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Partial purification and characterization of two aminotransferases from *Lactococcus lactis* subsp. *cremoris* B78 involved in the catabolism of methionine and branched-chain amino acids☆

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Abstract

Transamination of methionine and other amino acids followed by conversion of the resulting α-keto acids by enzymes from the mesophilic starter organism *Lactococcus lactis* subsp. *cremoris* B78 was studied. Two aminotransferases, displaying activity towards methionine, were partially purified and characterized. The enzymes most likely were branched-chain aminotransferases, since their activity towards valine, leucine and isoleucine was even higher than towards methionine. The enzymes, AT-A and AT-B, both showed a molecular mass of approximately 75 kDa and consisted of two identical subunits, each with a molecular mass of approximately 40 kDa. AT-A and AT-B also had a broad substrate specificity for the amino-group acceptor, α-ketoglutaric acid being the preferred cosubstrate. The enzymes catalyzed the conversion of methionine to 4-methylthio-2-ketobutyric acid, which was subsequently converted to methanethiol and dimethyldisulphide. The formation of these and other volatile sulfur compounds is considered to play an important role in the development of cheese flavour. Both AT-A and AT-B had a rather high optimum temperature, 45–50°C, and a pH optimum of 8. However, under simulated cheese-ripening conditions (10–15°C and pH 5.2–5.4) sufficient activity remained for conversion of methionine.

Keywords: Aminotransferases; *Lactococcus lactis*; Methionine; Cheese flavour

1. Introduction

Flavour formation in cheese is a complex process that in many respects is still poorly understood. In ripening cheese the breakdown of proteins is essential for formation of desirable flavour and texture. The process of proteolysis has been investigated extensively (Exterkate & Alting, 1995; Exterkate, Alting, & Slangen, 1995; Pritchard & Coolbear, 1993; Visser, 1993) and the products eventually formed are peptides and free amino acids (Engels & Visser, 1994; Olson, 1990; Visser, 1993).

Fractionation studies carried out with Cheddar (Aston & Creamer, 1986) and Gouda cheese (Engels & Visser, 1994) showed that low-molecular-mass components, to a large extent concentrated in the water-soluble fraction of cheeses, are of considerable importance for actual cheese flavour. Apart from amino acids and small peptides, which are considered to be of less importance for true cheese flavour (Engels & Visser, 1994), various volatile components were found in this fraction, e.g. amines, aldehydes, alcohols, acids, and sulphur compounds (Bosset & Gauch, 1993; Engels, Dekker, de Jong, Neeter, & Visser, 1997; Gallois & Langlois, 1990; Law, 1987; Visser, 1993). In part, these components are produced by degradation of amino acids (Alting, Engels, van Schalkwijk, & Exterkate, 1995; Engels & Visser, 1996; Hemme, Bouillanne, Métro, & Desmazeaud, 1982; Olson, 1990).

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The degradation of amino acids by non-starter organisms from surface-ripened cheeses, e.g. *Brevibacterium linens*, *Arthrobacter* sp. and *Corynebacterium* sp., and by secondary starter organisms from blue cheese, e.g. *Penicillium roqueforti*, has been reported (Dias & Weimer, 1998; Hemme et al., 1982; Law, 1987; Lindsay & Rippe, 1986). Research in our laboratory has shown that during the ripening of hard-type cheeses, such as Gouda, enzymes of mesophilic starter lactococci are also involved in the conversion of amino acids to aroma components (Engels & Visser, 1996). We detected degradation products of both methionine and leucine after incubation of these amino acids with cell-free extracts of *Lactococcus lactis* subsp. *cremoris* B78. Moreover, an enzyme from this organism, i.e. cystathionine β-lyase, capable of converting methionine into methanethiol was purified and characterized (Alting et al., 1995). A similar enzyme, cystathionine γ-lyase, was purified from *Lactococcus lactis* subsp. *cremoris* SK11 by Bruinenberg, de Roo, and Limsowtin (1997). Recently, Dias and Weimer (1998) and Gao, Mooberry, and Steele (1998) also reported the conversion of methionine to thiols by lactococcal enzymes. In the literature, various catabolic pathways for methionine have been considered (see Dias & Weimer, 1998).

The products of methionine breakdown are regarded to be important for cheese flavour. The presence of volatile sulphur compounds in various cheese types, e.g. Parmesan (Barbieri et al., 1994), Cheddar (Urbach, 1993), Gouda (Engels et al., 1997), and blue cheeses (Gallois & Langlois, 1990) has been reported. Until recently, their formation was usually attributed to the action of enzymes from non-starter organisms in cheese. Tanaka, Esaki, and Soda (1977) and Soda, Tanaka, and Esaki (1983) purified and characterized methionine γ-lyase from *Pseudomonas ovalis*, a pyridoxal 5′-phosphate (PLP)-dependent enzyme, which is regarded as a key enzyme in bacterial methionine metabolism. The same enzyme was reported by Collin and Law (1989) and by Lindsay and Rippe (1986) after its isolation from *Pseudomonas putida* and *Brevibacterium linens*, respectively. The enzyme catalyses the simultaneous deamination and dethiomethylation of methionine. Only the last few years, the possible role of aminotransferases in flavour formation in cheeses has received considerable attention. These PLP-dependent enzymes catalyze the transfer of the aminogroup from an α-amino acid to an α-keto acid (Ince & Knowles, 1986; Lee & Desmazeaud, 1985; Shipston & Bunch, 1989). The transamination of aromatic amino acids by *Brevibacterium linens* was investigated by Lee and Desmazeaud (1985), while aminotransferases from *Lactoccoccus lactis* strains converting aromatic amino acids were described by Yvon, Thirouin, Rijnen, Fromentier, and Gripon (1997) and Gao et al. (1998). Recently, the conversion of methionine via transamination was reported (Dias & Weimer, 1998; Engels, 1997; Gao et al., 1998).

In the present paper an enzymatic pathway of formation of methanethiol from methionine via 4-methylthio-2-ketobutyric acid (α-keto-γ-methylthiobutyric acid, KMBA) and of aldehydes from branched-chain amino acids is proposed. The purification and characterization of branched-chain aminotransferases from *Lactococcus lactis* subsp. *cremoris* B78 involved, are described.

2. Materials and methods

2.1. Materials

Amino acids, α-keto acids and the inhibitors hydroxylamine, DL-cycloserine, iodoacetamide, and DL-propargylglycine were purchased from Sigma Chemicals (St. Louis, Mo.). α-Keto glutaric acid was obtained from Janssen Chimica (Geel, Belgium), carboxymethoxylamine and KMBA from Aldrich Chemie GmbH (Steinheim, Germany), and *N*-ethylmaleimide from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.2. Organism and growth

Lactococcus lactis subsp. *cremoris* B78 was isolated from DL-type starter, code Bos, and was maintained and grown in milk (overnight at 20°C). Late-logarithmic phase cells were harvested from a 5-L culture and washed as described previously (Engels & Visser, 1996). The washed cells were resuspended to an $OD_{650\,nm}$ of approximately 80 in 20 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 20 μM PLP (20 mM KPi-EDTA-PLP). The suspension was stored overnight at −30°C.

2.3. Preparation of cell-free extract (CFE)

The −30°C cell suspension was defrosted and subsequently the cells were disrupted ultrasonically at 0°C for 15 cycles of 15 s (Heat Systems Sonicator XL 2020, NY, USA). The treated suspension was centrifuged (30 min, 30,000 *g*, 4°C) to remove intact bacteria and cell debris, and the supernatant (CFE) was collected. CFE was stored at −30°C until further use.

2.4. Determination of aminotransferase (AT) activities

AT activities, towards methionine, in CFE and in fractions thereof obtained during purification were determined routinely by incubating 100 μL samples in 20 mM or 50 mM (purified enzyme) KPi-EDTA-PLP, pH 7.5, with methionine (final concentration 10 mM) and cosubstrate α-ketoglutaric acid (final concentration 5 mM). The final volume of the incubation mixtures was 200 μL. The incubations were performed at 30°C for 2 h in the dark.

In a preliminary experiment it was established that the AT reaction rate was constant up to 3 h of reaction. The reaction was stopped by lowering the pH of the mixture to 2.5 via addition of 0.2 M HCl. This procedure will be further referred to as the standard assay.

The formation of KMBA during incubations was quantified by use of high-performance liquid chromatography (HPLC). The HPLC system consisted of an ISS-100 Perkin-Elmer automatic sample injector, two Waters M6000 A pumps, an AGC Waters type 680 gradient controller, and a Kratos 783 UV detector operating at 220 nm. Samples were chromatographed at 30°C on a Bio-Rad HiPore RP-318 reversed-phase column (4.6 mm × 250 mm) preceded by a Bio-Rad C_{18} cartridge guard column. The elution buffers were 5% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 90% acetonitrile, 0.08% TFA in water (solvent B). The components in the reaction mixture were separated isocratically at 0% solvent B for 5 min followed by a linear gradient from 0 to 70% solvent B over 2 min and isocratic elution at 70% solvent B for 5 min. The flow rate was 0.8 mL min^{-1}. KMBA eluted at 9.4 min. KMBA concentrations were determined using a standard curve with KMBA (0.02–5 mM) in 20 mM KPi-EDTA-PLP. Perkin-Elmer Nelson turbochrom 4.0 software (Cupertino, CA.) was used for processing raw HPLC data.

In preliminary studies a fast thin-layer chromatography (TLC) method for identification of KMBA as described by Alting et al. (1995) was used. Solutions of methionine and KMBA were used as standards. R_f values of methionine and KMBA were 0.57 and 0.71, respectively.

2.5. Determination of α-keto acid conversion

The conversion of KMBA to methanethiol, by CFE and fractions thereof, was monitored by determining the methanethiol oxidation product dimethyldisulphide (DMDS), using dynamic headspace gas chromatography with flame-photometric detection of sulphur compounds (Alting et al., 1995). Incubations with KMBA (final concentration 4 mM) were in 20 mM KPi-EDTA-PLP, pH 7.5, at 30°C.

Conversion of 3-methyl-2-ketobutyric acid to 2-methylpropanal by decarboxylating enzyme was measured after overnight incubation (35°C) of 3.5 mL CFE with 3-methyl-2-ketobutyric acid (10 mM) in a final volume of 5 mL 0.1 M Tris-Hepes buffer (pH 8) with and without addition of cofactor thiamine pyrophosphate (TPP, 100 μM). Headspace gas chromatography with flame ionization detection (GC-FID) was used for quantification of 2-methylpropanal formation.

2.6. Purification of aminotransferases

All purification steps were carried out at 4°C. Fractionation was performed by stepwise addition of solid ammonium sulphate to 80 mL CFE. Four ammonium sulphate fractions, i.e. 0–20% (w/v) pellet, 20–37% (w/v) pellet, 37–55% (w/v) pellet and 55% supernatant, were obtained by adding solid ammonium sulphate. Precipitated protein material was collected by centrifugation at 15,000 *g* for 5 min and dissolved in 20 mM KPi-EDTA-PLP, pH 7.5 (in the original CFE-volume). The solution displaying the highest AT activity towards methionine was extensively washed with the buffer and concentrated in a stirred-type Amicon ultrafiltration cell (Amicon, Danvers, MA, USA), operated under a nitrogen pressure of 300 kPa with a 10 kDa molecular mass cut-off Omega membrane (Filtron, Northborough, MA, USA). The washed and concentrated protein sample (15 mL, 6.7 mg protein mL^{-1}) was applied to a Q-Sepharose (Pharmacia Biotech, Uppsala, Sweden) anion-exchange column (50 mm × 140 mm) equilibrated with 20 mM KPi-EDTA-PLP, pH 7.5. Proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 20 mM KPi-EDTA-PLP buffer over a period of 100 min followed by isocratic elution at 0.5 M NaCl for 30 min. A flow rate of 5 mL min^{-1} was used and 10 mL fractions were collected. Absorbance was measured at 280 nm with a UV-MII detector (Pharmacia-LKB, Uppsala, Sweden). The active fractions were pooled and concentrated by ultrafiltration as described above. The concentrated enzyme solution (5 mL, 5 mg protein mL^{-1}) was loaded onto a MonoQ HR 5/5 anion-exchange column (Parmacia Biotech) equilibrated with the 20 mM KPi-EDTA-PLP buffer. Proteins were eluted with a linear gradient of 0–0.45 M NaCl in 20 mM KPi-EDTA-PLP buffer at 0.5 mL min^{-1} over a period of 90 min. Fractions with a volume of 1 mL were collected. Absorbance was measured at 280 nm. AT activity towards methionine was detected in fractions of two different regions of the MonoQ chromatogram. These will be referred to as AT-A and AT-B containing fractions, respectively. Each fraction was analyzed for purity by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). The purest active fractions from each of the two MonoQ peaks were pooled, washed with 50 mM KPi-EDTA-PLP, pH 7.5, and concentrated by ultrafiltration as described above. The sample containing AT-A was stored at −20°C. The concentrated sample containing AT-B was loaded onto a Superose 12 HR 10/30 gel filtration column (Pharmacia Biotech) and eluted with 50 mM KPi-EDTA-PLP, pH 7.5 at a flow rate of 0.3 mL min^{-1}. The AT active fractions were collected and stored at −20°C.

2.7. Analytical determinations

Purity of the enzymes was monitored by SDS-PAGE. For native PAGE, 8–25% Phastgel gradient gels were used with the Phast System (Pharmacia Biotech, Uppsala, Sweden) and 4–15% gradient Mini Protean II Ready Gels with a Mini Protean II cell (Bio-Rad

Laboratories, Hercules, CA). SDS-PAGE was performed on 12.5% polyacrylamide gels with either the Phast System or the Mini Protean II cell. Proteins from Pharmacia low- and high-molecular-mass calibration kits were used as molecular mass markers.

Isoelectric points were determined using linear pH gradient gels in the range 4–6.5 (Pharmacia Phastgel IEF 4–6.5) with the Pharmacia Phast System.

Proteins on both PAGE and IEF gels were detected by using Coomassie Brilliant Blue R-250 or silver staining (Pharmacia). Electrospray-ionization mass spectrometry (ESI-MS) (Micromass Quattro II, Cheshire, UK) was conducted on protein fractions obtained by reversed-phase (RP) HPLC. The HPLC equipment used was described above, with 10% acetonitrile and 0.1% TFA in water (buffer A) and 90% acetonitrile and 0.08% TFA in water (buffer B) as solvent system. Proteins were separated by a linear gradient of buffer B in buffer A from 20 to 50% buffer B over 30 min, from 50 to 70% buffer B over 5 min and isocratic at 70% buffer B for 5 min. The flow rate was 0.8 mL min^{-1}.

2.8. Protein determination

Protein concentrations were estimated by the method of Bradford (1976) with the Pierce test (Pierce, Rockford, IL, USA) using bovine serum albumin as standard (fraction V, Pierce).

2.9. Determination of temperature and pH dependence of activity

In the fractions obtained from MonoQ the activities of AT-A and AT-B towards methionine (amino donor) and α-ketoglutaric acid (amino acceptor) were determined at temperatures ranging from 10 to 70°C in 50 mM KPi-EDTA-PLP (pH 7.5) using the standard assay. Dependence on pH, with methionine and α-ketoglutaric acid as substrates, was assayed in the universal buffer described by Britton and Robinson (1931) at 30°C.

2.10. Substrate specificity

Activity of AT-A and AT-B towards α-ketoglutaric acid and the various amino acids tested was determined by incubation of AT-containing MonoQ fractions (20 μg protein) with the amino acids (final concentration 10 mM) and α-ketoglutaric acid (final concentration 5 mM) at 30°C for 2 h in the dark in 50 mM KPi-EDTA-PLP, pH 7.5. The decrease of the concentration of the amino acids, as a result of AT activity, was measured with a 4151 Alpha Plus amino acid analyser (Pharmacia-LKB, Uppsala, Sweden). The standard aminotransferase assay was used to measure activity of AT-A and AT-B (20 μg protein in assay) towards methionine and various α-keto acids. A final concentration of 5 mM was used for each α-keto acid.

2.11. Effects of inhibitors on the activity

The effects of several inhibitors were determined by studying AT-A and AT-B activity towards methionine and α-ketoglutaric acid, using the standard assay, after preincubation of the enzymes for 30 min with the inhibitors (each at a concentration of 1 mM).

3. Results

3.1. Formation of KMBA and DMDS from methionine by enzymes in CFE of Lactococcus lactis *subsp.* cremoris *B78*

Methionine was incubated, in the presence of α-ketoglutaric acid and PLP, with various CFE sub-fractions obtained by ammonium sulphate precipitation. Equal volumes of the CFE sub-fractions were used. Single sub-fractions, as mentioned in Materials and Methods, as well as combinations of these sub-fractions, were used in the incubations (Table 1). Production of the volatile sulphur compound DMDS was measured by gas chromatography.

The amount of DMDS formed during incubation of total CFE with methionine and α-ketoglutaric acid was about 3 μmol L^{-1} (Table 1), no single CFE sub-fraction being capable of forming similar amounts of DMDS. Only the combined 20–37 and 37–55% ammonium sulphate sub-fractions were able to generate nearly the same level of DMDS. TLC revealed formation of the α-keto

Table 1
Formation of DMDS from methionine or KMBA by CFE fractions obtained by precipitation with ammonium sulphate[a]

CFE fraction	DMDS formation (μmol L^{-1})	
	Substrate methionine (10 mM)	Substrate KMBA (4 mM)
0–20% $(NH_4)_2SO_4$ pellet	0.23	—[b]
20–37% $(NH_4)_2SO_4$ pellet	0.50	1.26
37–55% $(NH_4)_2SO_4$ pellet	0.41	0.08
0–20% + 20–37% $(NH_4)_2SO_4$ pellet	0.73	1.49
0–20% + 37–55% $(NH_4)_2SO_4$ pellet	0.71	0.18
20–37% + 37–55% $(NH_4)_2SO_4$ pellet	2.89	0.99
CFE	3.06	0.79

[a]Incubations performed at 30°C under standard assay conditions (see Materials and Methods) with α-ketoglutaric acid (5 mM) as cosubstrate. Pellets dissolved in equal volumes of 20 mM KPi-EDTA-PLP, pH 7.5, as described in Materials and Methods.
[b]Not detected.

acid KMBA only during incubation with these combined sub-fractions and during incubation with the single 37–55% ammonium sulphate sub-fraction. These data led to the conclusion that *Lactococcus lactis* subsp. *cremoris* B78 degrades methionine by a two-step mechanism in which KMBA is an intermediate. In our case the 37–55% sub-fraction was required for the formation of KMBA from methionine, whereas the 20–37% ammonium sulphate sub-fraction facilitated the breakdown of KMBA. The formation of KMBA, as well as the requirement of α-ketoglutaric acid, suggested involvement of aminotransferases, present in the 37–55% ammonium sulphate sub-fraction. A drastically reduced formation of KMBA after ultrafiltration of the 37–55% ammonium sulphate sub-fraction, which also removed the cofactor PLP, furnished further evidence for the involvement of aminotransferases (the amount of KMBA formed had decreased to less than 10% after removal of PLP).

Substantial formation of DMDS from KMBA (4 mM) only occurred in the presence of the 20–37% ammonium sulphate sub-fraction (Table 1). This sub-fraction apparently contains enzymes capable of converting α-keto acids, e.g. decarboxylases, although the direct product of decarboxylation of KMBA, methional by this sub-fraction could not be detected. In the presence of CFE from *Lactococcus lactis* subsp. *cremoris* B78, however, the formation of aldehydes from the α-keto acids from methionine and the branched chain amino acids valine, leucine and isoleucine could be detected with the aid of GC-MS (results not shown). The level of decarboxylation, as measured by GC-FID, of the α-keto acid of valine, 3-methyl-2-ketobutyric acid, to 2-methylpropanal by CFE of *Lactococcus lactis* subsp. *cremoris* B78 is shown in Fig. 1. The addition of TPP, a cofactor for decarboxylases, enhanced the formation of 2-methylpropanal.

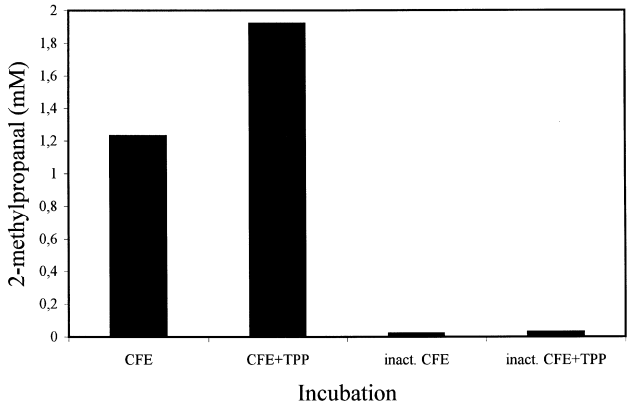

Fig. 1. Decarboxylation of 3-methyl-2-ketobutyric acid to 2-methylpropanal by CFE of *Lactococcus lactis* subsp. *cremoris* B78 with and without TPP. Inact. CFE = CFE inactivated by heating for 10 min at 100°C.

3.2. Purification and characterization of aminotransferases

The 37–55% ammonium sulphate fraction was subjected to further fractionation by Q-Sepharose chromatography and anion-exchange chromatography on a MonoQ HR 5/5 column (Fig. 2). Fractions 16–18 and 26–28 of the MonoQ chromatogram each contained an aminotransferase activity, referred to as AT-A and AT-B, respectively. The results of the purifications of the AT-A- and AT-B-containing fractions, based on the assay of methionine aminotransferase activity, are shown in Table 2. The level of purification of AT-A was 52-fold and the level of purification of AT-B, achieved with an additional gel filtration step, was 250-fold.

As judged by SDS-PAGE both AT-A- and AT-B-containing fractions were reasonably homogeneous, each showing a clear band corresponding to proteins with an apparent molecular mass near 40 kDa (Fig. 3). However, the presence of several components in the purified fractions was detected by RP-HPLC and by isoelectric

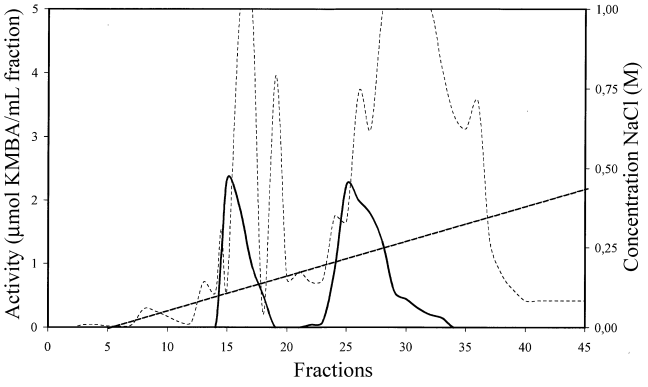

Fig. 2. Resolution on MonoQ HR 5/5 of two aminotransferase activities (—) towards methionine. Fractions (1 mL) were collected and assayed for activity using α-ketoglutaric acid as cosubstrate under standard assay conditions. Displayed are A_{280} (- - -) and concentration NaCl (– – –).

Table 2
Purification of AT-A and AT-B from *Lactococcus lactis* subsp. *cremoris* B78

Purification step		Total protein (mg)	Total activity (U)[a]	Specific activity (U mg^{-1})	Purification (fold)
Cell-free extract		859	503	0.6	1.0
Ammonium sulphate fraction 37–55%		95	74	0.8	1.4
Q-Sepharose		32	44	1.4	2.4
MonoQ	(AT-A)	0.3	9	31	51.7
	(AT-B)	1	15	15	25.0
Superose 12 (AT-B)		0.01	1.5	150	250

[a]One unit (U) catalyses the formation of 1 μg of KMBA per min.

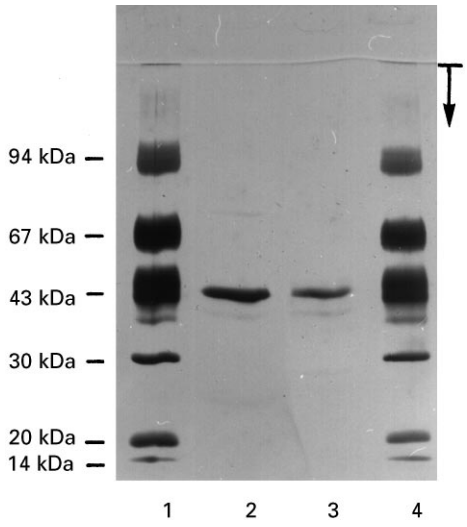

Fig. 3. SDS-PAGE in combination with silver staining showing purified AT-A (lane 3) and AT-B (lane 2). Lanes 1 and 4: Pharmacia low-molecular-mass markers (see Materials and Methods).

focusing (results not shown). After applying silver staining, the isoelectric focusing gel showed four bands for AT-A and three bands for the AT-B containing fraction. The bands were cut out of the gels, the protein was extracted with 50 mM KPi-EDTA-PLP, pH 7.5, and AT activity towards methionine was determined. The gel administered with the AT-A-containing fraction displayed AT activity in a protein band with a pI value of 4.45. With the AT-B-containing fraction, a protein band with a pI value of 4.55 showed AT activity.

For further purification of AT-A, the four subfractions obtained by IEF were subjected to RP-HPLC. In Fig. 4 the RP-HPLC chromatograms are shown as well as the chromatogram of the whole AT-A-containing MonoQ fraction. From the fact that trace A represents the only IEF fraction having AT-A activity, it was concluded that this activity should be ascribed to component 2 rather than to component 1, which was obviously copurified with AT-A (Fig. 4). Component 2 was isolated and its molecular mass was determined to be 41.98 kDa by ESI-MS. This value is in agreement with the molecular mass of the corresponding AT-A-containing MonoQ fraction, estimated by SDS-PAGE (with both components 1 and 2 appearing in one band, Fig. 3). On native PAGE a single 75 kDa band, displaying AT activity towards methionine, was observed (result not shown).

3.3. Temperature and pH dependence

For the transamination of methionine, the optimum pH was 8, for both AT-A and AT-B. AT-A and AT-B exhibited a rather broad range of maximum activity between the pH values 7 and 8.5. At the pH of cheese (5.2) about 20% of the enzyme activity found at the optimum pH was observed.

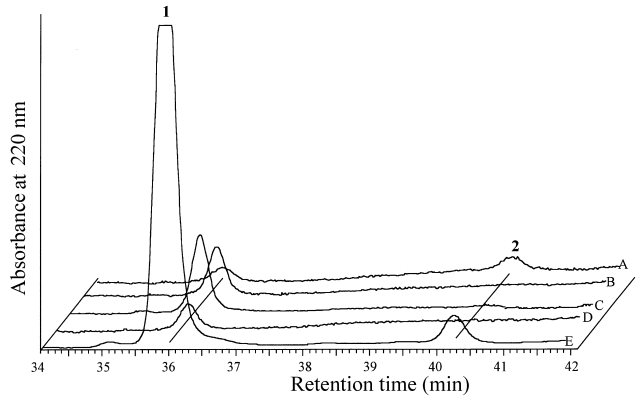

Fig. 4. Reversed-phase high performance liquid chromatograms of the total MonoQ AT-A-containing fraction (trace E) and of sub-fractions purified from the IEF gel (traces A–D). 1 = protein copurified with AT-A, 2 = AT-A.

Table 3
Substrate specificity of the aminotransferases AT-A and AT-B

Substrate	Relative activity (%)	
	AT-A	AT-B
Amino donor[a]		
L-valine	323	376
L-isoleucine	307	385
L-leucine	282	327
L-methionine	100	100
L-phenylalanine	12	38
L-tyrosine	—[b]	24
L-tryptophan	—	24
L-aspartic acid	—	1
L-histidine	—	<1
Amino acceptor[c]		
α-Ketoglutaric acid	100	100
α-Ketobutyric acid	78	75
α-Ketopentanoic acid	64	60
α-Ketoadipinic acid	45	43
Phenylpyruvic acid	38	44
Pyruvic acid	12	12
Oxalacetic acid	4	5

[a]Five millimolar α-ketoglutaric acid was used as amino-group acceptor. The concentrations of the amino acids were 5 mM, except for tyrosine, which was assayed at 2 mM. Activity on methionine was adjusted to 100%.
[b]Not detected.
[c]Ten millimolar methionine was used as amino-group donor. All α-keto acids were assayed at 5 mM. Activity on α-ketoglutaric acid was adjusted to 100%.

The optimum temperature for methionine transamination by AT-A and AT-B was 45–50°C. At temperatures above 50°C the activity decreased rapidly.

3.4. Substrate specificity

Table 3 shows the relative activities of AT-A and AT-B towards various amino acids and α-keto acids. The

Table 4
Effects of inhibitors on activity of AT-A and AT-B

Inhibitor	Inhibitor concentration	Remaining activity (%)[a]	
		AT-A	AT-B
Hydroxylamine	1 mM	2	5
Cycloserine	1 mM	13	16
Carboxylmethoxylamine	1 mM	2	3
Iodoacetamide	1 mM	85	80
N-ethylmaleimide	1 mM	99	87
Propargylglycine	1 mM	99	92
EDTA	4 mM	100	99
NaCl	0.17 M	98	101
	0.85 M	89	86

[a]The level of activity observed in the absence of inhibitor was defined as 100%.

enzymes showed a relatively broad specificity towards both α-keto acids (particularly α-ketoglutaric acid, α-ketobutyric acid, and α-ketopentanoic acid) and amino acids. Valine, isoleucine, leucine, and methionine were the preferred amino acids, suggesting mostly branched-chain aminotransferase activity. AT-B also showed substantial activity towards aromatic amino acids.

3.5. Inhibitors

AT-A and AT-B were strongly inhibited by the carbonyl binding agents hydroxylamine, carboxymethoxylamine and cycloserine (Table 4). The thiol reagents iodoacetamide and *N*-ethylmaleimide caused only a minor inhibition of AT-A and AT-B activities when utilized at a concentration of 1 mM. At a concentration of 3 mM, however, *N*-ethylmaleimide caused considerable inhibition. Propargylglycine and EDTA had no inhibitory effect, whereas NaCl showed (minor) inhibition of aminotransferase activity only when present at a high concentration (0.85 M).

4. Discussion

Methionine plays a central role in the catabolism of sulphur-containing amino acids. In vertebrates the transphulfuration pathway is known (Cooper, 1983; Soda, 1987). This route comprises the conversion of methionine to cystathionine through *S*-adenosylmethionine and homocysteine. For microorganisms a similar pathway has been characterized and one of the enzymes involved is cystathionine β-lyase. Previously, we reported the purification of this enzyme from the starter organism *Lactococcus lactis* subsp. *cremoris* B78 and its possible relevance for cheese flavour formation (Alting et al., 1995). Furthermore, we observed the formation of the transamination product KMBA from methionine with cell extracts of *Lactococcus lactis* subsp. *cremoris* B78 and we proposed a pathway for formation of methanethiol from methionine by enzymes from this organism (Engels, 1997). From the results of the present incubation experiments performed with single as well as combined ammonium sulphate precipitation sub-fractions of cells from this organism, we conclude that at least two enzymic steps are involved in transaminase-catalyzed degradation of methionine (Fig. 5). The first step, during which KMBA is produced, is the actual transamination step. The intermediate KMBA is, probably after decarboxylation, converted to methanethiol, which under aerobic conditions is converted rapidly to DMDS and/or DMTS. The enzymes involved in these two steps are also involved in degradation of the branched-chain amino acids leucine, isoleucine and valine (Fig. 5). Morgan (1976) described the conversion of branched-chain amino acids by transamination, and the subsequent conversion of the keto acids formed to aldehydes, due to the metabolic activity of *Lactococcus lactis* var. *maltigenes*. In the case of methionine the aldehyde formed, by decarboxylation of KMBA, would be 3-methylthiopropanal (methional). Methional has been detected in Cheddar (Urbach, 1993) and Emmental cheese (Preininger, Rychlik, & Grosch, 1994). Our experiments showed that decarboxylation of the α-keto acids of valine, leucine and isoleucine occurred in the presence of enzymes from *Lactococcus lactis* subsp. *cremoris* B78. It is possible that KMBA, like the other α-keto acids, was also enzymatically decarboxylated. On the basis of their results Gao et al. (1998) proposed a catabolic pathway of methionine by lactococci similar to that displayed in Fig. 5.

Two fractions with AT activity towards methionine, AT-A and AT-B, were resolved by anion-exchange chromatography. Both ATs were also able to transaminate various other amino acid substrates, such as leucine, isoleucine, valine, and phenylalanine, and displayed dependence on PLP and on an α-keto acid cosubstrate. The higher activity towards the branched-chain amino acids leucine, isoleucine, and valine indicated that we have purified branched-chain ATs (Kanda, Hori, Kurotsu, Ohgishi, Hanawa, & Saito, 1995; Lee-Peng, Hermodson, & Kohlhaw, 1979). A broad substrate specificity, however, is a common feature of ATs and conversion of methionine by branched-chain aminotransferases of other origin has been reported (Kanda et al., 1995; Kido, 1988; Scislowski & Pickard, 1993). This broad specificity implicates that the same enzymes may be used for conversion of various amino acids leading to different flavour compounds in cheese.

ATs are pyridoxal-5′-phosphate (PLP) dependent enzymes that catalyze the reversible transfer of the amino group from an α-amino acid to an amino acceptor, primarily an α-keto acid (Andreotti et al., 1994). Transaminations are physiologically important because they play a crucial role in the biosynthesis as well as the catabolism

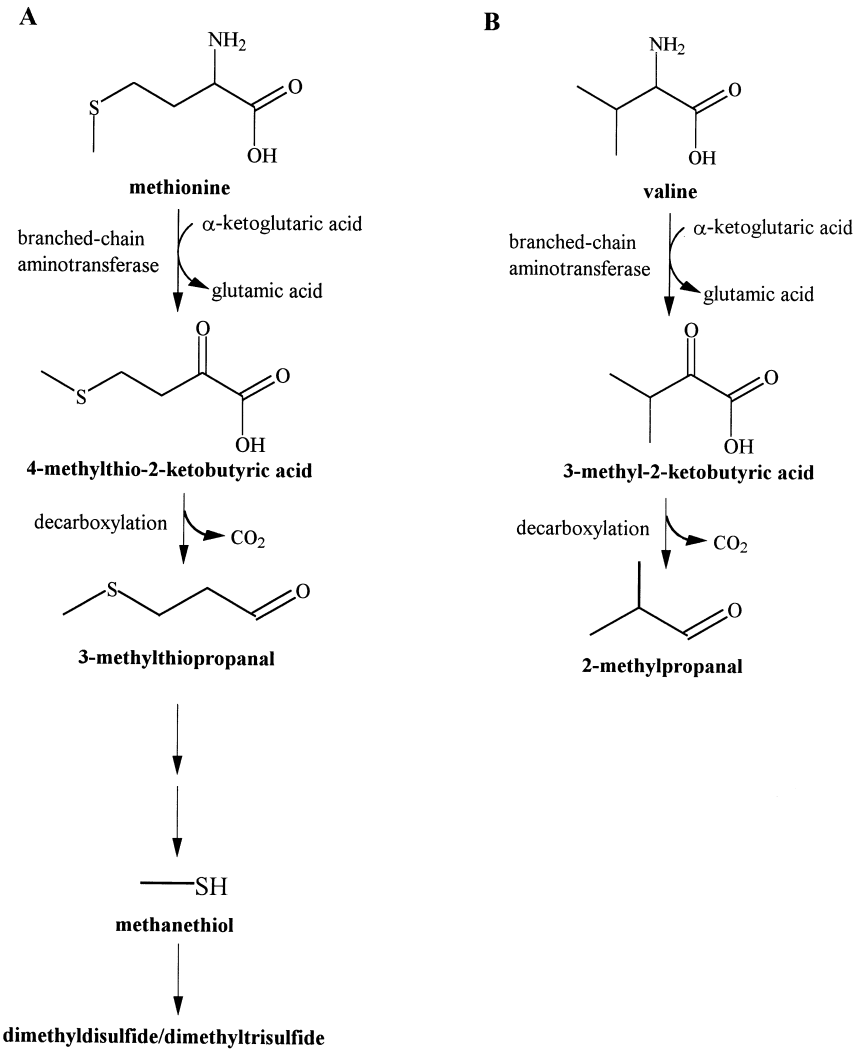

Fig. 5. Proposed pathway of the formation of methanethiol from methionine (A) and 2-methylpropanal from valine (B) by enzymes from *Lactococcus lactis* subsp. *cremoris* B78.

of amino acids (Fotheringham et al., 1986; Massey, Sokatch, & Conrad, 1976; Torchinsky, 1987). In nutritionally fastidious bacteria, like lactic acid bacteria, the interconversion of amino acids by ATs may be necessary when the organisms are grown on media not supplying the optimal proportion of amino acids.

Apart from the difference in elution behaviour during MonoQ anion-exchange chromatography, the AT-A and AT-B containing fractions differed in amino acid substrate specificity. AT-A was able to transaminate branched-chain amino acids, methionine and phenylalanine. In addition to these amino acids, tyrosine and tryptophan were used as a substrate by AT-B. Regarding the specificity towards the α-keto acids, there was hardly any difference between AT-A and AT-B, α-ketoglutaric acid being the most preferred cosubstrate for both enzymes. In contrast with a branched-chain aminotransferase purified from *E. coli* (Kanda et al., 1995), the lactoccal enzymes also utilized oxalacetate and pyruvic acid as amino acceptors. Both AT-A- and AT-B-containing fractions showed a 40 kDa band on SDS-PAGE, whereas with native gradient PAGE a 75 kDa band was seen. This suggests that the ATs are dimeric proteins with identical subunits. In *E. coli* (Kanda et al., 1995) and in mammals (Kido, 1988) branched-chain ATs with similar structural properties were found. With ESI-MS the sub-unit molecular mass of AT-A was estimated to be 41.98 kDa. Both AT-A and AT-B had a rather high optimum temperature and an alkaline pH optimum. These findings were in accordance with results found for other ATs (Kanda et al., 1995; Kido, 1988). The pI values of AT-A and AT-B were comparable with pI values reported for branched-chain ATs of *Methanococcus aeolicus* (Xing & Whitman, 1992) and *Bacillus brevis* (Kanda et al., 1995). Like most PLP-dependent enzymes the ATs were strongly inhibited by the carbonyl reagents hydroxylamine, cycloserine, and carboxymethoxylamine, presumably by their reaction with the aldehydic moiety

of PLP (Gentry-Weeks, Keith, & Thompson, 1993). Branched-chain ATs from *E. coli* (Kanda et al., 1995) and mammals (Kido, 1988) showed equal sensitivity towards these reagents. Propargylglycine, a strong inhibitor of cystathionine lyases (Alting et al., 1995) had no effect on the activity of the lactococcal ATs. Only a modest inhibition by thiol reagents (iodoacetamide and *N*-ethylmaleimide), EDTA, and NaCl was observed, which was in agreement with findings for other ATs (Ryan, Bodley, & Fottrell, 1972). The relatively small differences between the two AT-types reported in our study, might suggest that isomers of the same enzyme have been purified.

Transamination during cheese ripening has been reported. However, mostly organisms from surface-ripened cheese, e.g. *B. linens* (Hemme et al., 1982; Lee & Richard, 1984) were involved. A similar route of degradation of methionine, leading to the formation of ethylene, methanethiol, and carbon dioxide, has been proposed in *E. coli* by Ince and Knowles (1986) as well as by Shipston and Bunch (1989). In a paper published by Yvon et al. (1997) the purification and characterization of an aromatic amino acid converting aminotransferase from *Lactococcus lactis* subsp. *cremoris* NCDO 763 was described. The enzyme is able to convert aromatic amino acids, but also leucine and methionine. However, the specificity towards various amino acids, as well as the specificity towards α-keto acids, is clearly different from those of the enzymes described in this paper. For instance, the branched-chain amino acids isoleucine and valine did not appear to be suitable substrates for the aminotransferase described by Yvon et al. (1997). Recently, Gao and Steele (1998) purified 2 oligomeric species of an aromatic amino acid amino transferase from *Lactococcus lactis* subsp. *lactis* S3. Like the enzyme purified by Yvon et al. (1997) the enzymes primarily catalyzed the conversion of leucine and aromatic amino acids. They would remain active under conditions present in ripening cheese. The importance of the transaminase-catalyzed route of degradation of methionine is strongly supported by the recent finding of Fernández et al. (2000) that disruption of the gene encoding cystathionine-β-lyase in *Lactococcus lactis* MG1363 does not result in a decrease in formation of DMDS during incubation of CFE of this organism with methionine.

The formation of volatile sulphur compounds is considered to be very important for formation of cheese flavour (Cuer, Dauphin, Kergomard, Roger, Dumont, & Adda, 1979; Engels & Visser, 1996; Jollivet, Bezenger, Vayssier, & Belin, 1992). Previously, the breakdown of methionine, and other amino acids, was frequently attributed to non-starter organisms. Furthermore, in hard-type cheeses, like Cheddar, the breakdown of methionine, and the subsequent formation of sulphur volatiles, was considered mainly to be a non-enzymic process favoured by the low redox potential in the cheese interior (Manning, 1978). The present study demonstrates the role of enzymes from mesophilic lactococci in the process of methionine conversion. Together with the degradation of other amino acids by the ATs characterized, but also by other enzymes, this may lead to a myriad of breakdown products in the ripening cheese, many of them contributing to cheese flavour. A prerequisite for the above processes to take place is, of course, the availability of free amino acids. This requires a balanced proteolysis in cheese as well as lysis of starter cells to some extent (Visser, 1993). The conditions in the cheese interior, e.g. low oxygen, low moisture, high salt, will naturally influence the amino acid conversion processes. However, our results show that, at least in the case of the ATs, probably sufficient activity remains to allow a significant conversion of methionine.

Future research will further focus on the relevance of the pathways described and on the regulation of the enzymes involved for flavour formation.

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