BBA 66029

PROBLEMS IN PURIFICATION OF A *BACILLUS SUBTILIS* AUTOLYTIC ENZYME CAUSED BY ASSOCIATION WITH TEICHOIC ACID*

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(Received July 28th, 1969)

SUMMARY

1. Crude autolysin was isolated from postexponential cell walls of *Bacillus subtilis*. A radioactive assay was devised for detection of minute quantities of enzyme. This assay utilized small amounts of substrate and was 100-fold more sensitive than the commonly used spectrophotometric assay.

2. The crude autolysin was complexed with a large amount of teichoic acid. The teichoic acid was reduced to 5% of the original amount by ethanol precipitation and gel filtration on agarose. This fraction of teichoic acid persisted through repeated washings with ethanol and ether, electrophoresis in density gradients and on polyacrylamide gel, ion-exchange chromatography and gel filtration in 7 M urea.

3. The data show that the enzyme and teichoic acid are tightly bound.

INTRODUCTION

Recent reviews[1-3] have emphasized the universal occurrence of bacterial autolysins and revealed the paucity of procedures for recovery of the enzymes in a highly purified state. To date the classical methods of enzyme purification such as $(NH_4)_2SO_4$ precipitation, gel filtration, and ion-exchange chromatography have not resulted in extensive purification.

The autolysin from postexponential cell walls of *Bacillus subtilis* has been identified as an *N*-acyl muramyl-L-alanine amidase[4]. Experiments were designed to determine the role of this enzyme in the cell. During attempts to purify the amidase we encountered two major difficulties: (1) minute quantities of enzyme could not be detected by the usual technique of hydrolysis of cell walls, and (2) the amidase was strongly associated with teichoic acid which distorted its chemical and physical properties.

This report defines some of the problems in purification caused by association with teichoic acid and describes a simple quantitative radioactive assay for detection of minute quantities of enzyme.

Preliminary reports of this work were made[5,6].

* Part of this work was taken from a thesis submitted by W.C.B. for the Ph. D. degree in Microbiology at Oregon State University.

MATERIALS AND METHODS

Strains. Two highly transformable strains of *B. subtilis* were used: (1) the original 168 carrying *tryp*-2 and (2) BR290 carrying *tryp*-2 and *gta*B290 (ref. 7). Cell walls from BR290 were used as substrate because their rate of autolysis was much slower than that of 168 *tryp*-2.

Growth and preparation of cell walls. Spores were streaked onto tryptose blood agar base (Difco) and incubated overnight at 37°. One plate of each culture was used to inoculate 800 ml of minimal medium of SPIZIZEN[8], supplemented with 0.02% acid-hydrolyzed casein (Nutritional Biochemicals); 22 mM glucose, 5 mM $MgSO_4$ and 50 μg/ml L-tryptophan. After overnight growth at 37°, the contents of two flasks were added to 35 l of minimal medium in a commercial fermenter (Fermentation Design, Allentown, Pa.). The culture was incubated for 5 h at 37°. During this time the turbidity rose from 10–15 Klett units to 250–300 Klett units. Cells were harvested by Sharples centrifugation at 4°. When larger batches of culture (150 l) were prepared, cells were harvested with a Sharples high capacity centrifuge type AS 16P.

Cell walls were prepared by mechanical disintegration and differential centrifugation[9].

Autolysis. The autolytic enzyme was isolated from cell walls by autolysis in 20 mM $(NH_4)_2CO_3$ (pH 8.6). The suspension (5 mg/ml dry wt.) was incubated for 100 min at 37° and then centrifuged at 10 000 × *g* for 20 min. The supernatant liquid (crude autolysate) was stored at −20°. Autolysis was measured by following the decrease in turbidity of the cell wall suspension with a Klett–Summerson colorimeter (filter No. 66).

Enzyme assays. Enzymatic activity was measured by two methods. The first consisted of measuring the decrease in absorbance of a heat-inactivated suspension of cell walls of Strain BR290. Because this method consumed large amounts of material we devised a second method. The basis for this method was that [1-^{14}C]-glucosamine can be incorporated into BR290 cell walls with minimum dilution[10] and that all of the label could be accounted for in glucosamine, muramic acid and galactosamine. The enzymatic hydrolysis was measured by the release of radioactivity from heat-inactivated cell walls from BR290. The standard reaction mixture consisted of 12.5 μg/ml dry wt. of cell walls (specific activity, 40 000 counts/mg), 5 mM $MgSO_4 \cdot 6 H_2O$ and 10 mM Tris buffer (pH 9.2) in a final volume of 2.0 ml. The mixture was incubated at 42°. At various intervals after the addition of enzyme aliquots were removed and filtered through a 0.45-μ Millipore filter. After four washes with water the filters were immersed directly in scintillation fluid consisting of 22.5 g of 2,5-diphenyloxazole, 2.10 g of 1,4-bis-(5-phenyloxozolyl-2)benzene, 3.75 g of naphtalene and 200 ml of methanol. Measurements were made with a Packard Tri-Carb scintillation spectrometer. The results were expressed as the difference in counts/min per ml of the standard reaction mixture. Since the reaction was linear and not complete before 10 min, this time interval was used for all subsequent assays.

Chemical assays. The method of LOWRY *et al.*[11] was employed for the determination of total protein; bovine plasma albumin was the standard. Total and inorganic phosphorus was measured by the method of LOWRY *et al.*[12]. Teichoic acid was estimated from the organic phosphorus content[13].

Concentration. Preliminary studies indicated that lyophilization and evaporation

led to significant losses in enzyme activity. Thus, this method of concentration was impractical. Amicon ultrafiltration membranes were used (Amicon booklet No. 905). The UM-1 (Diffuse) membranes retained 50% of the enzyme whereas greater than 90% was retained by UM-1 (sharp). The latter membrane was used routinely.

Gel filtration. Agarose (Bio Gel A50m) was obtained from BioRad Laboratories. The gel was washed free of azide and thoroughly equilibrated with buffer prior to use. Sephadex G-100 and G-200 were obtained from Pharmacia, Inc. and prepared as directed by the manufacturer.

Ion-exchange chromatography. DEAE-cellulose (cellex D) and Ecteola-cellulose (cellex E) were obtained from BioRad Laboratories. Columns containing these materials were prepared as described by YOUNG[13].

Electrophoresis. Polyacrylamide gel electrophoresis of autolysin was performed under conditions described by the manufacturer (Shandon Scientific Corporation, Cat. No. SAE 2782) using Tris–glycine buffers at pH 9.5.

Isoelectric focusing was performed with LKB apparatus (Instruction manual No. 1-8100-E01) using ampholine solution pH 3–10. The sample was run at 4° for 48 h at 450 V.

RESULTS

Isolation of crude enzyme

Cell walls from postexponential phase cultures were suspended in buffer at 37°. Autolysis of this suspension was rapid and essentially complete in 40 min. This procedure routinely resulted in solubilization of 75–95% of the total mass of the cell walls.

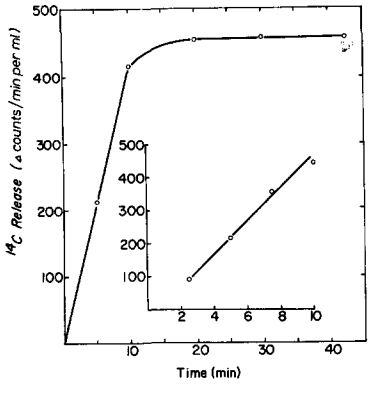
Enzymatic assays

Enzymatic activity was determined initially by following the decrease in absorbance of a heat-inactivated cell wall suspension. In this case, one unit was defined as the amount of enzyme giving an absorbance decrease of 0.001 in 10 min at 600 mμ. Subsequently, a radioactive assay was devised. During the standard conditions of assay the reaction was linear until approx. 90% of the radioactivity was released (Fig. 1). Spontaneous release of radioactivity during this period was less than 2%. This assay was 100-fold more sensitive than the spectrophotometric method, thereby making it extremely useful as an assay during the purification. The initial release of radioactivity from ^{32}P-labeled cell walls was also linear; but since the decay of ^{32}P is more rapid than that of ^{14}C, the ^{14}C method was more convenient.

Purification

During preliminary experiments, purification was attempted by fractional precipitation with $(NH_4)_2SO_4$ (ref. 14). Although some increase in specific activity could be obtained by this technique, the enzymatic activity was not confined to any of the fractions tested. Therefore, purification by fractionation with $(NH_4)_2SO_4$ was abandoned. Purification by gel filtration on Sephadex G-100 and G-200 was equally unsuccessful. The bulk of the enzymatic activity was restricted to the void volume in all cases. An example of this anomaly is shown in Fig. 2.

One significant observation made during these studies was that the enzyme



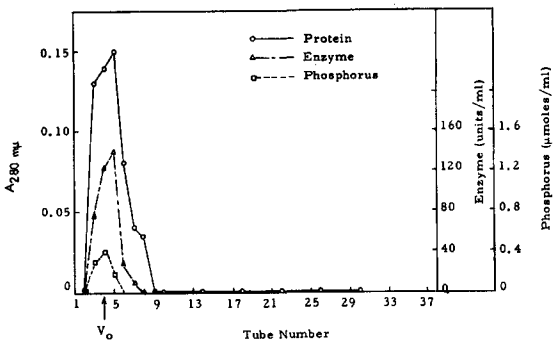
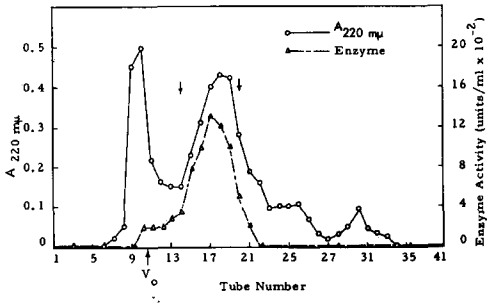


Fig. 1. Enzymatic release of ^{14}C from heat-inactivated cell walls of *B. subtilis* BR290. The assay mixture (2.0 ml) contained 0.2 ml crude autolysate (from 5 mg/ml dry wt. of cell walls), 25 μg of cell walls (specific activity, 40 000 counts/mg) and 1.6 ml of 50 mM Tris buffer (pH 9.2) containing 5 mM $MgSO_4$. The inset shows the reaction during the first 10 min.

Fig. 2. Gel filtration of partially purified autolysin on Sephadex G-100. The 65–85% $(NH_4)_2SO_4$ precipitate was concentrated to 1.0 ml and applied to a 1.2 cm × 25 cm Sephadex G-100 column. Elution was made with 1 mM Tris buffer (pH 7.1). 2-ml fractions were collected.

appeared associated with teichoic acid, a major polymer in *B. subtilis* cell walls. These findings were confirmed by the presence of teichoic acid constituents in the acid hydrolysate. The method which eliminated the bulk of the teichoic acid from the crude autolysate consisted of fractional precipitation with ethanol and gel filtration on agarose columns (Scheme 1). After precipitation the enzyme solution was applied to a column containing BioGel A50M. Four distinct peaks (A_{220} mμ) were observed having V_e (elution volume) to V_0 (void volume) ratios as follows: A = 1.0; B = 1.6; C = 2.5; and D = 3.0 (Fig. 3). Enzymatic activity was found only in Peak B. Peak B also contained the total teichoic acid of the sample. Only 32% of the initial activity was recovered with an 8-fold increase in specific activity. It is obvious, however, that



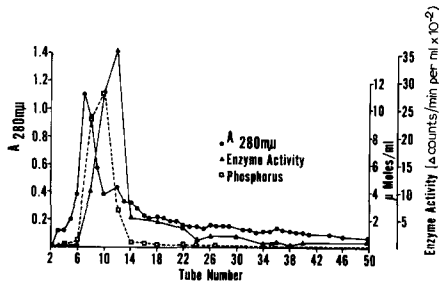
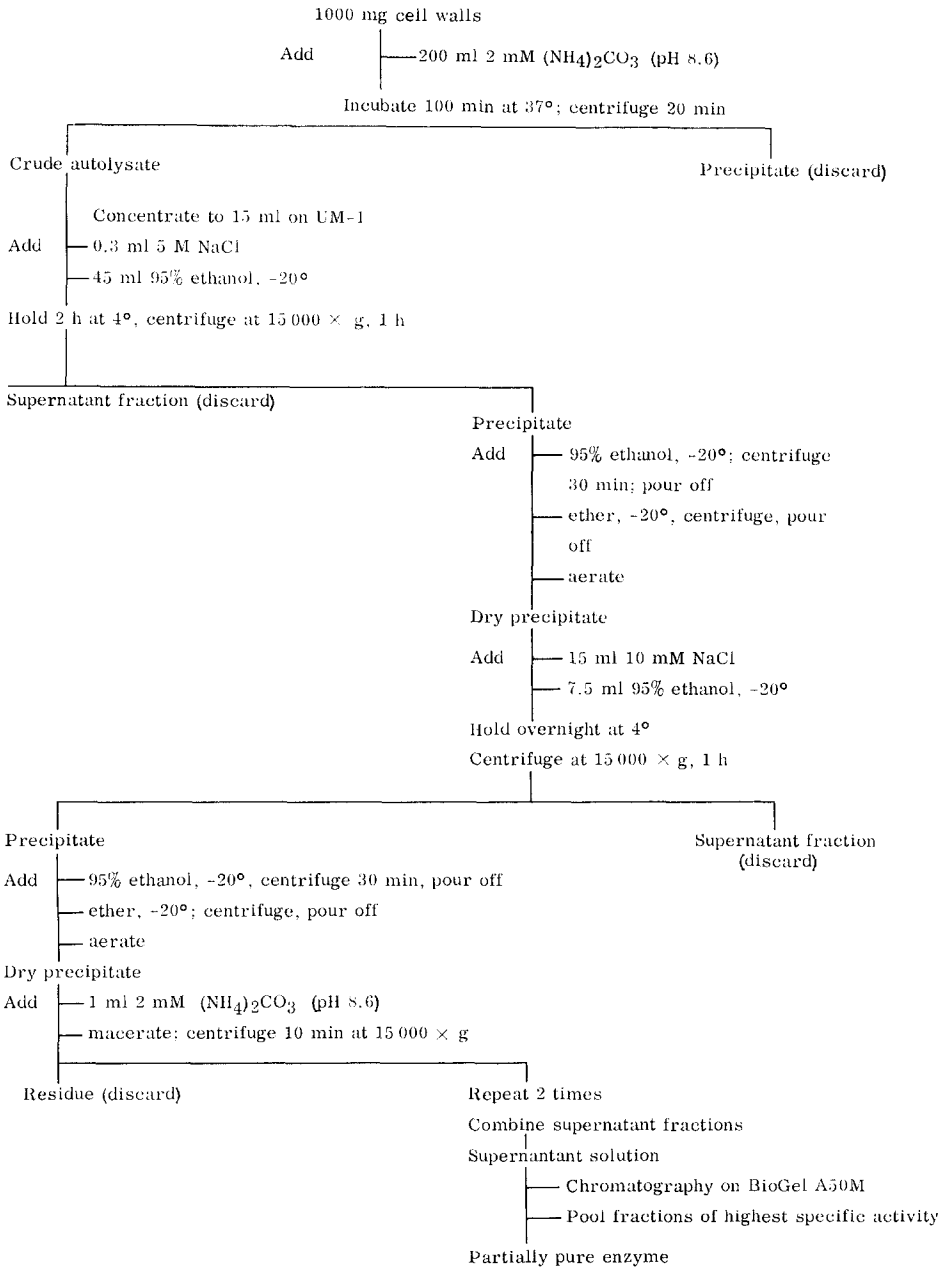


Fig. 3. Gel filtration of ethanol fractionated autolysin on BioGel A50M. Column dimensions were 1.2 cm × 50 cm. Elution was made with 1 mM Tris buffer (pH 8.0). Arrows refer to fractions pooled.

Fig. 4. Polyacrylamide electrophoresis of partially purified autolysin. Active fractions from the agarose column were concentrated to 1.0 ml, applied to the gel and subjected to electrophoresis as described in MATERIALS AND METHODS. The sample was eluted at a rate of 13 ml/h at 4°. 5-ml fractions were collected. The anode is on the left.



Scheme 1. Purification of crude autolysin by ethanol precipitation and gel filtration on agarose.

the purification factor would be much more impressive if values were based on whole cells rather than on cell walls. The importance of this method was its effectiveness in removing 95% of the teichoic acid found in the crude autolysate. Several experiments were designed to determine if the remaining teichoic acid (5%) was a contaminant or

more strongly associated with the enzyme. One such experiment was electrophoresis on polyacrylamide gel (Fig. 4). A discrete separation of the enzyme from teichoic acid was not observed. The extensive trailing could have resulted from fragmentation of the complex into enzyme fractions containing different chain lengths of teichoic acid. Other experiments included repeated washings with ethanol and ether, isoelectric focusing, column chromatography on Ecteola and DEAE-cellulose and gel filtration in 7 M urea. None of these treatments effected the complete separation of the enzyme from teichoic acid. These data raise the question as to whether this association is due to aggregation or covalent bonding.

DISCUSSION

The experiments presented here show clearly that purification of the autolytic enzyme is compounded by association with teichoic acid. This problem was first encountered during salt fractionation studies. It was initally assumed that teichoic acid was a contaminant, but another possibility was considered. The association of the enzyme with this strongly acidic molecule could explain the anomalous behavior observed during $(NH_4)_2SO_4$ fractionation. This view is supported by some findings by HEPPEL[15] which showed that large amounts of polyanions impede salt fractionation of enzymes. Additional evidence for a strong relationship between the enzyme and teichoic acid was provided by the gel filtration data. The inability of the enzyme to penetrate gels of wide pore sizes could be accounted for on this basis. Some heteropolymers such as glycoproteins isolated from epithelial cells have so large an effective molecular size that they are completely excluded from gels with the widest pore sizes available[16]. The aberrant behavior of glycoproteins was also observed by ANDREWS[17] during studies involving the determination of molecular weights of various proteins by gel filtration. It was reasoned that this behavior was exhibited because some glycoproteins have more expanded structures than typical globular proteins; this effect could be due to a greater hydration in solution of carbohydrate chains as contrasted to polypeptide chains. Recently, EVERSE AND KAPLAN[18] described unusual physical characteristics of diphosphopyridine nucleosidases of *B. subtilis* and *Neurospora crassa*. These enzymes contained as much as 80% polysaccharide. Thus, the high carbohydrate content of enzymes in *B. subtilis* is not without precedent.

The significance of the complex between the enzyme and teichoic acid is of considerable interest. One possibility is that this molecule serves a catalytic role. This hypothesis can be tested only if the two molecules can be separated by gentle procedures such as by digestion with glycosidases. Based on studies with many glycoprotein enzymes it seems unlikely that teichoic acid can be assigned a catalytic role. This view is supported by the example of ribonuclease A and B which are identical in amino acid composition, substrate specificity, and specific activity but differ in that ribonuclease B has a carbohydrate side chain[19,20]. Removal of the sialic acid from several other enzymes did not affect their enzymatic activity[21–23].

A more tenable explanation is that teichoic acid protects the enzyme against inactivation by proteases as well as against spontaneous inactivation. The remarkable stability of the amidase[6] under a variety of conditions is compatible with this interpretation.

It is also likely that bound teichoic acid localizes the enzyme for maximum

utilization of substrate. Focal degradation of cell walls has been demonstrated in several laboratories. MITCHELL AND MOYLE[24] observed hemispheral wall fragments produced by autolysis of *Staphylococcus aureus* and reasoned that this was due to localization of the enzyme at the equatorial ribbon of the cell. SHOCKMAN *et al.*[25,26] demonstrated by radioactive labeling and by a morphological analysis that the autolysin in *Streptococcus fecalis* was localized in the region of new cell wall synthesis (the coccal equators). Similarly, KAWATA *et al.*[27] showed lysis of *Clostridium botulinum* commenced at one end of the cell. CRIPPS AND WORK[28] showed that in *S. aureus* J.H.M. there was a localized rupture of the developing septum and an extrusion of high molecular weight material from this point. They proposed that the rupture was due to an autolytic system. Finally, some recent findings by SCHWARZ *et al.*[29] support our hypothesis. When cell division and cell wall synthesis in *Escherichia coli* were inhibited by low concentrations of penicillin, bulges appeared at the cell septum. They proposed that these bulges were caused by hydrolysis of the peptidoglycan by localized autolysins.

The data presented in this communication demonstrate that heteropolymers of the cell wall can influence the chemical and physical properties of autolysins. The findings that the enzyme and teichoic acid remained associated through salt fractionation, repeated ethanol precipitation, gel filtration in buffer and 7 M urea, ion-exchange chromatography, and electrophoresis, suggest that the two molecules are covalently bound. Obviously, further experiments are necessary to prove this hypothesis. Nevertheless, failure to seriously consider these factors in the interpretation of purification and characterization data might lead to erroneous conclusions.

ACKNOWLEDGMENTS

This investigation was started while one of us (W.C.B.) was a pre-doctoral fellow supported by a Public Health Service Training Grant 5T1AI 186. The work was continued while W.C.B. was a postdoctoral fellow supported by a fellowship GM36691-01 from the National Institute of General Medical Sciences.

F.E.Y. is a Faculty Research Associate of the American Cancer Society (PR8A). The study was aided by Public Health Service Research Grant GM12956 from the Institute of General Medical Sciences and E288E from the American Cancer Society.

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