidation products of methanethiol) from L-methionine by the action of CTL from strain B78.

It is now generally accepted that sulfur compounds are essential flavor components in many cheese varieties. Several low-molecular-weight sulfur compounds, including methanethiol and hydrogen sulfide, are found in the headspace profile of cheddar cheese (18, 26). The formation of methanethiol (18, 20, 21, 26) and hydrogen sulfide (12, 18, 21) in cheddar cheese appears to be important for the development of a balanced cheddar-like flavor. Interestingly, the enzymatic generation of methanethiol, via incorporation of L-methionine and the enzyme methioninase from *Pseudomonas putida* into cheddar cheese, resulted in accelerated development of cheese flavor (18). However, the results in this latter study also indicated that the presence of methanethiol alone is not sufficient to produce cheddar flavor. Methanethiol has also been recognized as an important contributor to the flavor of surface-ripened cheeses, including Brie and Camembert (18).

In conclusion, the results described in this paper indicate that SK11 γ-CTL may play an important role in the formation of flavor components during cheese maturation. Our further research is aimed at isolation of the gene encoding SK11 γ-CTL by using an oligonucleotide probe designed on the basis of the amino-terminal amino acid sequence of the purified enzyme. Cheese experiments involving lactococcal γ-CTL mutants will allow determination of the precise role of this enzyme in the cheese-ripening process.

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