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Detection of aminotransferase activity of *Propionibacterium freudenreichii* after SDS-PAGE

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Abstract

Aminotransferases (ATs) had previously been detected after native electrophoresis. We show now that aminotransferase(s) of *Propionibacterium freudenreichii* can be detected after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Moreover, it retained a high activity (84%) in the presence of 0.23% SDS, contrary to what was observed for aminotransferase(s) of *Bifidobacterium bifidum* (54%) and of six other cheese-related species (0–20%).

Keywords: Aminotransferase; Cheese-related bacteria; *Propionibacterium*; SDS-PAGE; Zymogram

1. Introduction

Numerous enzymatic activities have been detected in zymograms, after nondenaturing polyacrylamide gel electrophoresis (PAGE) (Gabriel and Gersten, 1992). However, some of these activities can also be detected after sodium dodecyl sulphate (SDS)-PAGE (Bischoff et al., 1998; Soler et al., 1999).

Aminotransferases (ATs) are ubiquitous enzymes involved in amino acid synthesis and catabolism. They catalyse the conversion of amino acids to the corresponding α-ketoacids, in the presence of an α-ketoacid acceptor of amino group (for example, α-ketoglutaric acid) which is converted into amino acid (glutamic acid). ATs of cheese-related bacteria have recently received an increase in interest, as they are responsible for the first step of degradation of amino acids to aroma compounds during cheese ripening. Nondenaturing zymograms have been used to detect AT activity in *Lactococcus lactis* (Gao et al., 1997; Yvon et al., 1997), as previously demonstrated for various plants (Driedger et al., 1994; Yamamoto and Duich, 1994). Since the use of a denaturing agent such as sodium dodecyl sulphate (SDS) improves resolution and allows molecular mass estimation, we assayed the detection of AT activity after SDS-PAGE. For that purpose, we determined first the effect of SDS toward AT activities in the cell-free extracts (CFE) of several cheese-related bacteria, and then directly after SDS-PAGE.

2. Material and methods

2.1. Strains and preparation of cell-free extracts

CFE were prepared of ten strains grown to late log phase on reference media (Table 1). Cells were

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Table 1
Origin of strains

Species	Strain	Collection	Medium
Bifidobacterium bifidum	ATCC29521 CIP 567	American Type Culture Collection, Rockville, MD Collection Institut Pasteur, Paris, France	MRS (Difco)
Brevibacterium linens	550	Provided by INRA, LGMPA, Thiverval-Grignon, France	TSB-YE[a]
Escherichia coli	NM514	Laboratory collection	LB[b]
Lactobacillus delbrueckii subsp. *lactis*	CIP101028	Collection Institut Pasteur, Paris, France	MRS
Lactobacillus helveticus	LH2	Industrial strain	MRS
Lactobacillus helveticus	LRTL430	Laboratory collection, commercial starter, France	MRS
Lactococcus lactis subsp. *cremoris*	CNRZ 105	Centre National de Recherche Zootechnique, Jouy-en-Josas, France	M17
Propionibacterium freudenreichii subsp. *shermanii*	TL34	INRA, Laboratoire de Recherches de Technologie laitière, Rennes, France	YEL[c]
Propionibacterium freudenreichii subsp. *shermanii*	ITGP23	Institut Technique Français du Fromage, Rennes, France	YEL
Streptoccccus thermophilus	CIP102303	Collection Institut Pasteur, Paris, France	M17 (Difco)

[a] Tryptone-Soy-Broth-Yeast Extract (Ferchichi et al., 1985).
[b] LB: Luria Bertani (Sambrook et al., 1989).
[c] YEL: Yeast Extract Lactate Agar (Malik et al., 1968).

harvested by centrifugation (8500 × *g*, 10 min, 4 °C), washed twice with sterile distilled water, and the suspension (OD_{650} ~ 20) subjected to a French Press apparatus (SLM Instrument, USA) (4 °C, 138 MPa, 10 min, two runs). Unbroken cells and cell walls were eliminated by centrifugation (30,000 × *g* for 20 min), and the supernatants sterilised by passing though 0.45-μm pore membranes. AT activity of CFE was assayed according to Yvon et al. (1997), with 4 mmol l^{-1} amino acid (Sigma). The final concentration of protein, determined by the method of Bradford (1976), with bovine serum albumin (Sigma) as standard, was standardised at 1350 μg ml^{-1}. Glutamic acid, which was produced from α-ketoglutaric acid, was enzymatically analysed with Boerhinger kit 139092 (Diffchamb, Lyon, France).

2.2. Assay of aminotransferase activity

AT activity was expressed as nmol glutamic acid produced per min per mg protein (U). To determine the effect of SDS (Carlo Erba) on AT activity, 20 μl of double-strength Laemmli buffer (Laemmli, 1970) without dithiothreitol, containing varying amounts of SDS (0.014% to 0.46% w/v final concentration), were added to 150 μl of the reaction mixture.

2.3. Electrophoresis

SDS-PAGEs were performed using the CFE of three species exhibiting different patterns of AT activity in the presence of SDS (*Propionibacterium freudenreichii*, *Bifidobacterium bifidum* and *Lactobacillus helveticus*), following the method of Laemmli (1970), using a Bio-Rad Maxi-PROTEAN II cell with a 12% separating gel topped with a 4% stacking gel (1-mm thickness). Acrylamide, bisacrylamide, ammonium persulfate *N*,*N*,*N*′,*N*′ tetramethylene diamine (Temed) were obtained from Bio-Rad. CFE were diluted in the same volume of double-strength Laemmli buffer containing 0.35% (w/v) dithiothreitol, and were not heated before loading. One hundred micrograms of protein (in 44 μl) was loaded per lane. Gels were run at room temperature at 60 V for 16 h then at 19 mA until the prestained standard of 25-kDa molecular mass migrated out of the gel. Standard proteins, prestained (ref. 161-0372 control No. 87922, Bio-Rad) or not (LMW, Pharmacia), were used for the estimation of molecular mass. Following electrophoresis, the gels were rapidly rinsed with distilled water and either stained in 0.1% (w/v) Coomassie brilliant blue in a methanol/acetic acid/water mixture (30:10:60) or assayed for AT activity after being cut into 2-mm slices. Each gel slice was incubated for 2 or 14 h at

37 °C in 150 μl transamination mixture containing 1% Triton X-100 and either leucine (Leu), isoleucine (Ile), valine (Val), phenylalanine (Phe), tyrosine (Tyr), methionine (Met), aspartic acid (Asp), or no amino acid (blank).

3. Results

Leucine AT activity varied depending on the species: the highest activity was found for *Lactococcus lactis* (13.6 U) and the lowest for *L. helveticus* LH2 (2.9 U).The presence of SDS reduced Leu AT activity for all the strains, but with very different patterns depending on species, *P. freudenreichii* ITGP23 keeping the highest activity (Fig. 1). Similar results were obtained for Phe AT activity, which was assayed for *P. freudenreichii* ITGP23 and *L. lactis* CNRZ 105: for example, at 0.23% SDS, *P. freudenreichii* kept 69% Phe AT activity (vs. 84% for Leu AT), and *L. lactis* < 1% (vs. 11%) (data not shown).

After SDS-PAGE, AT activities were detected only for the species exhibiting the highest activity in the presence of SDS, *P. freudenreichii*. For the two other species tested, *B. bifidum* and *L. helveticus* (two strains), no AT activity could be detected in gel slices

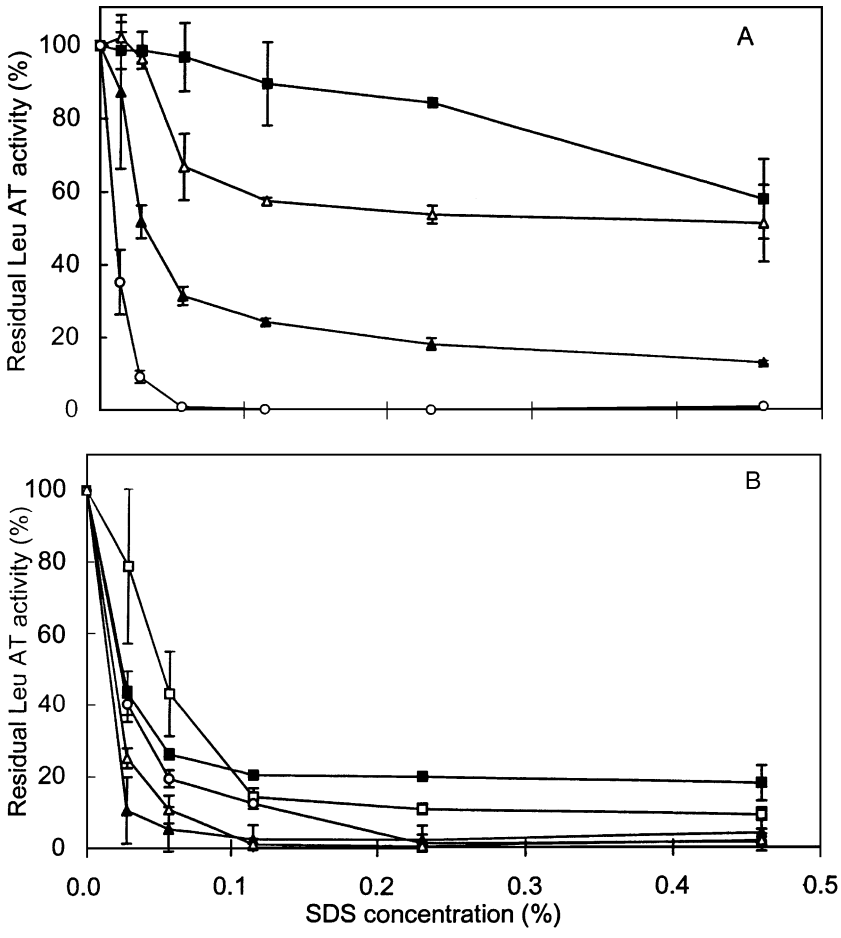

Fig. 1. Decrease in leucine aminotransferase activity in the presence of increasing amounts of SDS in the incubation mixture for the cell-free extracts of various bacterial species. A: ■, *P. freudenreichii* ITGP23; △, *B. bifidum*; ▲ *Brevibacterium linens*; ○, *Escherichia coli*. B: ■, *Streptococcus thermophilus*; □, *L. lactis*; ○, *Lactobacillus delbrueckii*; ▲ *L. helveticus* LBLH2; △, *L. helveticus* 430. Error bars indicate standard deviations of triplicate analyses.

after SDS-PAGE. With the two *P. freudenreichii* strains, only branched-chain amino acid AT activity was detectable after 2-h incubation of gel slices. This AT activity was detected in a narrow area of the gel (in two gel slices), as shown for Leu in Fig. 2. AT activity was detectable only after a prolonged incubation (14 h) for Met, Phe and Tyr, whereas Asp AT activity was not detected after SDS-PAGE (Table 2). Whatever the amino acid, AT activity was detected in the same area of the gel (Table 2). The corresponding molecular mass, estimated by regression analyses using standard proteins from 37 to 100 kDa, was 43 ± 3 kDa (Fig. 1; Table 2). Similar results were obtained for *P. freudenreichii* TL34 (data not shown). To estimate the recovery of AT activity after SDS-PAGE, we calculated the ratio of AT activity after SDS-PAGE (sum of the amount of glutamic acid produced by each "active" slice of gel) and before SDS-PAGE (amount of glutamic acid produced by the amount of CFE loaded in the gel lane). This ratio varied as a function of the amino acid used. Hence, ~ 80% AT activity was recovered after SDS-PAGE with branched-chain amino acids, and ~ 60% with Phe, Tyr and Met, whereas Asp AT activity was not recovered. Interestingly, the omission in transamination mixture of Triton X-100, which was added to help enzyme renaturation, did not decrease Leu AT activity.

4. Discussion

These results show for the first time that AT can be resistant to the drastic conditions of SDS-PAGE. Only *P. freudenreichii*, of the eight species tested, exhibited significant residual activity, suggesting that this property is not widespread for bacterial ATs, and raising the

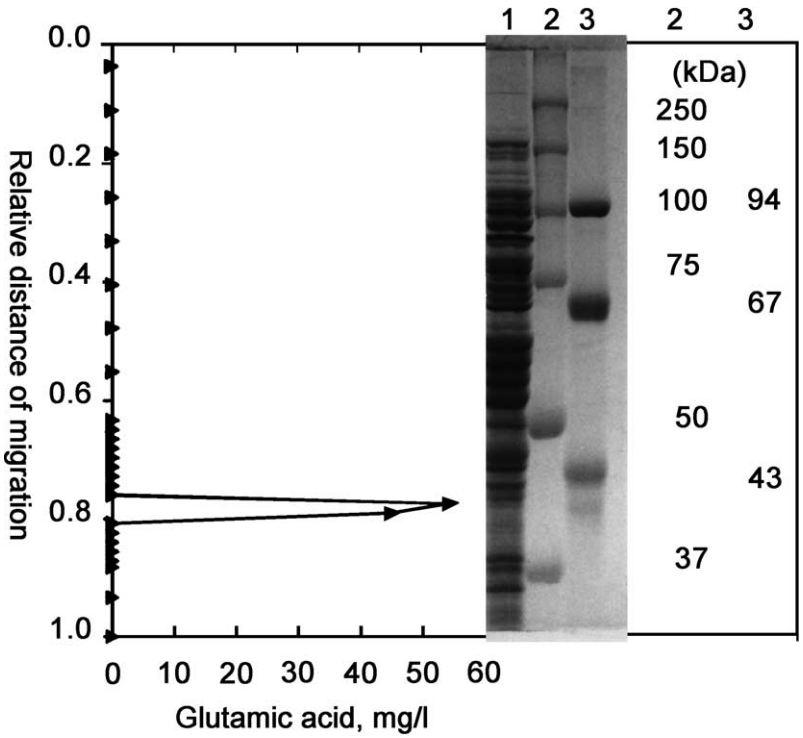

Fig. 2. Detection of leucine aminotransferase activity after SDS-PAGE of *P. freudenreichii* ITGP23 cell-free extracts. The activity is evaluated by the amount of glutamic acid (▲) produced from α-ketoglutaric acid by each gel slice (the entire lane was cut into 2-mm gel slices) after 2-h incubation, as function of the relative migration distance. Lanes: 1, cell-free extract; 2, prestained molecular mass standards; 3, low molecular mass standards.

Table 2
Recovery of aminotransferase activity of *Propionibacterium freudenreichii* ITGP23 cell-free extracts (CFE) after SDS-PAGE

	Glutamic acid (mg L^{-1}) produced by incubating[a]			
	Gel slices[b] after SDS-PAGE of CFE			CFE (before SDS-PAGE)
	Molecular mass range corresponding to gel slice (kDa)			
	46.2–44.9	44.9–43.7	43.7–42.6	
After 2-h incubation				
Ile	12 ± 7[c]	88 ± 9	41 ± 12	105 ± 6
Leu	6	89	71	111
Val	29	95	14	91
Phe	nd[d]	2	nd	5
Tyr, Met, Asp	nd	nd	nd	nd
After 14-h incubation				
Phe	6	33	27	101
Tyr	nd	8	nd	31
Met	8	43	35	130
Asp	nd	nd	nd	43

The activity was evaluated by the amount of glutamic acid produced from α-ketoglutaric acid by each gel slice incubated for 2 or 14 h in the presence of Leu, Ile, Val, Phe, Tyr, Met or Asp.

[a] The values of blank (without amino acid) were subtracted from the amount of glutamic acid formed in the presence of amino acid.

[b] Only the results for "active" gel slices are shown.

[c] Mean and standard deviation of three repeated assays of Ile AT activity.

[d] nd: not detected.

question of particular structural features of *P. freudenreichii* AT(s). A branched-chain amino acid AT activity was previously demonstrated in *P. freudenreichii* (Thierry et al., 2002), but this enzyme was not characterised. The in situ detection achieved here for two different strains allowed us to estimate its molecular mass. Moreover, we showed that the same enzyme, or enzymes with close molecular mass (i.e. in the same gel slice), was also able to transaminate aromatic amino acids and Met. By contrast, the Asp AT activity detected in the CFE was not recovered in the gel, indicating that the affinity for Asp of the AT found in the gel slice was more easily lost in denaturing conditions, or that another AT is involved, which did not withstand SDS-PAGE. The molecular mass found was consistent with the AT recently cloned and sequenced in lactococci (Rijnen et al., 1999; Yvon et al., 2000) and in other species (Hayashi et al., 1990). As the omission of Triton X-100, a nonionic detergent which has been proposed to help enzyme renaturation, (Russell, 1979; Gabriel and Gersten, 1992) did not modify the recovery of Leu AT activity of *P. freudenreichii*, it seems that the enzyme did not need to be renaturated after SDS-PAGE. This property could be used to purify it by preparative electrophoresis in denaturing conditions.

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