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Review

Autolytic systems in propionic acid bacteria

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Abstract — This review summarises the present knowledge of autolysis and autolytic systems of dairy propionibacteria. Details of physical and biochemical parameters affecting autolysis in media and buffer are presented. The effect of strain variation, temperature of incubation, growth phase, pH, ionic strength and cations is discussed. In addition, different methods to follow autolysis and specificity studies of autolytic enzymes are described. Autolytic enzymes in specific cell fractions were studied by renaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); all strains showed lytic activity by this method. © Inra/Elsevier, Paris.

Propionibacterium / autolysis / buffer studies / SDS-PAGE

Résumé — **Systèmes autolytiques des bactéries propioniques.** Cet article a pour but de faire un résumé des connaissances actuelles en ce qui concerne l'autolyse et les systèmes autolytiques des bactéries propioniques utilisées en laiterie. Les différents paramètres physiques et biochimiques qui affectent l'autolyse en bouillon et en solution tampon sont présentés en détail. Il est question de l'effet des paramètres suivants : variations de souches, température, phase de croissance, pH, force ionique et cations. En outre, différentes méthodes pour suivre l'autolyse, ainsi que des études de spécificité des enzymes autolytiques, sont mentionnées. La technique d'électrophorèse renaturante sur sodium dodécyl sulfate gel de polyacrylamide (SDS-PAGE) a été utilisée pour caractériser les enzymes autolytiques dans des fractions cellulaires spécifiques. Toutes les souches s'avèrent montrer une activité autolytique par électrophorèse renaturante SDS-PAGE. © Inra/Elsevier, Paris.

Propionibacterium / autolyse / solution tampon / SDS-PAGE

1. INTRODUCTION

Ripening of cheese is a complex process which involves the degradation of carbohydrate, fat and protein. The main changes

involve the formation of peptides and amino acids from caseins, fatty acids from milk fat, and the conversion of lactose to lactate or other fermentation products, such as CO₂, ethanol, acetate and other aroma compo-

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nents. It has been known for a long time that proteolysis of milk casein is a key factor in the ripening of cheese. Autolysis of bacterial cells has attracted interest owing to the possibility of accelerating proteolysis.

Bacterial autolysins are defined as endogenous enzymes that hydrolyse covalent bonds in the peptidoglycan causing self lysis of intact bacterial cells [21, 23]. The physiological role of these potentially lethal enzymes is not fully understood. It is suggested that the autolytic enzymes are involved in important biological processes such as cell division and separation, cell wall turnover, competence in genetic transformation, formation of flagella, and sporulation [21, 24, 26, 29]. Generally, autolytic enzymes have been considered to be synonymous with the peptidoglycan hydrolases. However, peptidoglycan hydrolases which hydrolyse bonds in the peptidoglycan that are not relevant for the mechanical stabil-

ity of the cell wall, or enzyme activities that hydrolyse only relatively few of the structurally essential bonds of the wall peptidoglycan, without release of soluble products, are not considered to be autolysins. The enzymes are classified on the basis of their cleavage specificities as N-acetylmuramidases, N-acetylglucosaminidases, N-acetylmuramyl-L-alanine amidases and endopeptidases (figure 1). The dairy propionibacteria (PAB) contain mainly L-diaminopimelic acid (L-DAP) as the diamino acid in the peptidoglycan of the cell wall; however, *Propionibacterium freudenreichii* contains meso-diaminopimelic acid (m-DAP) [4]. In strains with L-DAP, glycine is the bridging amino acid between DAP and the D-alanine of the adjacent chain. Most bacteria contain multiple autolytic enzymes. The presence of multiple autolysins within one organism have complicated the determination of the exact function of these enzymes [24].

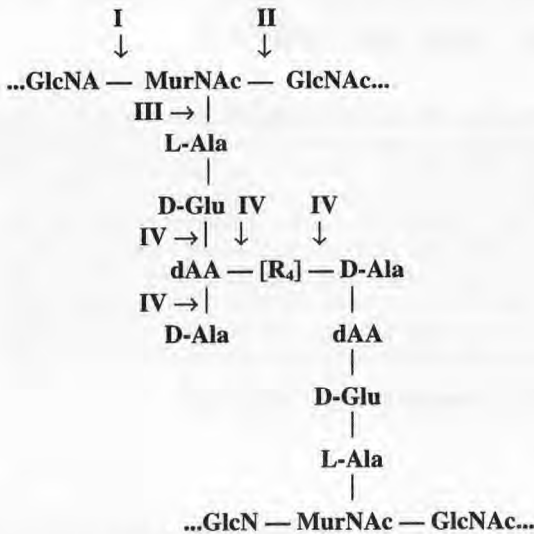


Figure 1. Schematic presentation of the sites of hydrolysis of the peptidoglycan by peptidoglycan hydrolases present in bacteria. I, N-acetylglucosaminidase; II, N-acetylmuramidase; III, N-acetylmuramyl-L-alanine amidase; IV, endopeptidase. Abbreviations: GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; dAA = diamino acid; $[\text{R}_4]$ = interpeptide bridge.

Figure 1. Présentation schématique des sites d'hydrolyse du peptidoglycane par les hydrolases peptidoglycaniques présentes dans les bactéries. I, N-acetylglucosaminidase; II, N-acetylmuramidase; III, N-acetylmuramyl-L-alanine amidase; IV, endopeptidase. Abréviations: GlcNAc, N-acetylglucosamine; MurNAc, acide N-acetylmuramique; dAA = diaminoacide; $[\text{R}_4]$ = pont interpeptidique.

Dairy PAB are important organisms in Emmental and other Swiss-type cheeses, where they convert lactate to propionate, acetate and CO₂. The acids and other volatile compounds contribute significantly to the characteristic flavour and aroma, and CO₂ is responsible for the characteristic eyes in these cheeses [7, 8]. In addition, amino acids, particularly proline, and small peptides are also assumed to contribute to the sweet, nutty flavour [10]. Autolysis of PAB may be important for the release of intracellular peptidases which may influence the ripening of Swiss-type cheeses.

The importance of autolysis in cheese ripening is becoming evident [1, 3, 30, 31]. However, the mechanisms of autolysis require further studies. In order to properly evaluate the possible technological applications to cheese ripening, the fundamental properties of the autolytic systems of the bacteria being used must be understood. Autolytic studies of PAB were first conducted in aqueous systems, such as media or buffer [12–14, 16, 17]. The main advantage of these studies is that different parameters are more easily defined than in cheese. The disadvantages are that the aqueous systems lack the complexity of cheese. The intention of this paper is to focus on recent work conducted on autolysis of the propionibacteria, with the main focus on work conducted in the authors' laboratory.

2. MEASUREMENT OF AUTOLYSIS

Several methods, such as optical density (OD), enumeration, electron microscopy, and detection of released intracellular markers, have been used to follow autolysis in broth [11, 16, 27, 28].

Autolysis monitored by OD can be characterised by the following two parameters: the rate of autolysis, expressed as the decrease in OD per minute during the first 60 min; and the extent of autolysis, expressed as percentage decrease of optical density after a certain time.

2.1. Autolysis of propionibacteria in media

Data on the autolysis of PAB in media are summarised in *table 1*. Growth and autolysis of 28 strains of PAB were measured in sodium lactate broth (SLB) by OD measurements [16]. Large variations in growth and maximum autolysis occurred between strains. Maximum growth varied from an OD₆₀₀ of 2.1 to 5.3 and maximum autolysis varied from 10 to 90%. Seventeen of the strains showed 70 to 90% autolysis. Maximum autolysis was observed after 13 to 72 d incubation at 30 °C. This observation is in contrast with the observation of Lemée et al. [13] who studied autolysis of 4 selected,

Table I. Autolysis of propionibacteria (PAB) in media.

Tableau I. Autolyse des bactéries propioniques (PAB) en bouillon.

Strains	Media	Maximal autolysis			Reference
		%	d [†]	temp	
28 PAB	SLB [‡]	10–90	13–72	30°	[16]
4 PAB	YEL [§]	60–80	8–9		[13]
1 PAB	YEL	80–88	8.3		[12]

[†] Days after inoculation / nombre de jours après l'inoculation.

[‡] Sodium lactate broth / bouillon de lactate de sodium.

[§] Yeast extract-sodium lactate / extrait de levure-lactate de sodium.

good autolytic strains in growth medium and observed spontaneous autolysis just after reaching maximum optical density. Their observation corresponded to 60 to 80 % autolysis after 8 to 9 d, respectively. These strains were also highly prone to autolysis in buffer. The spontaneous autolysis of *Propionibacterium freudenreichii* CNRZ 725 was shown to occur at pH 6 to 6.2, when the carbon source was depleted [14]. *P. freudenreichii* CNRZ 725 showed 88 % autolysis in yeast extract-sodium lactate broth (YEL) after 8.3 d of incubation at 30 °C [12].

Autolysis was monitored by measuring the release of proline (Pro)-iminopeptidase from 6 strains [16]. Four strains showed maximal Pro-iminopeptidase activity after 52 to 59 d incubation and maximal autolysis, measured by OD, after 49 to 59 d. Two strains showed an early unstable Pro-iminopeptidase peak after 8 to 9 d incubation; however, a second high Pro-iminopeptidase level was observed after 49 to 59 d incubation corresponding to maximum autolysis. This indicates a good correlation between Pro-iminopeptidase activity and maximum autolysis, which makes Pro-iminopeptidase a good marker for autolysis of most of the strains tested. Autolysis of two of the strains were studied in more detail by using OD, Pro-iminopeptidase, DNA and RNA as markers of autolysis. In addition, Pro production was followed. Results showed that the change in OD is the best marker for following autolysis. The release of Pro-iminopeptidase activity could also be used as a marker; however, some limitations, such as enzyme stability, degradation by intracellular proteinases, or the regulation of the lytic enzyme system during growth were noted. Both RNA and DNA were good markers for measuring initiation of autolysis but not for measuring autolysis over a longer period of time, probably because of degradation by RNases and DNAses. A major disadvantage of monitoring autolysis in broth is that it is very time consuming.

The influence of storage temperature on autolysis of 13 strains has also been tested. Maximum autolysis was observed at 30 °C. Three of the strains were little influenced by different storage temperatures and two of the strains showed high autolysis at 20 °C as well as 30 °C. These results may be of significance during cheese ripening as Swiss-type cheese is held at 20 to 25 °C in the warm room and at 10 to 12 °C in the cold room during the ripening process.

Langsrud et al. [9, 10] and Østlie et al. [16] found that propionibacteria released large amounts of proline when grown in media containing peptides. The release of proline from peptides coincided with the autolysis of propionibacteria.

2.2. Autolysis of propionibacteria in buffer systems

Propionibacterial cells autolysed spontaneously when they were transferred from SLB to a buffer solution. This phenomenon has been observed in many Gram-positive organisms [2, 15, 17, 22]. Autolysis in buffer occurs much faster than in broth.

Results regarding autolysis of propionibacteria in buffer are summarised in *table II*. Østlie et al. [17] studied autolysis of 21 strains of propionibacteria in buffer systems. Optimal conditions of autolysis were determined in potassium phosphate buffer (50 mmol·L⁻¹, pH 7.0). The effect of growth phase, temperature, pH, ionic strength and cations was investigated. The influence of the growth phase was tested on 6 strains and autolysis varied depending on the age of the cells. All strains showed maximum autolysis in the exponential growth phase at an OD of 0.2 to 1.4. The ability of the cells to autolyse decreased sharply at the end of the exponential growth phase and during the stationary growth phase. The influence of temperature on autolysis was tested for 13 strains. Autolysis was maximal at 30 °C for 5 of the strains and at 40 °C for the

Table II. Autolysis of propionibacteria (PAB) in buffer.**Tableau II.** Autolyse des bactéries propioniques (PAB) en solution tampon.

Strains	Buffer	Maximal autolysis					Specificity	Reference
		%	h [†]	temp	pH	I		
21 PAB	PPB [‡]	20–80	41–51	30–40°	7.2	0.3–0.5	glycosidase amidase/ endopeptidase	[17]
57 PAB	PPB [§]	38–86	24					[13]
1 PAB	PPB [∥]	65	3	40°	5.8		NAG [¶]	[12]

[†] Hours of incubation / heures d'incubation.

[‡] Potassium phosphate buffer (50 mmol·L⁻¹, pH 7.0) / tampon de phosphate de potassium (50 mmol·L⁻¹, pH 7.0).

[§] Potassium phosphate buffer (0.1 mol·L⁻¹, pH 6.2) / tampon de phosphate de potassium (0.1 mol·L⁻¹, pH 6.2).

[∥] Potassium phosphate buffer (0.1 mol·L⁻¹, pH 5.8) / tampon de phosphate de potassium (0.1 mol·L⁻¹, pH 5.8).

[¶] N-acetylglucosaminidase / N-acetylglucosaminidase.

remaining strains. Maximum autolysis varied from 20 to 80 % after 41 to 51 h of incubation. Seven strains autolysed between 50 to 80 % under the conditions of the experiment. The rate of autolysis was highest for all strains at 40 and 50 °C during the first 2 to 5 h of incubation. Autolysis was optimal at an ionic strength (I) of 0.3 to 0.5 depending on the age of the cells. This observation may be important when related to cheese ripening because I in the cheese usually is about 0.3. The effect of pH on autolysis was studied and an optimum pH of 7.2 was found. In addition, there was indication of a second optimal pH at 6.0. Autolysis was stimulated by Na⁺, K⁺ and NH₄⁺ and was inhibited by most of the divalent cations tested.

Lemée et al. [13] have studied autolysis of 57 strains of dairy PAB in potassium phosphate buffer (0.1 mol·L⁻¹, pH 6.2, 37 °C) harvested during exponential growth. They found that both the rate and extent of autolysis appeared to be strain-dependent. Two distinct clusters were observed. One cluster, containing 8 *P. freudenreichii* strains autolysed by 86 ± 9.4 %, and the other cluster, containing 49 strains, autolysed by 38.3 ± 9.4 % after 24 h. Optimal autolysis of

P. freudenreichii CNRZ 725 cells and isolated cell walls was observed in phosphate buffer at pH 5.8 and 40 °C [12]. The highest autolytic activity was observed in the early exponential growth phase at an OD of 0.3. The effect of various salts on autolysis of *P. freudenreichii* CNRZ 725 was also studied, and the presence of potassium and sodium salts led to significant increases in both the rate and the extent of autolysis.

The specificity of the autolytic enzymes from 5 strains of PAB was studied by Østlie et al. [17]. Muramidase or a N-acetylglucosaminidase would lead to an increase in the number of reducing sugar groups, while an increase in free amino groups would result from amidase or endopeptidase activity. Increases in both reducing sugars and free amino groups were observed for all strains, but to various extents. Lysis of the peptidoglycan seemed to result from a glycosidase and a N-acetylmuramyl-L-alanine amidase or an endopeptidase. Lemée et al. [12] reported only N-acetylglucosaminidase activity in the autolytic system of *P. freudenreichii* CNRZ 725. This result is different from our findings and may indicate strain variations.

3. CHARACTERISATION OF AUTOLYTIC ENZYMES BY RENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

The ability of autolytic enzymes to renature after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has made it possible to study their activity, substrate specificity and effect of physical and chemical treatments on activity [6, 20, 25]. Characteristics of autolytic enzymes of PAB studied by SDS-PAGE are summarised in table III. The use of renaturing gel electrophoresis to determine the autolytic enzyme profiles in specific cell fractions of the PAB have been studied by Østlie et al. [18]. Inactivated *Propionibacterium* cells were routinely used as substrate in the gels. The autolytic enzymes of 5 good autolysing strains (70–90 %) of PAB were studied by renaturing SDS-PAGE. Two different autolytic profiles were observed among the strains tested. *P. acidipropionici* ATCC 4965 showed 8 autolytic bands of molecular mass 25, 31, 39, 43, 55, 71, 97, and 122 kDa in the different cell fractions. The other 4 strains, *P. freudenreichii* INF- α , *P. freudenreichii* ISU P-59, *P. freudenreichii* ISU P-24, *P. jensenii* ISU P-50, showed one main autolytic band of molecular mass in the range 123–143 kDa in the different cell

fractions. In addition, a larger molecular weight band of weak intensity was often found in the cell wall fractions. Identical number of lytic bands were observed when inactivated cell walls were used as substrate instead of inactivated cells; however, lower contrast was observed in these gels. *Micrococcus luteus* cells were also tested as a substrate but no lytic activity was observed from any of the strains. Routinely, the gels were incubated in Tris-HCl buffer (0.025 mol·L⁻¹, pH 7.5) containing 1 % Triton X-100.

Lemée et al. [14] reported 8 lytic enzyme bands in *P. freudenreichii* CNRZ 725. The most intense band of this strain had an apparent molecular mass of 121 kDa; 6 other activities had molecular masses of 81, 87, 92, 100, 109 and 118 kDa; and a weak activity band at 34 kDa. Lemée et al. [14] tried several solutions for renaturing lytic activities. The optimum conditions were to incubate the gels in phosphate buffer (0.1 mol·L⁻¹, pH 5.8), KCl (0.1 mol·L⁻¹) or Emmental juice, all containing 1 % Triton X-100.

Both *P. freudenreichii* CNRZ 725 and *P. acidipropionici* ATCC 4965 had 8 lytic enzyme bands; however, the enzyme profile of *P. freudenreichii* CNRZ 725 was different from the lytic enzyme pattern of *P. acidipropionici* ATCC 4965. The multiplicity of the lytic bands does not necessarily reflect the exact number of cell wall hydrolases. Proteolytic processing of cell

Table III. Characteristics of autolytic enzymes of propionibacteria (PAB) by SDS-PAGE.

Tableau III. Caractéristiques des enzymes autolytiques des bactéries propioniques (PAB) par SDS-PAGE.

Strains	Substrate [†]	Samples [‡]	No. of bands	Mw (kDa)	Reference
1 PAB	cells, cw	if, cw, cm, s	8	25–122	[18]
4 PAB	cells, cw	if, cw, cm, s	1	123–143	[18]
1 PAB	cells	crude extract	8	34–121	[14]

[†] Substrate in the gel: propionibacteria cells or cell wall / substrat dans le gel : cellules ou paroi cellulaire de bactéries propioniques.

[‡] if, intracellular fraction; cw, cell wall fraction; cm, cell membrane fraction; s, supernatant fraction / if, fraction intracellulaire ; cw, fraction de paroi cellulaire ; cm, fraction de membrane cellulaire ; s, fraction de surnageant.

wall hydrolases may generate more than one polypeptide that may or may not retain enzymatic activity [5, 20]. Proteolysis of autolysins has been described as a method of autolysin regulation [19]. Proteolytic activity of 5 propionibacteria strains has been tested and preliminary results demonstrated that *P. acidipropionici* ATCC 4965 had the highest proteolytic activity (Østlie, unpublished results). The 8 lytic bands of *P. acidipropionici* ATCC 4965 may be generated by proteolytic processing of one or more autolytic enzymes.

4. CONCLUSION

The focus of this paper was the fundamental aspects of the autolytic systems of PAB. Conditions for growth and autolysis in media and buffers have been discussed. These conditions are very different from the complex conditions in a cheese. Therefore, further studies of autolysis of PAB in controlled model systems and in cheese are required to understand their behaviour in cheese during ripening. To what extent strain variations in autolysis are related to different enzymes or to different regulation mechanisms is not known. Further studies are needed to get a better understanding of these variations. The results described, especially the SDS-PAGE studies and specificity analysis, suggest that more than one autolytic enzyme are involved in the autolysis of dairy PAB. Purification and characterisation of autolysins need to be done to clarify the number and type of enzyme(s) involved in autolysis of PAB, and also to provide an insight into the function and regulation of autolysins, especially related to cheese ripening. PAB grow in synergism with lactic starter bacteria in the cheese, and thus, it is difficult to distinguish their individual contributions to proteolysis and the release of peptides and amino acids.

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