

An Aminotransferase from *Lactococcus lactis* Initiates Conversion of Amino Acids to Cheese Flavor Compounds

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The enzymatic degradation of amino acids in cheese is believed to generate aroma compounds and therefore to be involved in the complex process of cheese flavor development. In lactococci, transamination is the first step in the degradation of aromatic and branched-chain amino acids which are precursors of aroma compounds. Here, the major aromatic amino acid aminotransferase of a *Lactococcus lactis* subsp. *cremoris* strain was purified and characterized. The enzyme transaminates the aromatic amino acids, leucine, and methionine. It uses the ketoacids corresponding to these amino acids and α -ketoglutarate as amino group acceptors. In contrast to most bacterial aromatic aminotransferases, it does not act on aspartate and does not use oxaloacetate as second substrate. It is essential for the transformation of aromatic amino acids to flavor compounds. It is a pyridoxal 5'-phosphate-dependent enzyme and is composed of two identical subunits of 43.5 kDa. The activity of the enzyme is optimal between pH 6.5 and 8 and between 35 and 45°C, but it is still active under cheese-ripening conditions.

The enzymatic degradation of amino acids in cheese is believed to generate aroma compounds and therefore to be involved in the complex process of cheese flavor development.

Amino acids are released from casein through the action of proteolytic enzymes. While they are directly involved with small peptides in the basic taste of cheese, amino acids do not contribute directly (by themselves) to the typical cheese flavors which are associated with the volatile fraction of cheese (10, 34). However, they can contribute indirectly to these typical flavors since they are precursors of volatile aroma compounds such as aldehydes, acids, alcohols, esters, and thiols. Especially, aromatic, branched-chain, and sulfurous amino acids are precursors of compounds with, respectively, floral, cheesy, and sulfur flavors (11). Such compounds have been found in various cheeses, including semihard cheeses such as Gouda and Cheddar (8, 15, 28, 30). Moreover, some degradation products of aromatic amino acids participate to the development of off-flavors in cheese, especially in Cheddar (9).

Amino acid degradation in cheese is due mainly to the action of microbial enzymes although chemical degradation (Strecker degradation) can occur during ripening. Lactic acid bacteria (LAB), which are present in all types of cheeses, are known to play a major role in generating small peptides and amino acids in cheese (42). While proteinases and peptidases of LAB have been studied extensively (24), very little is known about amino acid catabolism in these bacteria. The degradation routes of amino acids in some cheese microorganisms have been studied (2, 25, 27, 29, 35), and, generally, they involve different reactions, including deamination, transamination, decarboxylation, and cleavage of the amino acid side chain (18).

In lactococci, transamination seems to be the first step of aromatic amino acid degradation, since no oxidative deamination or decarboxylation was detected in several strains of *Lactococcus lactis* subsp. *lactis* or *cremoris* (12, 39). Ketoacids produced by transamination can then either undergo spontaneous

degradation, as observed for indolepyruvic acid, which gave indoleacetic acid (12), or be enzymatically degraded to the corresponding aldehydes or carboxylic acids, as observed for phenylpyruvic acid (39). The transamination reaction is catalyzed by aminotransferases.

Aminotransferases which can transfer the α -amino group of aromatic amino acids to a ketoacid acceptor have been studied in various microorganisms, and some of them have been purified. Generally, two or more enzymes with overlapping specificities are found in the same microorganism. For example, in *Escherichia coli* there are two aminotransferases that utilize both aromatic amino acids and aspartate; one is encoded by the *tyrB* gene, and the other is encoded by the *aspC* gene (13). Aminotransferases of LAB have never been identified or purified, and the genes encoding these enzymes have not been identified either. It is essential to study the enzymes involved in the transformation of amino acids into aroma compounds to control or to improve their action during cheese ripening. As the first step of our work on the enzymatic conversion of amino acids to aroma compounds, here we have purified and characterized the major aromatic aminotransferase from *L. lactis* subsp. *cremoris* and have shown that this enzyme is essential for the transformation of aromatic amino acids to aroma metabolites by the cells. It is also able to initiate the degradation of leucine and methionine, which are also precursors of cheese aroma compounds.

MATERIALS AND METHODS

Chemicals. Amino acids, ketoacids, inhibitors, pyridoxal 5'-phosphate (PLP), EDTA, streptomycin sulfate, and lysozyme were obtained from Sigma Chemical Co., (St. Louis, Mo.). Q-Sepharose- and phenyl-Sepharose-Fast Flow gels and Mono-Q HR 10/10 and Superose 12 HR 10/30 columns were from Pharmacia Biotech (Uppsala, Sweden). L-[2,3,4,5,6-³H]phenylalanine was obtained from Amersham (Little Chalfont, England).

Strain, growth, and preparation of cellular extract. *L. lactis* NCDO 763, obtained from the National Collection of Food Bacteria (Shinfield, Reading, England), is designed as an *L. lactis* subsp. *cremoris* strain according to its genotype (14). It was grown in a chemically defined medium (CDM) (37) to an absorbance at 480 nm of 2.8.

Cells from a 5-liter culture were harvested by centrifugation at $8,000 \times g$ for 5 min and washed twice in 50 mM sodium β -glycerophosphate buffer (pH 7). The cells were resuspended in 250 ml of 50 mM triethanolamine buffer (pH 7) containing 20% sucrose and 0.4 mg of lysozyme/ml. After homogenization and

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incubation for 120 min at 30°C, the spheroplasts were separated from the soluble cell wall fraction by centrifugation at $12,000 \times g$ for 30 min and then lysed by being resuspended in 250 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM of β -mercaptoethanol 2 mM EDTA, and 0.1 mM of pyridoxal 5'-phosphate (buffer A). The cell particles were removed by centrifugation at $20,000 \times g$ for 30 min, and streptomycin sulfate was added to the supernatant to a final concentration of 5% (wt/vol). Insoluble material was removed by centrifugation at $20,000 \times g$ for 20 min, and the intracellular fraction was filtered through a 0.45- μ m-pore-size filter (Millipore Corp., Bedford, Mass.) and dialyzed overnight against buffer A.

Purification. The dialyzed cellular extract was loaded at 3 ml/min onto a Q-Sepharose Fast Flow column (gel bed, 83 ml) equilibrated with the buffer A. The column was washed with equilibration buffer until the absorbance at 280 nm returned to the baseline value, and retained proteins were eluted at 3 ml/min with a 150-min linear gradient of 0.1 to 0.5 M NaCl in buffer A. Fractions containing the L-phenylalanine: α -ketoglutarate aminotransferase (Phe-AT) activity, which eluted between 0.25 and 0.44 M NaCl, were pooled and concentrated to 25 ml with a 200-ml ultrafiltration cell equipped with a Diaflo YM-10 membrane (10-kDa cutoff) (Amicon Corp., Danvers, Mass.). Potassium phosphate was added to the concentrated active fraction to reach a final concentration of 1.5 M. After being stirred for 10 min at 0°C, the sample was centrifuged at $5,000 \times g$ for 10 min and the supernatant was loaded at a flow rate of 1 ml/min onto a column of phenyl-Sepharose Fast Flow (gel bed, 6.3 ml) which had been equilibrated with buffer A containing 1.5 M potassium phosphate (pH 7.5). The column was then washed with 1 bed volume of the equilibration buffer, and retained proteins were eluted with a 250-min linear gradient of 1.5 to 0 M potassium phosphate in buffer A at 1 ml/min. Fractions containing Phe-AT activity (which eluted between 0.45 and 0.22 M potassium phosphate) were pooled and dialyzed against buffer A. Dialysis buffer was replaced every 2 h for a total of three times. The dialyzed fraction was loaded in three steps at 3 ml/min onto a Mono-Q HR 10/10 column equilibrated with 25 mM Tris-HCl buffer (pH 8) (Tris buffer). The column was washed with equilibration buffer, and the enzyme was eluted with a 100-min linear gradient of 0.15 to 0.4 M NaCl in the same buffer at 3 ml/min. Eluent was collected in 3-ml fractions. The three most active fractions (which eluted around 0.3 M NaCl) were separately diluted 1:3 with 25 mM Tris buffer (pH 8) and reinjected onto the same Mono-Q column equilibrated with the same Tris buffer. The enzyme was then eluted with a 100-min linear gradient of 0.25 to 0.5 M sodium acetate in the Tris buffer at a flow rate of 2 ml/min, and eluent was collected in 2-ml fractions. The active fractions were pooled, concentrated by ultrafiltration on a Diaflo YM-10 membrane, desalted by gel filtration in a PD-10 column (Pharmacia), equilibrated with Tris buffer containing 0.05 mM PLP, and stored at -20°C. All steps were carried out at around 4°C.

Enzyme assays. To trace the enzyme activity during the different purification steps, we routinely used the test for Phe-AT coupled with the colorimetric L-glutamic acid assay of Boehringer (Mannheim, Germany). The reaction mixture for Phe-AT activity contained 70 mM Tris-HCl (pH 8), 3 mM L-phenylalanine, 10 mM of α -ketoglutarate, 0.05 mM PLP, and enzyme in a total volume of 0.250 ml. The stock solution of α -ketoglutarate was prepared daily, and its pH was adjusted to 6.0 to 7.0 with NaOH. The reaction was performed at 37°C for 15 min. The colorimetric L-glutamic acid assay was carried out on 20 μ l of the reaction mixture by the procedure described by Boehringer, except that the total volume was decreased to 250 μ l so that the assay could be performed in enzyme-linked immunosorbent assay plates. A Titertec Multiscan Plus detector (Flow Laboratories, Lugano, Switzerland) was used to measure the absorbance at 492 nm.

To determine specific activities, specificity for amino acids and α -ketoacids, and dependence on pH, temperature, and inhibitors, the formation of L-glutamic acid (or the amino acid corresponding to the α -keto acid used as amino group acceptor) was precisely measured by amino acid analysis with an LC3000 automatic analyzer (Biotronik, Maintal, Germany). Prior to amino acid analysis, the reaction for aminotransferase activity was performed as described above and stopped by adding sulfosalicylic acid to a final concentration of 3% (wt/vol). The mixture was kept for 10 min at 0°C prior to centrifugation at $20,000 \times g$ for 5 min. The supernatant was further diluted with the sample buffer (pH 2.2) (Biotronik) for amino acid analysis. One enzyme unit (1 U) is defined as producing 1 μ mol of glutamate per min from α -ketoglutarate.

For the specificity studies with the purified enzyme, other amino acids and α -keto acids were used as substrates under similar conditions. To investigate pH dependence, three buffer systems at a final concentration of 70 mM were used: acetate-phosphate in the pH range from 4.5 to 6.0, potassium phosphate in the pH range from 6.0 to 8.0, and phosphate-glycine in the pH range from 8.0 to 9.5. The temperature dependence was determined at the optimum pH (pH 8.0), and the stability of the enzyme was examined by keeping the pure preparation for 5, 15, 30, and 60 min at 0, 20, 37, 50, and 60°C prior to the assay. The effect of inhibitors was established after a 5-min preincubation of the pure enzyme preparation with the different inhibitors at final concentrations of 1 and 10 mM.

Protein determination. Protein concentrations were determined by the micro-method of Bradford (6) with the Coomassie protein assay reagent and bovine serum albumin fraction V as the standard, as specified by Pierce Chemical Co. (Rockford, Ill.).

TABLE 1. Purification steps for the Phe-AT activity of *L. lactis* subsp. *cremoris* NCDO763

Purification step	Total amt of protein (mg)	Total activity (U)	Sp act (U/mg)	Purification factor	Recovery (%)
Cell extract	336	33.6	0.1	1.0	100
Q-Sepharose	105	25.5	0.24	2.4	76
Phenyl-Sepharose	32	23.8	0.73	7.3	71
Mono-Q (NaCl)	2.2	5.1	2.3	23	15
Mono-Q (Na acetate)	0.047	1.3	27.6	276	4

Estimation of molecular mass. Molecular mass was estimated by gel filtration on a Superose 12 HR 10/30 column equilibrated with buffer A containing 0.15 M NaCl. Elution was performed at 0.3 ml/min. The column was previously calibrated under similar conditions with a mixture of marker proteins: tyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.35 kDa) (Bio-Rad Laboratories, Hercules, Calif.). Molecular mass was also estimated under denaturing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

PAGE. PAGE were performed with a Mini Protean II cell (Bio-Rad) as specified by the manufacturer. Native PAGE was carried out with discontinuous gels in which the running gel consisted of 10% acrylamide and the stacking gel consisted of 4% acrylamide. For SDS-PAGE, the running gel was made with 12.5% acrylamide and the stacking gel was made with 6% acrylamide. SDS-PAGE standards, low range (Bio-Rad), were used as molecular weight references. Proteins were visualized by Coomassie blue staining (Phastgel blue R; Pharmacia) or by silver staining as described by Blum et al. (5). Aminotransferase activities were detected in native PAGE by incubating thin gel bands (2 mm wide) in the reaction mixture for the enzyme assay and measuring the formation of L-glutamic acid by the colorimetric assay, as described above.

Phenylalanine catabolism by lactococci. The catabolism of [³H]phenylalanine by cells of *L. lactis* subsp. *cremoris* NCDO763 in five reaction mixtures containing different α -keto acids was compared. The five mixtures contained 100 mM Tris-HCl (pH 8) buffer, 0.3% glucose, 2 mM unlabelled phenylalanine, and 0.05 μ M L-[2,3,4,5,6-³H]phenylalanine (126 Ci/mmol). The first mixture did not contain any α -keto acid, while the other four contained 10 mM α -ketoglutarate, 10 mM α -ketoisocaproate, 10 mM oxaloacetate, and 10 mM α -ketoisovalerate. Cells from a 4-ml CDM culture, prepared and washed as described above, were added to 0.5 ml of reaction mixture and incubated at 37°C. Aliquots (100 μ l) of the reaction mixture were removed at 0, 2, 10, and 40 h of reaction time and centrifuged at $8,000 \times g$ for 5 min. The metabolites in the supernatants were separated by reverse-phase high-performance liquid chromatography (HPLC) and identified by comparison of retention times with those of appropriate standard compounds. The HPLC equipment consisted of a Waters 600 pump, a 717 plus auto sampler, a 486 UV detector (Waters Corp., Milford, Mass.), and a Radiomatic Flo-one/Beta A-515TR radio-HPLC detector (Packard Instrument Co., Meriden, Conn.). Data acquisition and processing were performed with the Flo-one for Windows radio-HPLC software (Packard). The separation was carried out on a Novapak C₁₈ column (2 by 150 mm; 4- μ m particles; Waters) equilibrated with 95% solvent A (0.115% trifluoroacetic acid)-5% solvent B (0.1% trifluoroacetic acid, 60% acetonitrile) at a flow rate of 0.3 ml/min. Metabolites were eluted with a linear gradient of 5 to 20% solvent B from 0 to 35 min; this was followed by a 5-min wash with 50% solvent B and a 15-min reequilibration under the initial conditions. UV detection was operated at 214 nm, and the eluent (flow rate, 0.3 ml/min) was then mixed with Ultima-Flo AP scintillation cocktail (0.7 ml/min; Packard) for radioactivity detection. The standard compounds used were phenylalanine, phenylethylamine, phenylpyruvate, phenylacetaldehyde, phenyllactate, phenylethanol, and phenylacetate.

RESULTS

Purification. Most of the Phe-AT activity (99%) was recovered in the intracellular extract. The results of purification of the intracellular extract are shown in Table 1, based on Phe-AT activity. The enzyme was purified 276-fold in a four-step procedure, with 4% recovery. With the Q-Sepharose chromatography, the Phe-AT activity was eluted as one major peak, which accounted for around 93% of the total activity. The residual activity was eluted earlier, in fractions also containing leucine and methionine aminotransferase (Leu-AT and Met-AT) activities. In the next three chromatographic steps, the Phe-AT activity was recovered as a single peak, which indicates that the purified aminotransferase accounted for most (more

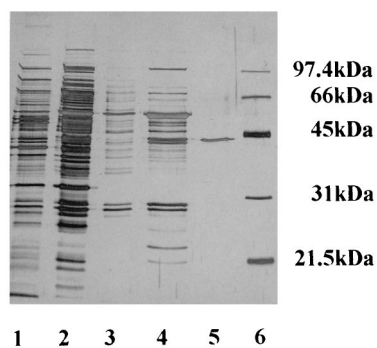


FIG. 1. SDS-PAGE (12.5% polyacrylamide) with silver staining showing the different purification steps of the Phe-AT activity. Lanes: 1, intracellular extract; 2 to 5, fractions from Q-Sepharose, phenyl-Sepharose, Mono-Q with sodium chloride as the counterion, and Mono-Q with sodium acetate as the counterion, respectively; 6, Bio-Rad low-molecular-mass markers.

than 90%) of the Phe-AT activity in the cell extract. Recovery from the Mono-Q chromatography step was poor because a large part (the beginning and the end) of the peak of activity, which was contaminated with other proteins, was discarded. The final fraction was judged to be pure by PAGE (data not shown) and SDS-PAGE with silver staining (Fig. 1). From a nonstained lane of native PAGE, gel bands 2 mm wide were cut out, and the Phe-AT activity was detected in the band corresponding to the single band visualized by silver staining (data not shown). The pure fraction, which had a specific activity of 27.6 U/mg of protein with phenylalanine and α -ketoglutarate as substrates, was used for characterization.

Characterization. The molecular mass of the enzyme was 87 kDa as determined by Superose 12 gel filtration and 43.5 kDa as determined by SDS-PAGE (Fig. 1). These data indicate that the enzyme is homodimer, which was confirmed by Edman degradation analysis of a blot from SDS-PAGE that produced a single sequence (data not shown).

The enzyme catalyzed the transamination of only L-amino acids. Table 2 shows that it exhibited a relatively broad substrate specificity. Among amino acids, its best substrate was leucine when α -ketoglutarate was used as the amino group acceptor, but it was also very active on the three aromatic amino acids and to a lesser extent on methionine. Other L-amino acids were not substrates. It can use all the ketoacids corresponding to its amino acid substrates as an amino group acceptor.

The activity of the enzyme determined at 37°C was optimal between pH 6.5 and 8 with phenylalanine and α -ketoglutarate as substrates and between pH 7 and 8 with leucine and α -ketoglutarate as substrates. The activities for phenylalanine and leucine in the pH range from 5 to 5.5 were 60 and 30% of activities at optimum pH, respectively. For both Phe-AT and Leu-AT activities, the optimum temperature determined at pH 8 was between 35 and 45°C. At 10°C, the activities were still 20 to 25% of the activities at the optimum temperature. The pure-enzyme preparation was not very stable. Storage of the purified enzyme in Tris-HCl buffer (pH 8) with 0.05 mM PLP at -20°C led to a progressive loss of its activity, which reached 40% after 4 months. However, it was rather stable up to 40°C over a period of 1 h but was partially inactivated by heating at 50°C (40% for 1 h) and still further inactivated by heating at 60°C (more than 80% for 30 min).

Table 3 summarizes the effect of some inhibitors and metal ions on the activity of the purified aminotransferase. The enzyme was not sensitive to sulfhydryl reagents such as iodoac-

etamide and iodoacetic acid. It was strongly inhibited by carbonyl reagents such as hydroxylamine, phenylhydrazine, DL-penicillamine, and, to a lesser extent, cysteine, which all are known inhibitors of PLP-dependent enzyme. Aminothiols, such as penicillamine and cysteine, react with free and bound PLP to yield thiazolidine derivatives, which are undoubtedly responsible for the inhibition of the enzyme. The fact that the thiazolidine formed from penicillamine is more than 10 times more stable than the thiazolidine formed from cysteine explains why penicillamine is a much more potent inhibitor than cysteine (36). However, 10 mM cysteine inhibited activity only by 64% when PLP was present at 0.05 mM in the reaction medium, which suggests that PLP is rather tightly bound to the enzyme. This was confirmed by the fact that when the pure preparation was desalted on a PD10 column with 50 mM potassium phosphate buffer, the enzyme lost only 45% of its initial activity, which was partially (80%) restored by adding 0.05 mM PLP. The enzyme was not metal ion dependent, since the chelating agent, EDTA, had no inhibitory effect and no divalent cation (Fe^{2+} , Co^{2+} , or Zn^{2+}) stimulated its activity. In contrast, Cu^{2+} and Ca^{2+} exerted an inhibitory effect, with Cu^{2+} being more potent in this respect. The activity of the enzyme was not affected by 0.4% NaCl but was reduced 40% by 4% NaCl.

Role of aminotransferases in the catabolism of phenylalanine. In the reaction medium containing α -ketoglutarate, the major metabolites produced by lactococcal cells from phenyl-

TABLE 2. Substrate specificity of the aminotransferase isolated from *L. lactis* subsp. *cremoris* NCDO 763

Substrate	Relative activity
Amino donor^a	
L-Leucine.....	100
L-Tyrosine.....	73
L-Phenylalanine	72
L-Tryptophan	37
L-Methionine.....	12
L-Isoleucine	— ^c
L-Valine	—
L-Histidine.....	—
L-Aspartate.....	—
L-Alanine.....	—
L-Cysteine.....	—
L-Lysine.....	—
L-Proline.....	—
D-Phenylalanine.....	—
D-Leucine.....	—
Amino acceptor^b	
α -Ketoglutarate (Glu).....	100, 100
α -Ketoisocaproate (Leu).....	112, ND ^d
α -Ketomethylvalerate (Ile).....	1.4, ND
α -Ketoisovalerate (Val).....	0.2, —
β -Phenylpyruvate (Phe).....	ND, 136
<i>p</i> -Hydroxyphenylpyruvate (Tyr).....	123, 121
Indole-3-pyruvate (Trp).....	128, 107
4-Methylthio-2-oxobutyrate (Met).....	94, 78
Oxaloacetate (Asp).....	—, —

^a α -Ketoglutarate (10 mM) was used as amino group acceptor. The concentrations of amino acids were 3 mM. The activity of leucine was adjusted to 100.

^b Phenylalanine (3 mM) (first number) and 3 mM leucine (second number) were used as amino group donors. All ketoacids were assayed at 10 mM. The activity on α -ketoglutarate was adjusted to 100. The amino acid corresponding to the amino group acceptor is given in parentheses.

^c —, not detected.

^d ND, not determined.

TABLE 3. Effect of protein-labeling reagents, transaminase inhibitors, and metal ions on the activity of the aminotransferase isolated from *L. lactis* subsp. *cremoris* NCDO 763^a

Compound	Concn (mmol/liter)	Inhibition (%) ^b
Iodoacetic acid	1	0
	10	3
Iodoacetamide	1	15
	10	18
Cysteine	1	37
	10	64
Hydrozylamine	1	100
	10	100
DL-Penicillamine	1	100
	10	100
Phenylhydrazine	1	100
	10	100
EDTA	1	0
	10	9
NaCl	10	2
	0.4%	2
	4%	41
CaCl ₂	1	26
	10	49
CuCl ₂	1	76
	10	100
FeCl ₂	10	0
CoCl ₂	10	0
ZnCl ₂	10	0

^a Purified enzyme was incubated with the inhibitors for 5 min at pH 8 before assaying for Phe-AT and Leu-AT activity with α -ketoglutarate as the amino group acceptor. NaCl, EDTA, CaCl₂, CuCl₂, FeCl₂, CoCl₂, and ZnCl₂ were added to the assay buffer.

^b The results are means of Phe-AT and Leu-AT activities.

alanine were identified as phenylpyruvate, phenyllactate, and phenylacetate (Fig. 2). Phenyllactate and phenylacetate are degradation products of phenylpyruvate. Their amounts gradually increased with incubation time until 40 h, while at the same time the amount of phenylalanine declined. Table 4 shows the effect of α -keto acid addition to the reaction medium on the degradation rate of phenylalanine. In reaction media lacking α -keto acid, 15 to 20% of phenylalanine was degraded to phenyllactate and phenylacetate for the first 2 h. Then phenylalanine and metabolite concentrations remained at the same level. The addition of oxaloacetate did not modify the phenylalanine degradation, and addition of α -ketoisovalerate accelerated it slightly. In contrast, adding α -ketoglutarate or α -ketoisocaproate sharply increased the degradation, which was complete after 40 h of incubation. No degradation occurred, even after 40 h, in blanks in which cells were not included.

DISCUSSION

Aminotransferases that catalyze the reversible transamination of aromatic amino acids in the presence of an α -keto acid

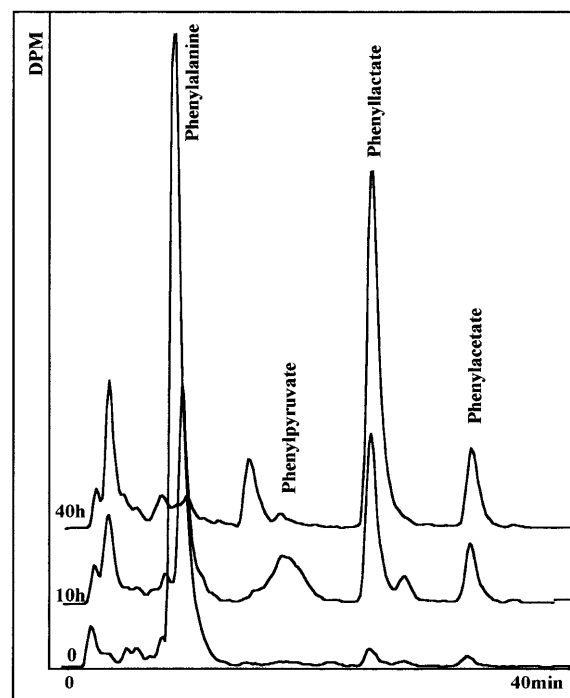


FIG. 2. HPLC separation and identification of [³H]phenylalanine metabolites produced by incubation for 0, 10, and 40 h of resting cells in reaction mixture containing α -ketoglutarate under the conditions described in Materials and Methods.

acceptor in a variety of microorganisms have been studied (3, 4, 21–23, 26, 31–33, 43, 44) but never in lactic acid bacteria. These enzymes play a major role in the metabolism of aromatic amino acids since they are involved in the last step of biosynthesis and take part in their catabolism. In this work, we have purified and characterized such an aminotransferase from *L. lactis* subsp. *cremoris* NCDO 763.

The purified aminotransferase was like other bacterial aromatic aminotransferases (Ar-AT) or aspartate aminotransferases (Asp-AT) previously described, but its specificity for substrates was unusual. Like almost all aminotransferases, it is a PLP-dependent and metal ion-independent enzyme. Like most Ar-AT and Asp-AT, our aminotransferase was not sensitive to sulfhydryl reagents, it was composed of two identical subunits, and its optimum pH for activity was between 6.5 and 8. Its N-terminal sequence (26 residues [not shown]) was not homologous to that of other aminotransferases or to that of other proteins, but we have identified an internal sequence of

TABLE 4. Effect of adding α -keto acids to the reaction mixture on phenylalanine degradation by lactococcal cells

α -Keto acid added ^a	Amt of phenylalanine degraded (% of initial) after:		
	2 h	10 h	40 h
None	16	19	13
Oxaloacetate (Asp)	14	12	20
α -Ketoisovalerate (Val)	ND ^b	37	47
α -Ketoglutarate (Glu)	16	68	97
α -Ketoisocaproate (Leu)	ND	76	95

^a The amino acid corresponding to the α -keto acid is given in parentheses.

^b ND, not determined.

22 residues which shares 50% identity and 60 to 70% homology with some bacterial Asp-AT (1, 19, 38, 41, 46). Like almost all Ar-AT, it acts on the three aromatic amino acids, and like some of them, such as the transaminase D (TrD) from *E. coli*, it is also active on leucine and methionine, suggesting that one of the two substrates (amino acid or ketoacid) must have a hydrophobic group attached to the β -carbon of the compound. However, in contrast to most Ar-AT from microorganisms, including TrD from *E. coli*, it was not active on aspartate and did not utilize oxaloacetate as an amino group acceptor. On the other hand, it must be pointed out that during purification, we isolated an Asp-AT which was not active on phenylalanine, in contrast to most bacterial Asp-AT including the transaminase A (TrA) of *E. coli* (26, 33, 43). The catalytic mechanism, as well as the substrate recognition mechanism, of TrA (from *E. coli*) has long been studied by using different methods, including X-ray crystallography and site-directed mutagenesis (16, 20, 40, 45). The comparison of TrA with TrD (17) gave some information on the distinctive feature of the active site which permits the recognition of aromatic substrates, although these two aminotransferases have overlapping specificities. Structural information on our Ar-AT from *L. lactis*, which does not utilize aspartate, could help to better understand the substrate recognition mechanism of Ar-AT.

From our results, we cannot conclude anything about the precise physiological role of the purified aminotransferase, but we can argue that the enzyme is probably involved in both biosynthesis and degradation of aromatic amino acids and only in catabolism of leucine and methionine. Indeed, since this strain of *L. lactis* subsp. *cremoris*, like all dairy strains of *L. lactis*, is auxotrophic for branched-chain amino acids and methionine, the reaction involving these amino acids must be exclusively catabolic. In contrast, aromatic amino acids are not essential for the growth of this strain (39), and the Phe-AT activity of extract of cells grown in medium lacking aromatic amino acids was similar to that of cells grown in CDM or in medium containing a high phenylalanine concentration. This result suggests that the purified aminotransferase, which was the major Phe-AT of cells grown in CDM, is constitutive. The physiological role of aminotransferases utilizing aromatic amino acids in different microorganisms has been studied (3, 21, 23, 26, 31, 32, 43). Generally, for a microorganism there are several enzymes capable of catalyzing the transamination of aromatic amino acids, and it has been demonstrated that some aminotransferases, which are constitutive, are involved mainly in biosynthesis while others, which are inducible, play a catabolic role (26, 43). The enzyme we have purified from *L. lactis* is undoubtedly responsible for the biosynthesis of phenylalanine and tyrosine, but it can also play a catabolic role in the presence of high concentrations of these aromatic amino acids.

Because of its role in the degradation of amino acids, the lactococcal Ar-AT is believed to play a decisive role in the formation of flavor compounds during cheese ripening. Indeed, it catalyzes the transamination of five amino acids (Leu, Phe, Tyr, Trp, and Met), which are precursors of aroma compounds, and transamination is the first step in the degradation of aromatic and branched-chain amino acids by *L. lactis*. We showed here that the presence of an α -keto acid acceptor was essential to initiate the degradation of phenylalanine by lactococcal cells. In the reaction medium lacking α -keto acid, a small amount of phenylalanine was degraded rapidly, probably due to the presence of residual α -keto acid in the cells and perhaps to the pyruvate produced by glycolysis, but the degradation of phenylalanine was rapidly stopped. Addition of α -keto acids, which are good substrates for the purified enzyme, to the reaction medium highly increased the production

of phenylalanine metabolites (phenyllactate and phenylacetate), whereas adding oxaloacetate, which is not a substrate, was without effect. Although α -ketoisovalerate was a poor substrate, its addition led to a small increase in the formation of phenylalanine metabolites. These results confirm the major role of the purified aminotransferase in the catabolism of aromatic amino acids by lactococci. However, 5 to 7% of the total Phe-AT activity in the cell extract was associated with a fraction containing Leu-, Ile-, and Met-AT activities. This enzyme(s) may also be slightly involved in aromatic amino acid transamination.

The possible role of the enzyme in the development of cheese flavors is also supported by the fact that it can be active under conditions of cheese ripening. Indeed, it is still active at cheese-ripening temperatures. Moreover, the intracellular medium is probably the best medium for enzyme activity, but amino acid transport or passive diffusion into the intact but energetically depressed cells could be a limiting factor. During cheese ripening, some lactococcal strains appear to be lysed, and different states of lysis have been distinguished (7). The first state corresponds to cell wall lysis, with only little alteration of the cytoplasmic membrane. In this case, the amino acid penetration into the cells is probably improved. The further states correspond to a rupture of the membrane and a progressive release of intracellular contents into the cheese. There, the enzyme could still act, since the cofactor appeared to be rather well bound to it and since it was still active at the pH of cheese and even at high NaCl concentrations. Although the enzyme activity was reduced markedly under these conditions, lysis could be beneficial because the release of intracellular peptidases accelerates the production of free amino acids (7), which are substrates for aminotransferases. However, the activities of enzymes which will further degrade α -keto acids into aroma compounds have still to be elucidated.

In conclusion, we have purified an aminotransferase from *L. lactis* which initiates the degradation of several amino acids, the precursors of aroma compounds, and which can be active under cheese-ripening conditions. Cloning of the gene coding for the enzyme is in progress, and genetically modified strains will be constructed to study the role and the importance of the enzyme in the development of cheese flavors.

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