Autolysis of *Clostridium acetobutylicum* ATCC 824

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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 autolise 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 NH$_2$-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 (Alcock *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, 10000 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

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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, 10000) to a final volume of 200 µl and applied to a Superose 12 HR 10/30 column (1 x 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 OD₆₂₀ min⁻¹ at 35°C.

For the fractionation study, the autolytic activity was measured using 1 µg [methyl-³H]choline-labelled, purified cell 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% (w/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 mass from 14400 to 94000 (low molecular mass standards) were used for the estimation of the molecular mass of the enzyme (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.

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**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 pH 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, the residual growth medium was removed with cotton tips. The unwashed pelleted 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 Ghuysen et al. (1968).

**Electron microscopy.** Bacterial morphometry 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) osmium tetroxide in acetone.

**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).
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 (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 × 10⁻³ 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.

<table>
<thead>
<tr>
<th>Cation*</th>
<th>Conc (m)</th>
<th>10^3 x k (min^-1)</th>
<th>Relative rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>5.1 ± 0.45</td>
<td>100</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>10^-5</td>
<td>1.06 ± 0.12</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>10^-4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10^-3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>10^-5</td>
<td>3.7 ± 0.39</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>10^-4</td>
<td>2.4 ± 0.3</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>10^-3</td>
<td>0.5 ± 0.1</td>
<td>10</td>
</tr>
<tr>
<td>Fe^{2+}</td>
<td>10^-5</td>
<td>4.8 ± 0.5</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>10^-4</td>
<td>4.0 ± 0.4</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>10^-3</td>
<td>2.0 ± 0.18</td>
<td>45</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>10^-5</td>
<td>5.1 ± 0.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^-4</td>
<td>4.2 ± 0.4</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>10^-3</td>
<td>2.6 ± 0.3</td>
<td>51</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>10^-5</td>
<td>5.15 ± 0.5</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>10^-4</td>
<td>5.1 ± 0.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^-3</td>
<td>3.4 ± 0.3</td>
<td>67</td>
</tr>
<tr>
<td>Mn^{2+}</td>
<td>10^-5</td>
<td>5.15 ± 0.45</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>10^-4</td>
<td>5.1 ± 0.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^-3</td>
<td>4.6 ± 0.5</td>
<td>90</td>
</tr>
</tbody>
</table>

*SO_4^{2-} 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.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (%)</th>
<th>Activation by 0.1% Brij (%)</th>
<th>Activation by 0.1% deoxycholate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall</td>
<td>34 ± 12</td>
<td>124 ± 6</td>
<td>110 ± 7</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>64 ± 15</td>
<td>116 ± 8</td>
<td>117 ± 6</td>
</tr>
<tr>
<td>Membrane</td>
<td>2 ± 2</td>
<td>115 ± 10</td>
<td>120 ± 11</td>
</tr>
</tbody>
</table>

* Relative activities with and without detergent for each fraction.
† Relative activities with and without detergent for each fraction.

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 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.
Autolysis of *Clostridium acetobutylicum* 865

Activity liberated into the supernatant

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NaOH treatment (+200 µg BSA ml⁻¹)</th>
<th>LiCl treatment (5 M)</th>
<th>Treatments at different pH (90 min incubation at 4 °C)</th>
<th>Ultrasonic treatment (pH 6)</th>
<th>Cell wall autolysis (7 h, 30 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[U ml⁻¹]</td>
<td>[U (mg protein)⁻¹]</td>
<td>pH 3</td>
<td>pH 4</td>
<td>pH 5</td>
</tr>
<tr>
<td>Autolytic activity (U ml⁻¹)</td>
<td>2.5</td>
<td>0</td>
<td>1.5</td>
<td>1.75</td>
<td>1.91</td>
</tr>
<tr>
<td>Specific activity [U (mg protein)⁻¹]</td>
<td>0.22</td>
<td>0</td>
<td>1.2</td>
<td>1.93</td>
<td>0.98</td>
</tr>
</tbody>
</table>

* Activity was measured after dialysis of the supernatant.

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.

Table 5. Purification of the autolysin of *Clostridium 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.

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

Table 6. Bond specificity of the autolysin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Purified cell walls solubilized by the autolysin [µmol (µmol glutamic acid)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muramic acid</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Muramitol</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Glucosaminol</td>
<td>0</td>
</tr>
<tr>
<td>Alanine</td>
<td>3 ± 0.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>Diaminopimelic acid</td>
<td>1 ± 0.4</td>
</tr>
</tbody>
</table>

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

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 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

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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.
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-acetylmuramoy-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 Mₐ was calculated to be 41000 (±1000) (Fig. 5) by SDS-PAGE using standard proteins and 44000 (±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.

NH₂-terminal amino acid sequence

The NH₂-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 *Clostridium 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 *Clostridium 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 *Clostridium saccharoperbutylacetonicum* (Yoshino et al., 1982). Unlike *Clostridium 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 *Clostridium 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 *Clostridium 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 *Clostridium 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 *Clostridium acetobutylicum* (Ogata & Hongo, 1974), *C. perfringens* (Williamson & Ward, 1979) and *Clostridium 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 (Slyetr & 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 *Clostridium 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 *Clostridium acetobutylicum*. 

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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 due to a limiting or at least low concentration of 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 absence 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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References


