Autolysis of *Clostridium acetobutylicum* ATCC 824

CHRISTIAN CROUX, BRUNO CANARD, GÉRARD GOMA and PHILIPPE SOUCAILLE*

Département de Génie Biochimique et Alimentaire, Unité Associée du Centre National de la Recherche Scientifique no. 544, Institut National des Sciences Appliquées, Avenue de Rangueil, F-31077 Toulouse Cedex, France

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The optimum conditions for autolysis of *Clostridium acetobutylicum* ATCC 824 were determined. Autolysis was optimal at pH 6.3 and 55 °C in 0.1 M-sodium acetate/phosphate buffer. The ability of cells to autolyse decreased sharply at the end of the exponential phase of growth. Lysis was stimulated by monovalent cations and compounds that complex divalent cations, and inhibited by divalent cations. The autolysin of *C. acetobutylicum*, which was mainly cytoplasmic, was purified to homogeneity and characterized as a muramidase. The enzyme was identical to the extracellular muramidase in terms of M_r , isoelectric point and NH₂-terminal amino acid sequence. The autolysin was inhibited by lipoteichoic acids and cardiolipin but not by phosphatidylethanolamine and phosphatidylglycerol. A mechanism of regulation and fixation involving lipoteichoic acid, cardiolipin and divalent cations is proposed.

Introduction

The anaerobic spore-forming Gram-positive bacterium *Clostridium acetobutylicum* is a well-known producer of acetone and butanol, two solvents of industrial interest (for a review see Jones & Woods, 1986). Butanol is highly toxic for the micro-organism (Soucaille *et al.*, 1987) and during the later stages of the fermentation degeneration and partial lysis of the cells are observed. A pleiotropic autolysin-defective mutant that produces decreased amounts of autolysin and was less sensitive to autolytic enzyme has also been reported to be more resistant to cell degeneration in the presence of butanol (Allcock *et al.*, 1981; Van Der Westhuisen *et al.*, 1982).

The physiological and physico-chemical conditions leading to the autolysis of *C. acetobutylicum* have not been extensively studied. Furthermore, only one enzyme involved in the autolysis of a solvent-producing *Clostridium* has been partially purified and characterized (Yoshino *et al.*, 1982). In order to investigate the regulatory mechanism that controls the autolytic enzymes of *C. acetobutylicum*, we have undertaken a study of the conditions promoting or inhibiting cell lysis and a purification of the autolytic enzyme(s) involved.

Methods

Organism and culture conditions. *C. acetobutylicum* ATCC 824 was obtained from the American Type Culture Collection. The strain was grown at 35 °C and pH 4.8, in a 2 litre (SGI, France) fermenter stirred at 200 r.p.m., using the synthetic medium previously described (Soni *et al.*, 1987). The fermenter was inoculated with a 1/10th volume of an early exponential phase culture, previously subcultured in the same medium.

For cell fractionation and autolysin purification, the fermentation broth was collected at the end of the exponential phase of growth (10 h of culture), centrifuged (15 min, 15000 g, 4 °C) and the pellet resuspended in 0.01 M-sodium acetate buffer, pH 4.

Cell fractionation. Cells were disrupted by three passages through a French press at 20000 p.s.i. (about 138 MPa). Unbroken cells were removed by centrifugation (10 min, 1000 g, 4 °C). Cell walls were pelleted (12000 g, 20 min, 4 °C), washed once and resuspended in 0.01 M-sodium phosphate buffer, pH 6 (cell-wall fraction). Membranes were recovered from the supernatant by high-speed centrifugation (220000 g, 2 h, 4 °C) and resuspended in 0.01 M-sodium acetate buffer, pH 4 (membrane fraction). The supernatant from this centrifugation served as the cytoplasmic fraction.

Autolytic enzyme purification. The cytoplasmic fraction and the product of autolysis of the cell-wall fraction after 7 h at 30 °C were combined.

(i) Ultrafiltration. The solution was concentrated to 9 ml and dialysed against 1 litre of 0.02 M-sodium acetate buffer, pH 5.2, using an ultrafiltration device (H1 P10 Amicon, cut-off M_r 10000), and filtered through a 0.2 µm membrane (Sartorius); 8 ml of filtrate were recovered and used as the crude enzyme preparation for the next steps of purification.

(ii) Column chromatography. All the purification steps were done on a Pharmacia FPLC system. Crude enzyme solution (8 ml, 1640 U) was applied to a prepacked Mono Q HR 10/10 column (1 × 10 cm), a

* Author for correspondence. Present address: Department of Chemical Engineering, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA; tel. (708) 491 2776; fax (708) 491 3728.

strong anion-exchanger, previously equilibrated with 0.02 M-sodium acetate buffer, pH 5.2. Elution was at a flow rate of 8 ml min⁻¹ using a linear NaCl gradient (0 to 0.3 M over 30 min). Fractions (8 ml) were collected and tested for autolytic activity as described below.

The autolytic-positive fractions from the anion-exchange chromatography were concentrated by ultrafiltration through a Centriprep-10 concentrator (Amicon, cut-off M_r 10000) to a final volume of 200 µl and applied to a Superose 12 HR 10/30 column (1 × 30 cm) previously equilibrated with 0.1 M-sodium acetate buffer, pH 5. The column was eluted with one bed volume of the same buffer at a flow rate of 0.3 ml min⁻¹. Fractions (0.5 ml) were collected and tested for autolytic activity.

Assay of autolytic enzyme activity. The standard assay was done at pH 4, in 0.1 M-sodium acetate buffer, by measuring the decrease of turbidity of a purified cell wall suspension, as previously described for the extracellular lytic enzyme (Croux *et al.*, 1992). One Unit (U) ml⁻¹ of lytic activity was defined as the amount of enzyme per ml which reduces the turbidity of a purified cell-wall suspension by 0.001 OD₄₅₀ min⁻¹ at 35 °C.

For the fractionation study, the autolytic activity was measured using 5 µg [methyl-³H]choline-labelled, purified cells walls of *C. acetobutylicum* NCIB 8052 (Croux *et al.*, 1992) in a total volume of 250 µl. In some cases the 250 µl volume also contained detergent (deoxycholate or Brij at 0.1% final concentration). One Unit of lytic activity was defined as the amount of enzyme that solubilized 1 µg cell wall min⁻¹ at 35 °C.

Protein concentration was estimated by the Lowry method with bovine serum albumin as the standard.

Inhibition of the autolytic enzyme by phospholipids and lipoteichoic acids. Phospholipids were first dissolved in methanol as a 100-fold concentrated solution before being added to the reaction mixture. Inhibition values were obtained by reference to a control run in the presence of 1% (v/v) methanol. Lipoteichoic acids were dissolved in water.

Molecular mass determination. The molecular mass of the autolytic enzyme was determined by SDS-PAGE in a Phast-Gel 8–25% gradient using the Pharmacia Phast-System.

Samples were previously heated at 100 °C for 5 min in the presence of 2.5% (w/v) SDS. Protein bands were stained with silver nitrate. Calibration proteins ranging in M_r from 14400 to 94000 (low molecular mass calibration kit; Pharmacia) were used for the estimation of the M_r of the lytic enzyme. To estimate the native M_r of the enzyme, gel-filtration chromatography was carried out on a Superose 12 HR 10/30 column calibrated with proteins of known molecular mass as standards (low molecular mass gel-filtration calibration kit; Pharmacia).

Isoelectric point determination. Analytical isoelectric focusing PAGE was performed in a Phast-Gel IEF 3–9 gradient using the Pharmacia Phast-System. Protein bands were stained with silver nitrate. The isoelectric point of the autolysin was determined with broad-range standard protein markers (Pharmacia) ranging from pI 3 to 10.

NH₂-terminal amino acid sequence. The NH₂-terminal amino acid sequence of lytic enzyme was determined by stepwise Edman degradation (Croux *et al.*, 1992) with a gas-phase sequencer (Applied Biosystems, model 470A) and high pressure liquid chromatography identification of the phenylthiohydantoin derivatives of amino acids obtained from the sequencer (Applied Biosystems model 120A).

Autolysis procedures. Cells were harvested by centrifugation (12000 g, 2 min, 4 °C) and after the supernatant fluid had been discarded, residual growth medium was removed with cotton tips. The unwashed pellet was then diluted to an initial OD₆₂₀ of 1 into preincubated (35 °C) 0.1 M-sodium phosphate buffer. The decrease in

optical density was monitored at 35 °C as a function of time. The first-order rate constant of autolysis, k (min⁻¹), was calculated from the slope of the log(OD₆₂₀) curve as a function of time.

Liberation of reducing sugars and free amino groups. Liberation of reducing sugars during the hydrolysis of cell walls was measured in heat-treated samples (3 min at 100 °C) by the method of Park & Johnson (1949) as modified by Thompson & Shockman (1968). Liberation of free amino groups was measured in heat-treated samples (3 min at 100 °C) by the method of Ghuyssen *et al.* (1968).

Specificity of the autolysin. Purified cell walls (2 mg ml⁻¹) were digested by the autolysin (10 U ml⁻¹) at 30 °C for 9 h. The reducing sugars liberated into the supernatant were reduced with NaBH₄ (10 mg ml⁻¹) at pH 9 in 0.1 M-borate buffer. The sample was dried *in vacuo*, washed three times with methanol and hydrolysed with 6 M-HCl at 100 °C for 4 h. After three washes in methanol the sample was dissolved in 0.1 M-citrate buffer and analysed on a Biotronic C200 amino acid analyser.

Electron microscopy. Bacterial morphology of autolysing cells was inspected after fixing with 2% glutaraldehyde (pH 7) for 10 min at room temperature. Samples were then applied as a thin film to 200 mesh Formvar-coated copper grids and briefly exposed to 2% (w/v) potassium phosphotungstic acid (pH 6.8).

Materials. The chemicals used were from Sigma except [methyl-³H]choline from Amersham.

Results

C. acetobutylicum cells taken from the exponential phase of growth spontaneously autolysed when they were suspended in sodium phosphate buffer, pH 6.3 (Fig. 1). Concomitantly, UV-absorbing material was released. The autolysis of *C. acetobutylicum* followed first-order kinetics. Electron microscopy of autolysing cells revealed that digestion of the cell walls occurred first at the polar regions (Fig. 2).

Fig. 1. Kinetics of autolysis of *C. acetobutylicum* ATCC 824. Autolysis of exponential phase cells was assayed in 0.1 M-sodium phosphate, pH 6.3, as loss of OD₆₂₀ of the suspension (●) and increase of absorbance of supernatants at 260 (▲) and 280 (■) nm.

Fig. 2. Transmission electron micrograph of *C. acetobutylicum* ATCC 824. Cells were fixed and postfixed as described in Methods. (a) Control exponential phase cells; (b) exponential phase cells harvested and transferred to 0.1 M-sodium phosphate, pH 6.3, at 35 °C for 1 h. Bars, 1 μ m.

Effect of pH, temperature and phase of growth

The initial rate of autolysis was dependent upon the pH of the sodium acetate/phosphate buffer; the optimum was at pH 6.3.

The initial rate of autolysis was also clearly dependent on the temperature at which the cells were incubated with an optimum at 55 °C. The Arrhenius law held true between 20 and 45 °C, indicating competition between kinetic activation and denaturation of the enzyme system at higher temperature. The activation energy for the autolysis of cells was calculated to be 34 kJ mol⁻¹.

The rate constant of autolysis (k) reached a maximum for cells from the end of the exponential phase of growth, a minimum for cells from the stationary phase and increased again in the lysis phase (Fig. 3).

Effect of different ions and chelating agents

All the monovalent cations used (except NH₄⁺) increased the rate constant of autolysis at concentrations higher than 0.1 M (Table 1). On the other hand, at concentrations higher than 10⁻⁴ M, all the divalent cations tested (except Mn²⁺) inhibited the autolysis of *C. acetobutylicum*.

Fig. 3. Autolysis of cells and extracellular lytic activity during different stages in the growth cycle. ●, OD₆₂₀; ▲, autolysis constant, k ; △, extracellular lytic activity.

Table 1. Effect of monovalent cations on the autolysis of *C. acetobutylicum* ATCC 824

Cells were harvested at the end of the exponential phase of growth and autolysis was assayed in 0.1 M-sodium phosphate, pH 6.3, as loss of OD₆₂₀.

Cation*	Concn (M)	10 ³ × k (min ⁻¹)	Relative rate (%)
None		5.1 ± 0.45	100
Na ⁺	0.1	5.3 ± 0.5	104
	0.2	10.6 ± 0.6	208
	0.3	13.8 ± 0.7	271
K ⁺	0.1	5.2 ± 0.5	102
	0.2	9.5 ± 0.6	186
	0.3	12.5 ± 0.6	245
NH ₄ ⁺	0.1	5.05 ± 0.5	99
	0.2	5.1 ± 0.4	100
	0.3	5.6 ± 0.5	110

* Cl⁻ was the anion in all the experiments.

butylicum (Table 2). Chelating agents, at a concentration of 5 × 10⁻³ M increased the rate constant of autolysis by 80% for EDTA, 70% for EGTA and 120% for citrate. The effect of EDTA, at a concentration of 5 × 10⁻³ M, could be completely overcome by the addition of 5 × 10⁻² M (final concentration) MgSO₄.

Effect of amino acids, amino sugars and amino alcohols

Among the amino acids and amino sugars found in the peptidoglycan of *C. acetobutylicum* only glucosamine (acetylated or not) inhibited cell autolysis (20% inhibition at a concentration of 1 mM). At a concentration of 0.3 M both ethanolamine (relative constant rate of 270%) and choline (relative constant rate of 280%) activated cell autolysis.

Table 2. Effect of divalent cations on the autolysis of *C. acetobutylicum* ATCC 824

Cells were harvested at the end of the exponential phase of growth and autolysis was assayed in 0.1 M-sodium phosphate, pH 6.3, as loss of OD₆₂₀.

Cation*	Concn (M)	10 ³ × <i>k</i> (min ⁻¹)	Relative rate (%)
None		5.1 ± 0.45	100
Cu ²⁺	10 ⁻⁵	1.06 ± 0.12	21
	10 ⁻⁴	0	0
	10 ⁻³	0	0
Zn ²⁺	10 ⁻⁵	3.7 ± 0.39	73
	10 ⁻⁴	2.4 ± 0.3	47
	10 ⁻³	0.5 ± 0.1	10
Fe ²⁺	10 ⁻⁵	4.8 ± 0.5	94
	10 ⁻⁴	4.0 ± 0.4	79
	10 ⁻³	2.0 ± 0.18	45
Mg ²⁺	10 ⁻⁵	5.1 ± 0.5	100
	10 ⁻⁴	4.2 ± 0.4	82
	10 ⁻³	2.6 ± 0.3	51
Ca ²⁺	10 ⁻⁵	5.15 ± 0.5	101
	10 ⁻⁴	5.1 ± 0.5	100
	10 ⁻³	3.4 ± 0.3	67
Mn ²⁺	10 ⁻⁵	5.15 ± 0.45	101
	10 ⁻⁴	5.1 ± 0.5	100
	10 ⁻³	4.6 ± 0.5	90

* SO₄²⁻ was the anion in all the experiments.

Table 3. Distribution of autolytic activity in different cell fractions

Activity for each fraction is given as a percentage of the total activity and was measured by solubilization of labelled cell walls in 0.1 M-sodium acetate buffer, pH 4.

Fraction	Activity (%)	Activation by 0.1% Brij (%)*	Activation by 0.1% deoxycholate (%)†
Cell wall	34 ± 12	124 ± 6	110 ± 7
Cytoplasm	64 ± 15	116 ± 8	117 ± 6
Membrane	2 ± 2	115 ± 10	120 ± 11

* Relative activities with and without detergent for each fraction. Activities were measured by solubilization of labelled cell walls in 0.1 M-sodium acetate buffer, pH 4.

† Relative activities with and without detergent for each fraction. Activities were measured by solubilization of labelled cell walls in 0.1 M-sodium phosphate buffer, pH 6.5.

Distribution of autolytic enzyme(s) in different cell fractions

In order to purify the autolytic enzyme(s), their localization was first examined after breakage of the cells at pH 4 with a French press. Only small amounts of autolytic activity were detected in the membrane

Fig. 4. Autolysis of native cell walls at 35 °C. Native cell walls (20 mg) were suspended in 5 ml 0.1 M-sodium phosphate, pH 6, without (a) and with (b) 1 mM-phosphoramidone. ●, Relative OD₆₂₀; ▲, liberated reducing groups; ■, liberated -NH₂ groups.

Fig. 5. SDS-PAGE of the autolysin (lane A) and the extracellular muramidase (lane B) of *C. acetobutylicum* ATCC 824.

fraction, most activity being either cytoplasmic or associated with the cell wall (Table 3). Measurement of autolytic activity in the presence of deoxycholate or Brij, detergents that suppress inhibition of the autolysin by lipoteichoic acid, led to only slightly increased activity in the different fractions.

Fig. 6. Comparison of NH₂-terminal amino acid sequences of the autolysin from *C. acetobutylicum* and muramidases from *C. acetobutylicum* (Croux *et al.*, 1992), *Chalaropsis* (Felch *et al.*, 1975) and Cp-1 phage of *S. pneumoniae* (E. Garcia *et al.*, 1988). * Amino acid involved in the active site of the *Chalaropsis* muramidase.

Table 4. Extraction of the autolysin from the cell wall

Native cell walls were used at a concentration of 10 mg ml⁻¹ in all experiments.

Activity liberated into the supernatant	NaOH treatment (+200 µg BSA ml ⁻¹)	LiCl treatment (5 M)*	Treatments at different pH (90 min incubation at 4 °C)						Ultrasonic treatment (pH 6)			Cell wall autolysis (7 h, 30 °C)	
			pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	2 min	15 min	60 min	pH 4	pH 6
Autolytic activity (U ml ⁻¹)	2.5	0	1.5	1.75	1.91	2.58	4.08	4.1	2.7	2.9	5	7	13
Specific activity [U (mg protein) ⁻¹]	0.22	0	1.2	1.93	0.98	0.33	0.43	0.42	0.42	0.42	0.42	2	1.5

* Activity was measured after dialysis of the supernatant.

Table 5. Purification of the autolysin of *C. acetobutylicum* ATCC 824

The starting point for purification was a mixture of the cytoplasmic fraction and the cell wall fraction after treatment at pH 6 for 7 h at 30 °C. Cells were taken from 1.5 litres of a 10 h culture of *C. acetobutylicum* ATCC 824.

Purification step	Total activity (U)	Protein (mg)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (-fold)
Crude extract	1640	550	3	100	1
Concentration, dialysis and filtration	1320	87	15.2	80.5	5.1
Anion-exchange chromatography	820	0.094	8700	50	2920
Gel filtration	690	0.033	20800	42	6980

Extraction of autolysins from the cell wall

Different methods of extraction of the autolytic enzyme(s) were tested (Table 4). Treatment with high concentrations of LiCl did not release any active autolysins. Treatment with NaOH in the presence of

Table 6. Bond specificity of the autolysin

Compound	Purified cell walls [µmol (µmol glutamic acid) ⁻¹]	Purified cell walls solubilized by the autolysin*
Muramic acid	1.2 ± 0.1	0.32 ± 0.1
Muramitol	0.02 ± 0.02	0.8 ± 0.2
Glucosamine	2.2 ± 0.2	2 ± 0.3
Glucosaminitol	0	0
Alanine	3 ± 0.4	3.1 ± 0.4
Glutamic acid	1 ± 0.3	1 ± 0.2
Diaminopimelic acid	1 ± 0.4	1.2 ± 0.3

* Solubilized peptidoglycan was reduced with NaBH₄ and analysed after hydrolysis as described in Methods.

bovine serum albumin led to only a low recovery of activity, probably due to significant denaturation of the autolysin at high pH.

Although the amount of extracted autolysin after incubation of cell walls at 4 °C for 90 min was higher at basic pH, the specific activity of the supernatant was lower than at acid pH. This indicated that the extraction of autolysin was more specific at low pH. Autolysis of

Fig. 7. Inhibition of the autolysin from *C. acetobutylicum* by lipoteichoic acids and cardiolipin. Autolytic activity ($1 \mu\text{g}$ autolysin ml^{-1}) was measured at 35°C in 0.1 M -sodium acetate buffer, pH 4. ●, *S. faecalis* lipoteichoic acid; ○, *Bacillus subtilis* lipoteichoic acid; ▲, cardiolipin.

native cell walls for 7 h at 30°C was the most effective treatment for the release of autolysin. As observed above, extraction at pH 4 gave the highest specific autolytic activity while the greatest amount of autolysin was released at pH 6.

Nature of the enzyme involved in the autolysis of native cell walls

In sodium acetate/phosphate buffer at 35°C , the maximum rate of autolysis of native cell walls occurred at pH 6. Autolysis of the walls was accompanied by an increase in the reducing power and in the number of free amino groups liberated (Fig. 4a). During autolysis of the walls, autolysin (18 U ml^{-1}) and proteinase (41 U ml^{-1}) were also released. The proteolytic activity was due to an acidic metalloprotease inhibited by phosphoramidone (Croux *et al.*, 1990). Autolysis of the walls, in the presence of this specific inhibitor, did not lead to liberation of free amino groups (Fig. 4b). The rate of liberation of reducing groups was not affected but the rate of wall autolysis was lower. Thus, lysis of the peptidoglycan appeared to result only from the action of a glycosidase(s) and not from *N*-acetylmuramoyl-L-alanine amidase or endopeptidase activities.

Purification of the autolysin

The autolysin was purified starting from a mixture of the cytoplasmic fraction and the cell-wall fraction after treatment at pH 6. After concentration and dialysis, chromatography of the crude enzyme sample through the Mono Q anion-exchanger showed a single peak of autolytic activity. The autolytic enzyme was further purified by gel-permeation chromatography on Superose 12. The enzyme appeared homogeneous when analysed by SDS-PAGE (Fig. 5). The results of the overall

purification procedure are presented in Table 5. A 6980-fold purification was achieved with a final yield of 42%.

The M_r was calculated to be $41\,000 (\pm 1000)$ (Fig. 5) by SDS-PAGE using standard proteins and $44\,000 (\pm 2000)$ by calibrated Superose 12 column. These results indicate that the autolysin is monomeric. The isoelectric point of the autolysin, estimated by isoelectric focusing, was 3.8.

NH_2 -terminal amino acid sequence

The NH_2 -terminal amino acid sequence of the autolysin of *C. acetobutylicum* was determined and compared with those of other lytic enzymes. The 23 amino acids of the autolysin showed 100% similarity with the extracellular muramidase of *C. acetobutylicum*, 39% with the first 24 amino acids of the muramidase of the fungus *Chalaropsis* and 18% with the first 28 amino acids of the muramidase of the Cp-1 bacteriophage of *Streptococcus pneumoniae* (Fig. 6).

Effect of pH

The autolysin was active over the pH range 2.5–7 with an optimum at pH 4.5 on boiled native cell walls, 3.5 on SDS-treated walls and 3 on purified cell walls.

Bond specificity

Cell walls were digested by the autolysin to 75% of the initial turbidity with a concomitant release of reducing groups. In order to study the bond specificity, digested cell walls were reduced, hydrolysed with 6 M-HCl and analysed with an automatic amino acid analyser (Table 6). Compared to undigested cell walls, the sample showed a 70% decrease in muramic acid which was reduced to muramitol, indicating that the autolysin was a muramidase.

Effect of phospholipids and lipoteichoic acids on autolysin activity

Among the three phospholipids tested, only cardiolipin was inhibitory (50% inhibition at a concentration of 20 nmol ml⁻¹) (Fig. 7a). Phosphatidylglycerol and phosphatidylethanolamine at concentrations as high as 200 nmol ml⁻¹ had no effect on autolysin activity.

Two lipoteichoic acids, isolated from *Bacillus subtilis* and *Streptococcus faecalis*, had an identical inhibitory effect which was much greater than the effect of cardiolipin (Fig. 7b).

Discussion

Cells of *C. acetobutylicum* spontaneously autolysed when they were transferred from culture medium to a buffer solution. This phenomenon is known for many Gram-positive (Kawata *et al.*, 1968; Mohan *et al.*, 1965; Ogata & Hongo, 1974; Shockman *et al.*, 1961) and Gram-negative (Hebeler & Young, 1975; Leduc & van Heijenoort, 1980; MacLeod & Matulat, 1961; Mohan *et al.*, 1965) bacteria and is due to the action of autolytic enzyme on the peptidoglycan (Joseph & Shockman, 1974; Leduc & van Heijenoort, 1980; Mohan *et al.*, 1965; Wegener *et al.*, 1977).

We purified the autolysin of *C. acetobutylicum* ATCC 824 to homogeneity and characterized it as a muramidase. The enzyme was identical to the extracellular muramidase (Croux *et al.*, 1992) in term of M_r , isoelectric point and NH₂-terminal amino acid sequence. A similar result has been suggested for the lytic enzyme of *C. saccharoperbutylacetonicum* (Yoshino *et al.*, 1982). Unlike *C. acetobutylicum* NCIB 8052 (J. L. Garcia *et al.*, 1988), no amidase activity could be detected either as liberation of NH₂ groups during autolysis of native cell walls or after purification of the autolysins. Moreover, in the presence of choline, strain ATCC 824 neither gave rise to the formation of long chains of cells nor was cellular autolysis inhibited, contrary to what has been observed for strain NCIB 8052 (J. L. Garcia *et al.*, 1988) and for *C. saccharoperbutylacetonicum* (Podvin *et al.*, 1988). Furthermore we were unable to radiolabel the cell wall of strain ATCC 824 with [*methyl*-³H]choline, suggesting that this strain does not contain choline in its cell wall contrary to what has been described for strain NCIB 8052.

Most of the *C. acetobutylicum* ATCC 824 autolysin was cytoplasmic, a lower proportion being associated with the cell walls in contrast to what has been shown for *Bacillus subtilis* (Brown, 1972) and *Streptococcus faecalis* (Shockman & Cheney, 1969), where the enzyme was associated with the cell wall through the teichoic acids.

The amidase of *Streptococcus pneumoniae* was mainly cytoplasmic (Briese & Hakenbeck, 1984, 1985), and did not possess any signal peptide, suggesting a special mechanism for its translocation (P. Garcia *et al.*, 1986; E. Garcia *et al.*, 1988). A similar mechanism could exist for the muramidase of *C. acetobutylicum*, as it has been recently shown that this enzyme does not possess a signal peptide (Croux & Garcia, 1991).

The rate of cellular autolysis had an optimum at pH 6.3, a value close to the one obtained for *C. saccharoperbutylacetonicum* (Ogata & Hongo, 1974), *C. perfringens* (Williamson & Ward, 1979) and *C. botulinum* (Kawata *et al.*, 1968; Kawata & Takumi, 1971). However, there was an important difference between the optimum pH, close to 6, for cellular or native cell-wall autolysis and the optimum pH of 3 for lysis of purified cell walls by the autolysin. Highly acidic cell wall proteins have already been described for numerous *Clostridium* strains (Sleytr & Messner, 1983) and could decrease the local pH near to the cell wall. The fact that the elimination by SDS of protein from boiled native cell wall led to a decrease in the optimum pH of the autolysin from 4.5 to 3.5 is in agreement with this hypothesis.

The physiological conditions leading to the most effective autolysis were associated with the end of the exponential phase of growth, as has been previously reported for other Gram-positive bacteria (Allcock *et al.*, 1981; Mohan *et al.*, 1965; Ogata & Hongo, 1974; Shockman *et al.*, 1961). This could be attributed to variations (i) in the amount of cell-wall associated autolysin, (ii) in the sensitivity of the peptidoglycan to the autolysin or (iii) in the inhibition of cell-wall associated autolysin. The first hypothesis is in agreement with the liberation of the autolysin into the extracellular fluid at the end of the exponential phase of growth, a period in which the ability of the cells to autolyse decreased. The second hypothesis could be related to a change in the degree of acetylation of the peptidoglycan, as the muramidase of *C. acetobutylicum* ATCC 824 is active only if the glucidic part of the peptidoglycan is not *N*-acetylated (Croux *et al.*, 1992). The third hypothesis has been confirmed for *Streptococcus faecalis* (Carson *et al.*, 1981; Sayare *et al.*, 1972) and is associated with change in the amount of lipoteichoic acid.

Monovalent cations such as Na⁺ and K⁺ increase the rate of autolysis at concentration higher than 0.1 M. Such an effect has already been described for other species of *Clostridium* (Ogata & Hongo, 1974) as well as for *Streptococcus faecalis* (Shockman *et al.*, 1961), a teichoic-acid-negative mutant of *Staphylococcus aureus* (Gilpin *et al.*, 1972), and the Gram-negative bacterium *Neisseria gonorrhoeae* (Hebeler & Young, 1975).

In contrast, all the divalent cations tested (except Mn²⁺) inhibited the autolysis of *C. acetobutylicum*.

Furthermore, autolysis was activated by the addition of compounds that complex divalent cations, e.g. citrate or EDTA. Similar effects have been reported for some Gram-positive (Ogata & Hongo, 1974) and Gram-negative (Eagon & Carson, 1965; Elmros *et al.*, 1976; Leduc & van Heijenoort, 1980) bacteria. The role of complexing agents as well as the role of monovalent cations could be to eliminate divalent cations from the cell wall and/or the plasma membrane. We have shown that in common with several other autolysins (Cleveland *et al.*, 1976), the muramidase of *C. acetobutylicum* is inhibited by cardiolipin and lipoteichoic acid. These compounds are highly negatively charged and lipoteichoic acids are able to bind divalent cations (Hughes *et al.*, 1973). The autolysin of *C. acetobutylicum* is an acidic protein (pI 3.8), negatively charged under the physiological conditions of growth. The autolysin could be inhibited and fixed to lipoteichoic acid and/or cardiolipin by ionic interactions that involve divalent cations. An association between autolysin and particular membrane fractions has already been shown for *Streptococcus pneumoniae* (Briese & Hakenbeck, 1985). When cells of *C. acetobutylicum* are suspended in buffer, the loss of divalent cations associated with the dilution could lead to liberation of part of the autolysin which would no longer be inhibited, and so autolysis could take place. In the presence of monovalent cations or compounds that complex divalent cations, this phenomenon could be amplified. The partial reversibility of autolysis during addition of divalent cations implies that the autolysin remains within the proximity of the site of fixation and regulation, most probably at the external surface of the cytoplasmic membrane. During growth of the microorganism, the liberation of the autolysin into the culture medium could be due to a limiting or at least low concentration of divalent cations.

One of the experimental results is not in agreement with the hypothetical mechanism of regulation proposed: during cell fractionation of the autolysin, only a small portion of the enzyme was found to be associated with the membrane fraction. However, since the breakage of the cells was done with a French press and at a pH close to the isoelectric point of the autolysin, an *in vitro* redistribution of the enzyme could have occurred. Immunocytology experiments will be needed to further confirm the distribution of the autolysin *in vivo*.

Currently, biochemical experiments are in progress to study the autolysis profile and the secretion of the autolysin in the presence of excess concentrations of different divalent cations in the culture medium.

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