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Characterisation of autolytic enzymes in *Lactobacillus pentosus*

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ABSTRACT

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Aims: To characterize autolysis and autolytic system of the lactic acid bacterium *Lactobacillus pentosus.*

Methods and Results: Autolysis of nine *Lact. pentosus* strains was evaluated in buffer solution. Their peptidoglycan hydrolase profiles were examined by renaturing SDS-PAGE and revealed two major activity bands at 58 and 112 kDa. Specificity analysis indicated the presence of at least two different types of peptidoglycan hydrolase activities in *Lact. pentosus* 1091.

Conclusions: Autolysis of *Lact. pentosus* was shown to be strain dependent and involvement of at least two different autolysins was evidenced.

Significance and Impact of the Study: The autolytic system of *Lact. pentosus* was characterized for the first time and the data obtained could be used in the selection of strains of technological interest.

Keywords: autolysis, *Lactobacillus pentosus*, nonstarter lactic acid bacteria, peptidoglycan hydrolase, renaturing SDS-PAGE.

INTRODUCTION

Bacterial autolysis results from the action of endogenous enzymes that hydrolyse covalent bonds of the peptidoglycan, the main component of the cell wall. According to their cleavage specificities, the peptidoglycan hydrolases (PGH) are classified as: (i) β-*N*-acetylmuramidase, (ii) β-*N*-acetyl-glucosaminidase, (iii) *N*-acetylmuramyl-L-alanine amidase, and (iv) peptidases (Shockman and Höltje 1994).

Autolysis of lactic acid bacteria used as starters in dairy fermentations was shown to have a prominent effect on biochemical reactions involved in flavour development (Chapot-Chartier *et al.* 1994; Wilkinson *et al.* 1994; Crow *et al.* 1995; Valence *et al.* 2000). Indeed, the release of intracellular enzymes into the cheese curd consequently to autolysis, favours their action on milk-component-derived substrates. In particular, it was shown that the release of intracellular peptidases accelerates amino acid production and results in bitterness decrease by hydrolysis of large hydrophobic peptides. In addition, it was evidenced recently, that amino acid catabolism leading to aroma formation can also be stimulated by autolysis (Bourdat *et al.* 2003).

The nonstarter lactic acid bacteria (NSLAB) found in cheese are either adventitious contaminant bacteria originating from factory environment and milk, or adjuncts added to cheese milk. These NSLAB include mainly homofermentative and heterofermentative mesophilic lactobacilli and pediococci. They are present at very low cell density at the day of cheese manufacture and they grow during ripening to reach maximal counts after several weeks or months. NSLAB were shown to contribute to cheese flavour development (Crow *et al.* 2001). Their autolysis could also have a beneficial impact on cheese quality especially in cheeses with extended ripening time, by releasing their intracellular content in the cheese curd after inactivation of starter enzymes (Crow *et al.* 1995). *Lactobacillus pentosus* is one of the facultative heterofermentative LAB, isolated as adventitious NSLAB from several cheese varieties produced

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mainly form raw milk according to traditional techniques (De Angelis *et al.* 2001; Nikolaou *et al.* 2002; Duthoit *et al.* 2003). In the present study, we compared several *Lact. pentosus* strains for their ability to lyse and we provide a first characterization of the peptidoglycan hydrolase system of *Lact. pentosus*, obtained by activity detection in renaturing SDS-PAGE and specificity analysis.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus pentosus strains used in the present study were obtained from the CNRZ culture collection (INRA, Jouy-en-Josas, France) and are listed in Table 1. The strains were stored at −18°C in MRS broth (de Man *et al.* 1960) containing 15% glycerol (v/v) and grown in MRS broth at 30°C.

Autolysis of whole cells in buffer solution

Exponential phase bacterial cells ($O.D._{650}$: 1·5–2) were harvested by centrifugation (3000 *g* for 10 min at 4°C) and washed in potassium phosphate buffer (50 mmol l^{-1}, pH 6·5). The cells were resuspended in the same buffer and the initial $O.D._{650}$ was adjusted to 0·6–0·8. The suspension was incubated at 30°C. The extent of autolysis was expressed as percentage decrease in $O.D._{650}$ after 24 h of incubation.

Preparation of cell walls and cytoplasmic extract

Exponential phase bacteria were washed and resuspended in cold potassium phosphate buffer (50 mmol l^{-1}, pH 6·5) supplemented with 1 mmol l^{-1} phenylmethylsulphonyl fluoride (PMSF). Cells were broken by 10 ultrasonication cycles (20 s) at 4°C with 20-s intervals. Unbroken cells were discarded by centrifugation (3000 *g* for 20 min at 4°C). The supernatant was then centrifuged at 20 000 *g* for 20 min at 4°C. The following supernatant was used as cytoplasmic fraction and the pellet (containing native cell walls) was washed two times with cold distilled water before storing at −20°C.

Table 1 Autolysis and peptidoglycan hydrolase profiles of *Lact. pentosus* strains*

Strains (CNRZ collection)	Autolysis extent† (%)	Peptidoglycan hydrolase bands A 112 kDa	B 58 kDa	C 31 kDa
1563	63	+	+	–
1245	54	+	+	+
1543	62	+	+	–
1861	48	+	+	–
1559	34	+	+	–
1865	56	+	+	–
1549	82	+	+	–
1553	74	+	+	–
1091	94	+	+	+

*Data derived from two independent experiments. The variation of the autolysis extent was less than 10%.

†Bacterial cells were incubated in 50 mmol l^{-1} potassium phosphate buffer, pH 6·5. The autolysis extent was defined as the percentage decrease of $O.D._{650}$ after 24-h incubation at 30°C.

Preparation of cellular fractions for renaturing SDS-PAGE analysis

Whole cell SDS extract was prepared as follows: the cell pellet from 4 ml culture was resuspended in 40 μl SDS-PAGE sample buffer [50 mmol l^{-1} Tris–HCl (pH 6·8), 1% SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0·0025% (w/v) bromophenol blue] and boiled at 100°C for 3 min.

For LiCl extract, the cell pellet from 50 ml bacterial culture was resuspended in 500 μl of a solution composed of 5 mol l^{-1} LiCl, 50 mmol l^{-1} Tris–HCl (pH 6·8) and 1 mmol l^{-1} PMSF. The suspension was incubated at 4°C for 1 h and centrifuged (3000 *g* at 4°C for 15 min) to eliminate the cells. The supernatant was dialysed overnight at 4°C against 1000 volumes of 100 mmol l^{-1} LiCl and 50 mmol l^{-1} Tris–HCl (pH 6·8).

Cell walls and cytoplasmic fraction were prepared as described above. All the samples were resuspended in SDS-PAGE sample buffer and boiled at 100°C for 3 min before loading on polyacrylamide gels.

Renaturing SDS-PAGE

Renaturing SDS-PAGE electrophoresis was performed essentially as described by Lepeuple *et al.* (1998) using 12·5% (w/v) polyacrylamide separating gels containing 0·2% (w/v) autoclaved cells of *Micrococcus lysodeikticus* ATCC 4698 (Sigma) as substrate. Following electrophoresis, gels were incubated in 200 ml renaturation buffer [50 mmol l^{-1} potassium phosphate buffer (pH 6·5) containing 0·1% Triton-X-100] at 37°C for 16 h under gentle agitation. The gels were stained for 2 h in 0·01% KOH containing 0·1% methylene blue and destained in distilled water.

Specificity of autolytic enzymes

Native cell walls (10 mg ml^{-1}) were resuspended in 50 mmol l^{-1} potassium phosphate buffer (pH 6·5) and the suspension was incubated at 30°C. Unhydrolysed cells walls were eliminated by centrifugation (16 000 *g* for 15 min). The reducing sugars and free amino groups liberated during cell wall hydrolysis were determined by the methods of

Thompson and Shockman (1968) and Ghuysen *et al.* (1966) respectively.

RESULTS

Autolysis of *Lact. pentosus* in buffer solution

The autolysis extent of nine *Lact. pentosus* strains was evaluated after transfer of exponential phase bacteria into potassium phosphate buffer (50 mmol l^{-1}, pH 6·5) at 30°C. The results are presented in Table 1. The autolysis extent ranges between 34 and 94% revealing the strain dependency of the autolytic behaviour.

Peptidoglycan hydrolase profile by zymogram

The PGH activities present in the nine *Lact. pentosus* strains were detected by renaturing SDS-PAGE using *M. lysodeikticus* cells incorporated in the gel as a substrate. Two bacteriolytic bands with apparent molecular masses of 112 kDa (A) and 58 kDa (B) were revealed in the whole SDS cell extracts of all the tested strains (Table 1). The band B appeared generally rather as a lytic zone composed of two or three very close bands. In addition, a 31-kDa activity band (C) was detected only in the strains 1245 and 1091 (Fig. 1, lane a). Because of its high autolysis extent and original PGH profile, strain 1091 was selected for further study. Different cellular fractions were prepared and analysed by renaturing SDS-PAGE. In LiCl extract, three extra bands with lower intensity and apparent molecular masses of 37, 43 and 77 kDa appeared on the gel (Fig. 1, lane b). Examination of native cell walls revealed a band at 95 kDa in addition to the profile of the LiCl extract and an increase of the intensity of the band at 43 kDa (Fig. 1, lane c). Interestingly, in the cytoplasmic fraction the band at 31 kDa was the major one, while the others were absent or very faint (Fig. 1, lane d), suggesting that the cellular localization of this activity is different from the others. Only very faint lytic bands were observed when the culture supernatant was analysed (data not shown).

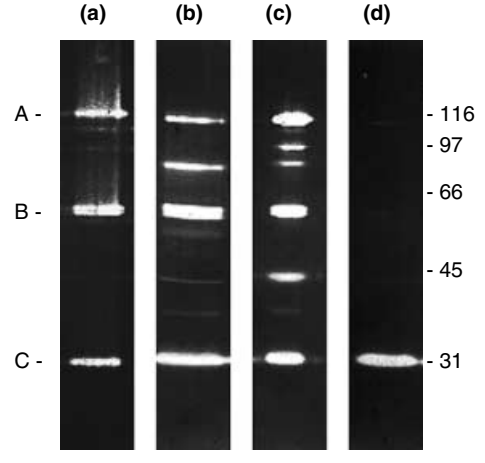

Fig. 1 Renaturing SDS-PAGE evaluation of peptidoglycan hydrolases in different fractions of *Lact. pentosus* 1091 on a gel containing autoclaved cells of *M. lysodeikticus*. Whole cell SDS extract (lane a), LiCl extract (lane b), cell wall fraction (lane c) and cytoplasmic fraction (lane d)

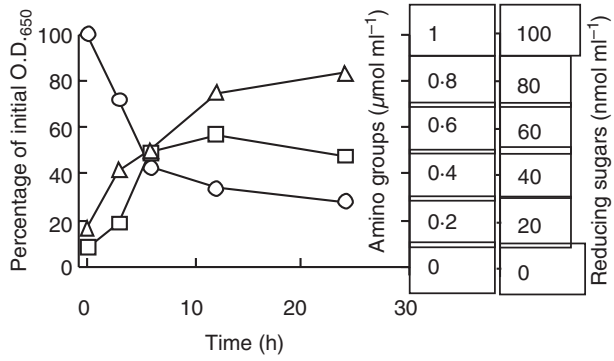

Fig. 2 Specificity analysis of peptidoglycan hydrolases of *Lact. pentosus* 1091. Autolysis of native cell walls (○) analysis of reducing sugars (△) and free amino groups (□)

Hydrolytic specificity of autolysins

Characterization of newly exposed chemical groups of the peptidoglycan after spontaneous autolysis of cell walls enables determination of the specificity of the autolytic enzymes. Hydrolysis of native cell walls of strain 1091 resulted in an increase of both reducing groups and free amino groups (Fig. 2). This result indicates the presence of a glycosidase (muramidase or glucosaminidase) and an amidase or a peptidase, which are involved in peptidoglycan hydrolysis.

DISCUSSION

In the present study, autolysis and PGH content of the lactic acid bacterium *Lact. pentosus* was characterized for the first time. As previously reported for several other species of lactic acid bacteria (Lortal *et al.* 1997a; Cibik and Chapot-Chartier 2000; Ouzari *et al.* 2002) a great level of variability of the autolysis extent was observed among *Lact. pentosus* strains, which indicates that the autolytic efficiency is a strain-dependent character and not a characteristic of the bacterial species.

In renaturing SDS-PAGE, two major PGH activities were detected at 112 and 58 kDa, in the nine strains tested. In addition, the strains 1091 and 1245 presented an

additional band with apparent molecular mass of 31 kDa. It is worth noting that after cell fractionation, this 31-kDa band was the only one present in the cytoplasmic fraction. These observations suggest that, as reported for other lactic acid bacteria such as *L. lactis* and *Streptococcus thermophilus* (Lepeuple *et al.* 1998; Husson-Kao *et al.* 2000), this activity band could correspond to the endolysin of a resident prophage present only in the two strains 1091 and 1245. In *L. lactis* and *S. thermophilus*, the presence of an additional prophage-encoded bacteriolytic enzyme could be linked with a highly autolytic phenotype in certain conditions. In the present case and in the conditions tested, strain 1091 exhibits the highest autolysis extent of the tested strains but strain 1245 presents only moderate level of autolysis extent.

Previous studies have reported that the PGH profile is conserved within a species (Chapot-Chartier 1996; Lortal *et al.* 1997b). Especially in the case of *Lactobacillus* species, the PGH profile detected in zymogram was proposed as a classification tool. Regarding *Lact. pentosus*, the two major PGH bands at 112 and 58 kDa are characteristic of the species and constitute a PGH pattern different from the one found in several other lactobacilli (Lortal *et al.* 1997b). However, the conservation of the PGH pattern is moderated by the fact that an additional 31 kDa band can be detected in some *Lact. pentosus* strains.

Supplementary bands were detected in LiCl extract and native cell walls compared with SDS whole cell extract. However, it is not possible to conclude whether these activity bands correspond to additional enzymes or whether they are degradation products of the main 112- and 58-kDa bands, which retain their activity. For example, in *L. lactis* several activity bands detected in zymogram were shown to be degradation products of the major autolysin AcmA as *acmA* gene inactivation led to their disappearance (Buist *et al.* 1995).

A better understanding of the role of NSLAB autolysis in the ripening process and the contribution of autolytic strains to the flavour development could be achieved by the comparative utilization of high and low lysing strains of *Lact. pentosus* as adjunct strains in cheese making.

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