***Lactococcus lactis* contains only one glutamate decarboxylase gene**

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Glutamate decarboxylase, which is associated with a glutamate-dependent acid-resistance mechanism, was purified from *Lactococcus lactis* subsp. *lactis* by a three-step procedure. The specific activity was increased about 114-fold with a yield of 16%. The N-terminal amino acid sequence of the enzyme was determined. The gene encoding this enzyme was cloned in *Escherichia coli*, and its nucleotide sequence was determined. The deduced amino acid sequence suggests that the enzyme is produced as a mature form (466 amino acid residues), not as a precursor protein. The subunit molecular mass of *L. lactis* glutamate decarboxylase was calculated to be 53 926 Da. The enzyme was maximally active at pH 4.7 and reacted only with L-glutamate among 20 α -amino acids. The apparent K_m value was calculated to be 0.51 mM. The activity was stable at acidic pH values; there was no activity in the neutral pH range. At pH 4.1 the enzyme activity was retained at temperatures up to 70 °C in 10 min incubations. *L. lactis* glutamate decarboxylase behaved as a single protein when the enzyme was purified. A single band corresponding to the glutamate decarboxylase gene was detected on Southern blot analysis. These data suggest that there is one glutamate decarboxylase gene in *L. lactis*.

Keywords: *Lactococcus lactis*, glutamate decarboxylase, acid resistance, *gadB*

INTRODUCTION

Glutamate decarboxylase (GAD; EC 4.1.1.15) catalyses the irreversible decarboxylation of L-glutamate to GABA. GAD has been isolated from a wide variety of sources and its biochemical properties have been characterized. *Escherichia coli* GAD (Fonda, 1985) is a homohexameric enzyme with a subunit size of 50 kDa. Each subunit contains a pyridoxal phosphate bound to a lysyl residue. *E. coli* contains two copies of GAD structural genes that map to different positions on the chromosome (Smith *et al.*, 1992). They are 98% similar at the nucleotide level. Induction of GAD is one of the acid stress responses in bacteria (Bearson *et al.*, 1997).

Lactococcus lactis is a Gram-positive coccus that forms pairs or chains and produces L-lactate from glucose.

Abbreviations: GABA, γ -aminobutyric acid; GAD, glutamate decarboxylase; CBB, Coomassie brilliant blue; PGI, phosphoglucose isomerase.

The DDBJ accession number for the sequence reported in this paper is AB010789.

Lactococcal strains are essential to milk fermentation, especially in the cheese-making process, to provide optimal conditions for curd formation and for the development of texture and flavour. Since *L. lactis* increases the acidity through its anaerobic fermentation, it is likely to have acid-resistance mechanisms to maintain viability under low-pH conditions. Amino acid decarboxylation decreases the acidity by the consumption of hydrogen ions. The generation of carbon dioxide by glutamate decarboxylation can cause cheese-eye formation during cheese ripening (Zoon & Allersma, 1996).

Sanders *et al.* (1998) sequenced the *L. lactis* *gadCB* gene and suggested that it encoded a glutamate-dependent acid-resistance mechanism comprised of glutamate-GABA antiporter and GAD. As it is difficult to purify GAD protein, the biochemical characteristics of the *L. lactis* enzyme have not yet been elucidated. In this study, we purified *L. lactis* GAD 114-fold by a three-step procedure and investigated its biochemical characteristics. Additional experiments indicated that there was only one GAD structural gene in *L. lactis*.

METHODS

Materials. An M3A-Sepharose column (Nomura *et al.*, 1997) was used for the purification of *L. lactis* GAD. The monoclonal antibody (mAb) M3A is specific to *L. lactis* phosphoglucose isomerase (PGI). The mAb was bound to EAH-Sepharose 4B (Pharmacia) by carbodiimide coupling.

Bacterial strains and growth conditions. *Lactococcus lactis* subsp. *lactis* biovar diacetylactis 01-7 was isolated from a cheese starter (Nomura *et al.*, 1998). *L. lactis* was maintained in sterile litmus milk and subcultured once a week. TYG medium (pH 6.8) consisted of 0.5% tryptone (Difco), 0.5% yeast extract (Difco), 1% glucose and 0.5% sodium succinate. An actively growing culture was obtained by transferring 1% inoculum to TYG medium and incubated at 30 °C for 16 h. *Escherichia coli* XL-1 Blue (Stratagene), used as the cloning host, was grown in LB medium. For screening of recombinants, LB medium was supplemented with ampicillin (50 µg ml⁻¹), X-Gal (100 µg ml⁻¹) and IPTG (0.5 mM).

Purification of GAD activity. All steps were done at 4 °C. Actively growing culture of *L. lactis* 01-7 (20 ml) was transferred to 2 litres of TYG medium. After incubation for 14 h at 30 °C, cells were harvested by centrifugation at 1800 g for 20 min. Pellets were washed once with 20 mM sodium phosphate buffer (pH 7.2) and resuspended with 100 ml of the same buffer containing lysozyme (0.1 mg ml⁻¹, Wako Pure Chemicals), *N*-acetylmuramidase (0.1 µg ml⁻¹, Seikagaku-kogyo) and DNase I (4 U ml⁻¹, Takara Shuzo). After 2 h incubation at 37 °C, the cell suspension was sonicated using a Tomy UR-150P ultrasonic cell breaker for 5 min. Cell debris was removed by centrifugation at 11000 g for 10 min. Ammonium sulfate was added to the cell-free extract to give a final concentration that was 80% saturated. After 2 h incubation, the precipitates were collected by centrifugation at 8000 g for 20 min and redissolved with PBS (10 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.4). The supernatant was collected by centrifugation at 11000 g for 10 min, applied to a Sephacryl S-300 column (4 × 80 cm) and the column developed with PBS. Active fractions were pooled and loaded onto an M3A-Sepharose column (2 ml) which had been equilibrated with PBS. The column was washed with PBS and eluted by a stepwise gradient of the following NaCl concentrations in PBS (10 ml each): 0.20, 0.25, 0.30, 0.35 and 0.40 M. The active fraction was dialysed against 50 mM sodium acetate buffer (pH 4.6) and stored at -20 °C until use. The experiments were done several times; results of a typical example are given.

Protein concentration was determined by the BCA protein assay reagent (Pierce).

Electrophoresis. SDS-PAGE on a 10% polyacrylamide gel was carried out under reducing conditions as described by Laemmli (1970). Native PAGE was performed using the standard pore formation of Davis at pH 7.5 (Gabriel, 1971). After electrophoresis, gels were stained with 0.1% Coomassie brilliant blue R-250 (CBB) and destained with 30% methanol.

GAD assay. GAD activity was measured as described previously (Nomura *et al.*, 1998). The reaction mixture consisted of 1 ml 50 mM sodium acetate buffer pH 4.7 containing 2 mM L-glutamate, 0.1 mM pyridoxal phosphate and aliquots of the enzyme preparation. The amount of GABA produced was determined using a Hitachi L-8500 amino acid analyser. One unit of GAD was defined as the amount of enzyme required to produce 1 µmol GABA from L-glutamate per min at 30 °C and pH 4.7.

N-terminal amino acid sequencing. GAD protein (5 µg) was separated on a 10% SDS-PAGE gel and electrotransferred to a PVDF membrane. The membrane was stained with 0.1% CBB, destained with 60% methanol, washed in distilled water and dried. The GAD band was cut out and was subjected to micro-sequencing on a Beckman LF-3400 gas-phase sequencer.

DNA methods. Unless specified otherwise, all manipulations of DNA were performed as described by Sambrook *et al.* (1989). Plasmid DNA from *E. coli* was isolated by the alkaline lysis method (Sambrook *et al.*, 1989). Genomic DNA from *L. lactis* was isolated by the method of Saito & Miura (1963); plasmid DNA from *L. lactis* was isolated according to Anderson & McKay (1983). pBluescript II SK(-) (Stratagene) was used as the cloning vector. Southern blots were transferred to Hybond-N (Amersham) and hybridized with probes that were labelled with digoxigenin-dideoxyUTP using a DIG DNA labelling and detection kit (Boehringer). Hybridization was performed at 42 °C in a buffer containing 50% formamide. The filter was washed twice in 2×SSC/0.1% SDS at room temperature and then twice in the same buffer at 68 °C.

DNA sequence analysis. DNA fragments inserted into the polylinker of pBluescript II were deleted unidirectionally by using a kilo-sequencing deletion kit (Takara Shuzo). Nested deletion clones were sequenced as described by Sanger *et al.* (1977) using a *Taq* dye-primer cycle sequencing kit and M13 universal dye primer (Applied Biosystems). Both strands were sequenced. Sequence data were assembled and analysed using the GENETYX Mac version 9.0.1 software (Software Development).

RESULTS

Purification of GAD from *L. lactis*

Purification of *L. lactis* GAD was first attempted with a procedure consisting of salting out, hydrophobic batch fractionation, gel filtration and anion-exchange chromatography. The purified GAD fraction, however, still manifested PGI activity. N-terminal amino acid

Fig. 1. M3A-Sepharose chromatography of *L. lactis* GAD. The partially purified GAD fraction (6.8 mg protein) was applied to the column (2 ml) which had been equilibrated with PBS. The elution conditions and the detection of GAD were as described in Methods. PGI activity assay was as described previously (Nomura *et al.*, 1997). Fractions of 2 ml were collected. —, NaCl concentration; ●, A_{280} ; ○, GAD activity; △, PGI activity.

Table 1. Summary of GAD purification from *L. lactis*

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (-fold)
Crude extract	197	424	0.466	100	1
Ammonium sulfate	184	412	0.446	93	1
Sephacryl S-300	47.6	6.84	6.96	24	15
M3A-Sepharose	31.2	0.584	53.2	16	114

sequencing of this fraction indicated that a major protein of the fraction was *L. lactis* PGI rather than GAD. Therefore, a column with an anti-PGI mAb bound to Sepharose (M3A-Sepharose) was used for the purification of GAD. GAD activity, which was partially fractionated from the cell-free extract by salting out and subsequent gel filtration, was applied to the antibody column. Both GAD and PGI activities were adsorbed onto the column at a concentration of 0.15 M NaCl (Fig. 1). All of the GAD activity was eluted from the column as a single peak with an NaCl concentration of 0.35 M, whereas PGI activity was eluted at 0.30 M NaCl. No PGI activity was detected in the GAD fraction. This indicated that GAD could be completely separated from PGI using an M3A-Sepharose column. It seemed that GAD adsorbed to the column better than PGI, although PGI was the original antigen for mAb M3A. As M3A had specific affinity for PGI in a Western blot analysis (Nomura *et al.*, 1997), GAD may have a form similar to the epitope for M3A in its surface structure.

The purification of GAD from *L. lactis* 01-7 is summarized in Table 1. The enzyme was purified so that it accounted for almost all the protein in the fraction. The specific activity of GAD was increased about 114-fold, with a yield of 16%. The activity was observed as a single peak on both Sephacryl S-300 gel filtration and M3A-Sepharose chromatography. A GAD preparation from M3A-Sepharose chromatography was subjected to native PAGE to examine its purity. One major protein was observed, although a few minor contaminants were visible. GAD activity in slices of an unstained gel showed a single peak coinciding with a major band of the CBB-stained gel. No other GAD activity was observed at the positions corresponding to any minor bands. PGI activity could not be detected in any slices of the gel. These data suggested that GAD activity of *L. lactis* resides in a single kind of protein. We used this fraction as the purified enzyme preparation for further studies.

Properties of *L. lactis* GAD

The pH dependence of GAD activity was examined. The activity was detected at pH 4.0–5.5 and was maximal at pH 4.7. The enzyme reacted only with L-glutamate among the 20 α -amino acids (including proline) tested. The apparent K_m value calculated from a Lineweaver–Burk plot was 0.51 mM. The effect of pH on stability of the enzyme was examined at 30 °C and 50 °C for 5 h at various pH values. The enzyme was

stable at acidic pH values ranging from 3.4 to 5.9 at 30 °C and to 4.6 at 50 °C. It showed no activity in the neutral pH range. The thermal stability of the enzyme was subsequently tested at pH 4.1 for 10 min. The activity remained at 90% or more from 30 to 70 °C, but was rapidly lost at 80 °C. The purified enzyme preparation was subjected to an SDS-PAGE analysis. One major band was observed, suggesting that *L. lactis* GAD, like other bacterial GADs, consisted of identical subunits. The apparent molecular mass of the subunit was estimated to be about 54 kDa. Attempts to determine the native molecular mass of the *L. lactis* GAD by gel filtration gave unreliable results and were abandoned.

N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the purified GAD was determined. Edman degradation provided a sequence of 15 residues from the N-terminus: MLYGKENRDEAEFLE. This agreed with the deduced amino acid sequence of the product of the *gadB* gene of *L. lactis* subsp. *lactis* MG1363 (Sanders *et al.*, 1998). However, alignment of this sequence with those of the GADs of *E. coli* (Smith *et al.*, 1992), *Streptococcus pneumoniae* (García & López, 1995), cat (Kobayashi *et al.*, 1987), drosophila (Jackson, 1990) and petunia (Baum *et al.*, 1993) failed to find significant similarity. It was previously reported that the sequences of the N-terminal region of various GADs were not similar, while the sequence of a 55-residue segment around the active-centre lysine was significantly conserved (Maras *et al.*, 1992).

DNA cloning and analysis

The gene for the GAD of strain 01-7 was cloned from a partial *Hind*III library in pBluescript II SK(–) by reverse genetics using a degenerate oligonucleotide 5'-TATGGAAAAGAAAATCGNGATGAA-3' designed on the N-terminal amino acid sequence (residues 3 to 10). The N-terminal sequences deduced from the open reading frame and determined from the purified enzyme were identical, and no prepro-sequence was observed. This indicated that the enzyme is produced as a mature form, not as a precursor protein. The sequence contained the active-site lysyl residue forming a Schiff base with pyridoxal phosphate and consensus amino acid residues conserved in GAD from mammalian and

Fig. 2. Restriction map and deduced amino acid sequence of the gene for GAD of *L. lactis* strain 01-7. (a) Restriction map of the 3.6 kb *HindIII*-*SalI* fragment of cloned 01-7 DNA. The dashed arrow indicates that *gadC* is incomplete. The hybridization probe for Southern blotting is shown by a long double-headed arrow below the map. (b) Primary structure alignment of GAD from *L. lactis* strains 01-7 and MG1363. A dot indicates a residue identical to both sequences. The sequence corresponding to the N-terminal amino acid sequence of the purified enzyme is underlined. Residues conserved in GAD from many sources are marked by asterisks. The pyridoxal-phosphate-binding lysine is arrowed.

bacterial sources (Maras *et al.*, 1992) (Fig. 2b). As expected, the gene and its deduced amino acid sequence were highly similar to *gadB* and GadB, respectively, of *L. lactis* MG1363 (Sanders *et al.*, 1998). Seventy-six of 1398 bases were substituted. Fourteen of these affect the amino acid encoded, resulting in the substitutions of 13 residues of 466 amino acids.

Southern blot analysis

A 2.4 kb *XbaI*-*EcoRI* fragment was labelled and used as a hybridization probe for the GAD gene (Fig. 2a). *L. lactis* 01-7 DNA was digested with restriction enzymes and subjected to Southern blotting (Fig. 3). One or two cross-hybridizing bands of sizes agreeing with expectations based on the restriction map were observed in all the digests. A single band was detected with the *EcoRI*, *XbaI* and *EcoRI/XbaI* digests, as the probe contained no sites for these enzymes. An *EcoRV* site was contained in the probe; two hybridizing bands were thus observed with this digest. Although the probe contained two internal *HindIII* sites, they were so near to each other that the probe did not hybridize efficiently to this small fragment under these conditions. These results indicated that there must be a single cross-hybridizing gene in *L. lactis* 01-7. Although strain 01-7 contained five or six plasmids, no hybridization signal was observed with these (data not shown), indicating that *gadB* is located on the chromosome. A Southern blot of another eight strains of *L. lactis* also suggested that *gadB* is a single-copy gene (unpublished result).

Fig. 3. Southern blot analysis of *L. lactis* subsp. *lactis* strain 01-7 DNA. The DNA was digested with *EcoRI* (lane 1), *XbaI* (lane 2), *XbaI/EcoRI* (lane 3), *HindIII* (lane 4) or *EcoRV* (lane 5). The blot was probed a digoxigenin-labelled 2.4 kb *XbaI*-*EcoRI* fragment (Fig. 2a).

DISCUSSION

In this study, we purified a protein with glutamate decarboxylation activity from *L. lactis*, demonstrated that this enzyme was GAD, and investigated its enzymic characteristics. The properties of *L. lactis* GAD were similar to those of *E. coli* GAD (Fonda, 1985). *L. lactis*

GAD reacted only with L-glutamate at pH 4.7, with a K_m of 0.51 mM. It consisted of 54 kDa subunits. The *E. coli* GAD also reacts only with L-glutamate at pH 4.6, with a K_m of 0.5–1.0 mM, and is composed of 50 kDa subunits. This comparison suggests that *L. lactis* GAD is a typical bacterial GAD.

The role of bacterial amino acid decarboxylases has been proposed to be in the maintenance of acidic pH by the consumption of hydrogen ions as decarboxylation proceeds (Gale, 1946). Three amino-acid-dependent acid-resistance systems have been described for enterobacteria (Bearson *et al.*, 1997). These systems involve the inducible amino acid decarboxylase, lysine decarboxylase, arginine decarboxylase and glutamate decarboxylase. When growth of *L. lactis* in skim milk stops, the cytoplasmic pH would be decreased to about pH 5.4–5.7 (Kashket, 1987; McDonald *et al.*, 1990); subsequently acids diffuse into cells across the membrane, resulting in the lowering of the intracellular pH (Lee & Wong, 1998). In this pH range GAD is able to decarboxylate the substrate better than lysine or arginine decarboxylases because their activities are optimal at pH 5.7–5.8 (Sabo *et al.*, 1974; Soda & Moriguchi, 1969) and pH 5.2 (Blethen *et al.*, 1968), respectively. During milk fermentation L-glutamate, substrate for GAD, will be available in larger quantities than the other amino acids, because the milk protein casein contains a high proportion of glutamate. These facts suggest that GAD is likely to play a more significant role in acid resistance of *L. lactis* than the other amino acid decarboxylases.

Since *L. lactis* tolerates low pH conditions better than many other bacteria, it must contain effective acid-resistance mechanisms. However, the present study indicated that *L. lactis* (a Gram-positive coccus) contains a single GAD gene (*gadB*), while the Gram-negative rods *E. coli* (Smith *et al.*, 1992) and *Shigella* spp. (Small & Waterman, 1998) contain two GAD genes. The functional properties of the two *E. coli* isozymes were identical (De Biase *et al.*, 1996). Expression of these genes confers resistance to low pH (Waterman & Small, 1996; Hersh *et al.*, 1996; Sanders *et al.*, 1998). As the properties of the enzymes were similar to each other, the function of the *E. coli* and *L. lactis gadB* genes seems likely to be similar. It is interesting that *L. lactis* shows the stronger acid-resistance phenotype, although it contains only one *gadB* gene.

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