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Purification and Characterization of an Autolysin from *Clostridium acetobutylicum*

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A proteinaceous substance with antibiotic-like activity, resembling that of a bacteriocin, was isolated from an industrial-scale acetone-butanol fermentation of *Clostridium acetobutylicum*. The substance, purified by acetone precipitation, diethylaminoethyl cellulose chromatography, and polyacrylamide gel electrophoresis, was characterized as a glycoprotein with a molecular weight of 28,000. The glycoprotein was partially inactivated by certain protease enzymes. It had no effect on deoxyribonucleic acid, ribonucleic acid, or protein synthesis, and it did not result in the loss of intracellular adenosine triphosphate. The glycoprotein lysed sodium dodecyl sulfate-treated cells and cell wall preparations, and therefore it is referred to as an autolysin. The autolysin gene appeared to be chromosomal since plasmid deoxyribonucleic acid was not detected in the *C. acetobutylicum* strain.

As a result of the world energy shortage the production of solvents from renewable substrates by *Clostridium acetobutylicum* is being reexamined by a number of laboratories. A fermentation plant for the production of acetone and butanol from molasses is operating in South Africa (15). We previously reported that *C. acetobutylicum* produced a bacteriocin which affected the solvent yields obtained during the fermentation process (1). The producer cells were sensitive to the bacteriocin. The release of bacteriocin towards the end of the exponential growth phase was accompanied by lysis of the culture and inhibition of the production of solvents. We now describe the purification and characterization of the bacteriocin and conclude that it is an autolysin. Although autolysins have been studied in other *Clostridium* strains (5, 9, 12), this is the first characterization of an autolysin produced by *C. acetobutylicum*.

MATERIALS AND METHODS

Bacterial strains and media. The *C. acetobutylicum* strains and the bacterial strains used in the activity spectrum studies (Table 1) were described previously (1). The potato medium used for the maintenance of the *C. acetobutylicum* strain and the molasses fermentation medium (CFM) used for the production of the autolysin were described by Barber et al. (1). The *Clostridium* basal medium of O'Brien and Morris (13) was used for the well assay (11) described below. Plate cultures were incubated in GasPak (BBL Microbiology Systems) jars at 34°C.

Autolysin assay. The autolysin was assayed by either the well method (11) described by Barber et al. (1) or the decrease in optical density of whole cells or cell wall preparations at 600 or 660 nm, respectively. Autolysin titers in arbitary units were expressed as the reciprocal of the highest doubling dilution that gave a zone of inhibition surrounding the well. Cell walls were prepared from exponential-phase cells by the method of Kawata et al. (6). The crude cell walls were treated with 2% (wt/vol) sodium dodecyl sulfate (SDS) for 30 min and washed five times with 50 mM phosphate buffer, pH 6.0, to remove the SDS. The cell walls were resuspended in the phosphate buffer and stored at 4°C until required.

Autolysin production and purification. Crude autolysin was prepared by 60% (vol/vol) acetone precipitation, at 4°C, of supernatant samples from 48-h *C. acetobutylicum* cultures in CFM medium. The crude autolysin was lyophilised and reconstituted by the addition of 10 mM sodium acetate buffer (pH 4.5), added to a diethylaminoethyl cellulose (Whatman) column (1.5 by 15 cm) and eluted with the sodium acetate buffer at 4°C. Fractions (2 ml) were collected and assayed for autolysin activity by the well plate technique and for protein by absorption at 280 nm. Fractions showing autolysin activity were concentrated by dialysis against polyethylene glycol at 4°C and then added to 5% (wt/vol) polyacrylamide disc gels (0.8 by 8 cm) in 50 mM phosphate buffer, pH 6.0 (ca. 250 μg of protein per gel).

The gels were electrophoresed at 4°C towards the cathode in 10 mM phosphate buffer, pH 6, at 27 V per gel for 5 h. The gels were then cut in half lengthwise, and one-half was stained with Coomassie blue for 2 h and destained overnight in 45% (vol/vol) methanol containing 7% (vol/vol) acetic acid. The remaining half of the gel was stained for either lipid (2) or carbohydrate (17) or stored at −20°C until the complementary gel (stained for protein) was destained. The two halves were then matched, and the corresponding sections opposite stained protein bands were cut out. The same unstained sections from three gels were macerated and eluted with 2 ml of 10 mM sodium acetate buffer, pH 4.5, for 6 h at 4°C. The gel was removed by centrifugation, and the supernatant was

TABLE 1. *Activity of concentrated crude and partially purified autolysin from C. acetobutylicum*[a]

Organism	Activity[b]		
	Heat-inactivated crude autolysin	Crude autolysin (8,192 AU[c])	Partially purified autolysin (256 AU)
Clostridium butyricum	−	+	−
C. perfringens strain 1	−	+	−
C. perfringens strain 2	−	−	−
C. sporogenes strain 1	−	+	−
C. sporogenes strain 2	−	−	−
C. histolyticum	−	+	−
C. oedematiens	−	−	+
Bacillus subtilis	−	+	+
B. polymyxa	−	+	+
B. laterosporus	−	+	+
B. coagulans	−	+	+
B. cereus	−	+	+
B. licheniformis	−	+	+
B. megaterium	−	+	+

[a] Autolysin activity was assayed by the well method in seeded agar plates.

[b] +, Any observable zone of inhibition; −, no zone of inhibition.

[c] AU, Arbitrary units.

assayed for autolysin activity by the well plate technique. As a control, three sections of unstained gels not containing proteins were eluted and tested for activity. The purity and molecular weight of the eluted protein were determined by SDS-polyacrylamide gel electrophoresis.

Molecular weight determination. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (8). The purified protein obtained by elution from the preparative polyacrylamide disc gels was concentrated by the addition of 2 volumes of acetone. After standing at −20°C for 18 h, the precipitate was removed and resuspended in dissociation buffer (8) and electrophoresed on a 4% (wt/vol) acrylamide stacking gel with a 12% (wt/vol) acrylamide resolving gel. The following proteins were used as molecular weight markers and were electrophoresed simultaneously in adjacent wells; ovalbumin, aldolase, carbonic anhydrase, trypsin inhibitor (Pharmacia Fine Chemicals, Inc.), chymotrypsinogen (Miles-Seravac), and myoglobin (Sigma Chemical Co.).

Protein and nucleic acid syntheses. Protein synthesis in exponential-phase *C. acetobutylicum* cells was assayed by the incorporation of [^{14}C]leucine (0.25 μCi/ml) in trichloroacetic acid-precipitable material. Ribonucleic acid and deoxyribonucleic acid (DNA) syntheses were determined by difference after incorporation of [^{3}H]adenine (2 μCi/ml) into trichloroacetic acid-precipitable material (total nucleic acid) and into NaOH-hydrolyzed trichloroacetic acid-precipitable material (DNA) (4). The effect of the autolysin on macromolecular synthesis was determined by the addition of 1,638 arbitrary units to exponential-phase cells. Heat-inactivated autolysin was used as a control.

Estimation of intracellular levels of ATP. Intracellular levels of adenosine triphosphate (ATP) were determined before and after (10 min) the addition of autolysin or heat-inactivated autolysin to exponential-phase cells in *Clostridium* basal medium. ATP was measured by counting the flashes of light emitted by mixtures of cell extract and firefly lantern extracts (Sigma) (3). A Beckman scintillation counter was used, and 0.05 nmol of ATP produced 100,000 cpm.

Enzyme inactivation studies. Trypsin (Difco), pepsin (Merck & Co., Inc.), pronase (Miles Laboratories), papain (Sigma; 1 mg/ml), and proteinase K (Boehringer Mannheim Corp.; 0.1 mg/ml) were added to partially purified autolysin and assayed for activity after 30 min at 20°C.

Plasmid studies. Cultures, grown overnight in *Clostridium* basal medium broth at 37°C, were diluted into fresh broth containing [^{3}H]thymidine (10 μCi/ml) and incubated for 2 h. The cultures were lysed and analyzed for plasmid DNA on ethidium bromide-CsCl gradients by the method of Lacey and Grinsted (7). The presence of plasmid DNA was also investigated by nick translation (10). Exponential-phase cultures were lysed and centrifuged as above, and the fractions corresponding to a plasmid band were removed. The DNA was labeled by nick translation, using [^{32}P]ATP, electrophoresed on an agarose slab gel, and autoradiographed.

RESULTS

Mode of action. Crude and partially purified (after diethylaminoethyl cellulose chromatography) autolysin produced zones of inhibition when tested against *C. acetobutylicum*. The addition of crude and partially purified autolysin to exponential-phase cells in *Clostridium* basal medium broth resulted in a decrease in the growth rate of the bacterium but did not cause lysis of the cells (Fig. 1). However, SDS-treated whole cells and cell walls were lysed by both the crude and partially purified autolysin (Fig. 2). Studies on the effect of the autolysin on macromolecular synthesis in exponential-phase cells indicated that it did not inhibit DNA, ribonucleic acid, or protein synthesis. In addition, the autolysin did not decrease the intracellular levels of ATP.

Purification and molecular weight determination. Crude autolysin was eluted as a single peak of activity after diethylaminoethyl cellulose chromatography. The partially purified autolysin after diethylaminoethyl cellulose chromography was unstable, and only 1% of the activity was retained after 24 h at 4°C. Preparative purification of the autolysin on polyacrylamide disc gels yielded a single stained protein band. This band, after elution from the unstained half of the gel, possessed autolysin activity and produced zones of inhibition in the well test. Carbohydrate staining of the complemen-

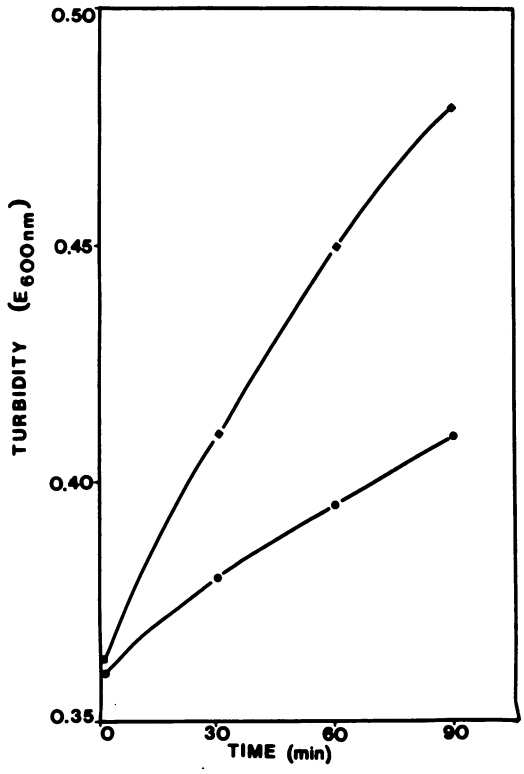

FIG. 1. *Effect of partially purified autolysin on the growth of C. acetobutylicum. Symbols: Heat-inactivated autolysin control (■) and autolysin (●) added at time zero time.*

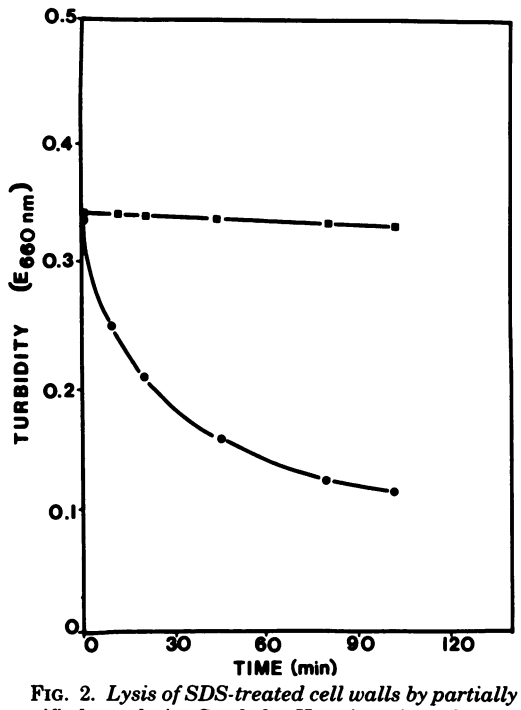

FIG. 2. *Lysis of SDS-treated cell walls by partially purified autolysin. Symbols: Heat-inactivated autolysin control (■) and autolysin (●) added at time zero time.*

tary gels indicated that the autolysin was a glycoprotein. Lysozyme was added to the gels as a positive control for the detection of carbohydrate moieties. No lipid component was detected in the autolysin after staining the gels with Sudan black.

SDS-polyacrylamide gel electrophoresis of the concentrated autolysin after elution from the preparative gels showed a single band and indicated that the autolysin had been purified to homogeneity. SDS-polyacrylamide gel electrophoresis studies with known markers indicated that the autolysin had a molecular weight of 28,000 (Fig. 3).

Enzyme inactivation studies. The partially purified autolysin was partially inactivated by the nonspecific proteases pronase and proteinase K but was not inactivated by the specific proteases, trypsin, pepsin, and papain.

Activity spectrum. Crude autolysin, which was concentrated by acetone precipitation, was very active against *C. acetobutylicum* and had a titer of 8,192 arbitrary units. This autolysin preparation inhibited four strains of *Clostridium* and seven *Bacillus* species (Table 1). Two additional strains of *Clostridium* species tested, a *C. per-*

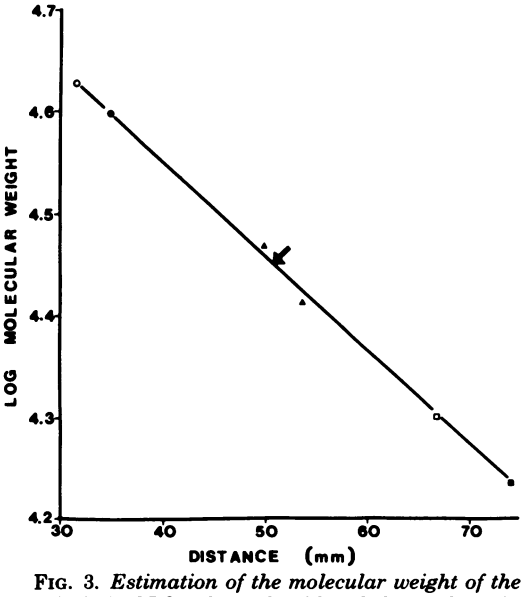

FIG. 3. *Estimation of the molecular weight of the autolysin by SDS-polyacrylamide gel electrophoresis. The gel was calibrated with (○) ovalbumin (43,000 molecular weight); (●) aldolase (40,000 molecular weight); (△) carbonic anhydrase (30,000 molecular weight); (▲) chymotrypsinogen (25,700 molecular weight); (□) trypsin inhibitor (20,100 molecular weight); and (■) myoglobin (17,200 molecular weight). Arrow indicates the position of the autolysin.*

fringens strain and a *C. sporogenes* strain, were resistant to the autolysin. All of the *Clostridium* strains except *C. oedematiens* were resistant to the partially purified autolysin, whereas all *Bacillus* strains were sensitive.

Plasmid studies. No plasmid DNA was detected by either dye-buoyant density gradient centrifugation or gel electrophoresis of labeled DNA.

DISCUSSION

C. acetobutylicum produced an autolytic glycoprotein in an industrial fermentation. The autolysin was similar to a bacteriocin, and the similarity between bacteriocins and lytic agents has been described by Tagg et al. (16). The evidence that the strain does not contain a plasmid suggests that the autolysin is chromosomal, but genetic crosses are required to confirm the observation.

The lytic activity could only be assayed on whole cells and cell wall preparations after SDS treatment. However, the autolysin did affect growth in vivo. There are examples of other autolysins which are similar in that lytic activity can only be demonstrated under certain conditions (5, 9). The autolysin did not affect DNA, ribonucleic acid, or protein synthesis and did not cause the loss of intracellular ATP by affecting the membrane. In our previous report (1) we noted that the bacteriocin was unusual in that it affected the donor strain. This activity is typically associated with the action of an autolysin, although it has been reported for a few bacteriocins (14).

The concentration and purification of the autolysin resulted in activity spectra different from those obtained previously with culture supernatants (1). The wide spectrum of activity, which included strains from other gram-positive genera, also suggests that the protein is an autolysin rather than a bacteriocin.

The induction of large amounts of an autolysin has important implications for the industrial production of solvents. The control of the induction of the autolysin and the isolation of autolysin-negative mutants is being investigated with a view to improving the fermentation process.

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LITERATURE CITED

1. **Barber, J. M., F. T. Robb, J. R. Webster, and D. R. Woods.** 1979. Bacteriocin production by *Clostridium acetobutylicum* in an industrial fermentation process. Appl. Environ. Microbiol. **37**:433–437.
2. **Crowle, A. J.** 1961. Immunodiffusion, p. 306. Academic Press, Inc., New York.
3. **Foulds, J.** 1971. Mode of action of a bacteriocin from *Serratia marcescens*. J. Bacteriol. **107**:833–830.
4. **Friesen, J. D. (ed.).** 1968. Measurement of DNA synthesis in bacterial cells. Methods Enzymol. **12B**:625–635.
5. **Kawata, T., and K. Takumi.** 1971. Autolytic enzyme system of *Clostridium botulinum*. 1. Partial purification and characterization of an autolysin of *Clostridium botulinum* type A. Jpn. J. Microbiol. **15**:1–10.
6. **Kawata, T., K. Takumi, S. Sato, and H. Yamashita.** 1968. Autolytic formation of spheroplasts and autolysis of cell walls in *Clostridium botulinum* type A. Jpn. J. Microbiol. **12**:445–455.
7. **Lacey, R. W., and J. Grinsted.** 1972. Linkage of fusidic acid resistance to the penicillinase plasmid in *Staphylococcus aureus*. J. Gen. Microbiol. **73**:501–508.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
9. **Mahony, D. E., M. E. Butler, and R. G. Lewis.** 1971. Bacteriocins of *Clostridium perfringens*. 2. Studies on mode of action. Can. J. Microbiol. **17**:1435–1442.
10. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. U.S.A. **72**:1184–1188.
11. **Mayr-Harting, A., A. J. Hedges, and R. C. W. Berkely.** 1972. Methods for studying bacteriocins, p. 330–333. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 7A. Academic Press, Inc., London.
12. **Nakamura, S., I. Okada, J. Mizuno, and S. Nishida.** 1977. *Clostridium perfringens*-specific lysin. Can. J. Microbiol. **23**:601–606.
13. **O'Brien, R. W., and J. G. Morris.** 1971. Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J. Gen. Microbiol. **68**:307–318.
14. **Reeves, P.** 1972. The bacteriocins, p. 16, 44. Chapman and Hall, London.
15. **Spivey, M. J.** 1978. The acetone/butanol/ethanol fermentation. Proc. Biochem. **13**:2–5.
16. **Tagg, J. R., S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40**:722–756.
17. **Upreti, G. C., and R. D. Hinsdill.** 1973. Isolation and characterization of a bacteriocin from a homofermentative *Lactobacillus*. Antimicrob. Agents Chemother. **4**:487–494.