AN ABSTRACT OF THE THESIS OF

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Title: STUDIES ON PURIFICATION AND CHARACTERIZATION OF AN AUTOLYTIC ENZYME FROM CELL WALLS OF BACILLUS SUBTILIS

Abstract approved:

Dr. Dorothy K. Fraser

The purpose of this investigation was to purify and characterize the autolytic enzyme from cell walls of Bacillus subtilis 168. The crude enzyme was obtained by autolysis of purified cell walls in buffer at 37°C. Two purification methods were developed. The first involved fractional precipitation with ammonium sulfate. The enzyme activity was found in the 30-85% fraction. Purification by this method was 3.2 fold while the recovery was 35%. A second, more efficient, method was developed using ethanol as a precipitant in the presence of 0.1 M NaCl. Crude autolysates were precipitated with 75% cold ethanol. The precipitate was dissolved and exposed to 33% ethanol. This fraction was further purified by chromatography on Bio Gel A50m. Active fractions were pooled and concentrated. This scheme resulted in a purification of 14 fold and a yield of 31%.

With heat-inactivated cell walls as substrate the partially purified autolytic enzyme was active at temperatures from 30°C to 62°C with maximum activity at 54°C. The pH optimum was broad (7-10); maximum activity occurred at pH 9-9.5.

Divalent cations were required for activity. Activation occurred with Ba^{++}, Ca^{++}, Mg^{++}, and Mn^{++}. The reaction was inhibited by Fe^{++} and Cu^{++}.

Solutions of enzyme were stable for several hours at room temperature and for at least two months at -20°C. Activity was unaffected by freezing and thawing during this period. Lyophilization caused a 50% reduction in activity.

No evidence for proteolytic activity was found.

The partially purified enzyme contained 3% tightly bound organic phosphorus which was assumed to be in teichoic acid. This complex was not dissociated by several physical methods such as electrophoresis, ion-exchange chromatography, and gel filtration. These findings permitted tentative characterization of the enzyme as an acidic glycoprotein.

Studies on Purification and Characterization of an Autolytic Enzyme from Cell Walls of *Bacillus Subtilis*

by

Willie Claiborne Brown

A THESIS

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in partial fulfillment of
the requirements for the
degree of

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June 1968

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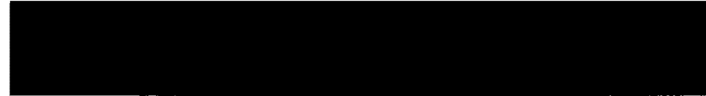

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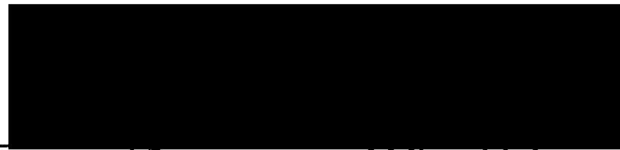

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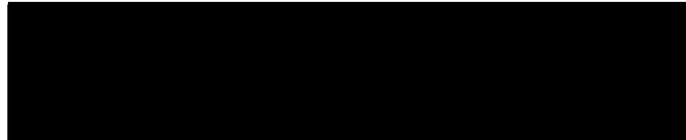

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STUDIES ON PURIFICATION AND CHARACTERIZATION OF AN AUTOLYTIC ENZYME FROM CELL WALLS OF *BACILLUS SUBTILIS*

INTRODUCTION

Autolytic enzymes are intracellular enzymes capable of degrading the cell walls of the same organism in which they are found (Stolp and Starr, 1965). One of the earliest observations of this phenomenon was made with the pneumococci (Dubos, 1937). The enzyme complex isolated from autolysates of these organisms were able to lyse heat-killed suspensions of the same bacterium.

Since then autolytic activity has been observed among both Gram positive and Gram negative organisms. Nomura and Hosada (1956) isolated an "autolysin" from *Bacillus subtilis* H by ammonium sulfate precipitation of whole cell or cell wall autolysates and found that it consisted of both cell wall lytic and proteolytic activities. A lysozyme like enzyme was also found in autolysates of *Staphylococcus aureus* (Mitchell and Moyle, 1957). In the same year, a cell wall lytic enzyme was found associated with spores of *Bacillus cereus* and *Bacillus anthracis* (Strange and Dark, 1957). This enzyme, different from lysozyme in its mode of action, was partially purified by treatment with clupein sulfate and ammonium sulfate. Autolytic enzymes have also been found associated with vegetative cell walls of *Bacillus cereus* (Singer and Church, 1964; Mohan, *et al.*, 1965);

however, comparisons between the spores and vegetative enzymes have not been made.

A lytic complex associated with cell walls of *Eschericia coli* was reported by Pelzer (1963a). Partial purification of this complex was achieved by chromatography on DEAE-Sephadex (Pelzer, 1963b). In another report, the autolytic system in this organism was used for the production of spheroplasts.

One of the most extensively studied autolytic systems has been that of *Streptococcus faecalis*. In a series of papers, (Shockman, 1963, 1965; Shockman, Kolb and Toennies, 1958; Toennies and Shockman, 1958; Conover, Thompson and Shockman, 1966) it was reported that cells from exponential phase cultures of *S. faecalis* 9790 autolyzed rapidly when placed under conditions which prevented the synthesis of cell wall polymers. This inhibition could be caused by addition of a specific inhibitor, for example, penicillin or cycloserine, or by deprivation of an essential precursor of cell wall polymers such as L-lysine, D-alanine, aspartic acid, glutamic acid or glucose. Isolated and purified cell walls of these cells would autolyze slowly when placed in phosphate buffer. From the autolysate an enzyme was recovered which was capable of dissolving intact cell walls.

In more recent studies (Shockman, Thompson and Conover, 1967), partial purification of the enzyme was achieved by

chromatography on Bio Gel P-60. It is interesting that none of the common purification procedures such as treatment with protamine sulfate, streptomycin, magnesium chloride, ammonium sulfate nor DEAE-cellulose batch absorption would increase the specific activity above that of the partially purified enzyme. Among the properties observed were activation by trypsin, pH optimum of 6.7, and a limited substrate specificity. Further, it was shown that the enzyme was markedly influenced by ionic strength, and displayed variable activity during storage at -10°C.

Conditions needed to obtain autolysis in _B_. _subtilis_ 168 have been studied in some detail (Young and Spizizen, 1963; Young, 1964a, 1966a). The novel feature of this system is that the autolytic enzyme is highly active in transformable strains but lower in activity in numerous nontransformable and transformable strains grown under conditions unfavorable for the development of maximal competence. The crude enzyme isolated from cell walls was stimulated by Na^+ , K^+ and divalent cations of Mg, Mn, Ca and Sr, and was inhibited by Cu^{++}, Fe^{++} and Ni^{++} . It was also shown that the enzyme had a broad pH optimum with a maximum at 9.5, and displayed a linear reaction between 24° and 45°C.

Any discussion of the mechanism of hydrolysis of cell walls by autolytic enzymes requires a brief description of cell wall structure (Strominger and Ghuysen, 1967). The bacterial substrate hydrolyzed

by all lytic enzymes studied so far is a rigid polymer located within the cell wall and is built up of linear polysaccharide chains of alternating β-1, 4-linked N-acetyl-glucosamine and N-acetylmuramic acid residues. Peptide chains substitute either all of the acetylmuramic residues in some organisms or some of them in others through N-acetymuramyl-L-alanine linkages. These peptides usually contain L- and D-alanine, D-glutamic acid, and one dibasic amino acid such as L-lysine or one of the isomers DD- meso or LL- diaminopimelic acid. This tetrapeptide is cross-linked thus making up of a network of two or three dimensions. It is generally assumed that the terminal carboxyl group of D-alanine and the ϵ-amino group of lysine or one of the amino groups of diaminopimelic acid are involved in the cross-links either directly or by means of additional peptide chains. Recently, the name "murein" has been proposed to denote this complex polymer, while "muropeptide" is suggested for the constituent low molecular weight subunits (Weidel and Pelzer, 1964).

In addition to "murein" the cell walls of all bacteria contain other polymers. These materials may or may not be covalently linked to the "murein"; but, unlike "murein", none of these compounds appear necessary for the maintenance of shape and mechanical strength of the cell wall. One of the most thoroughly investigated among this group is a class of compounds called teichoic acids

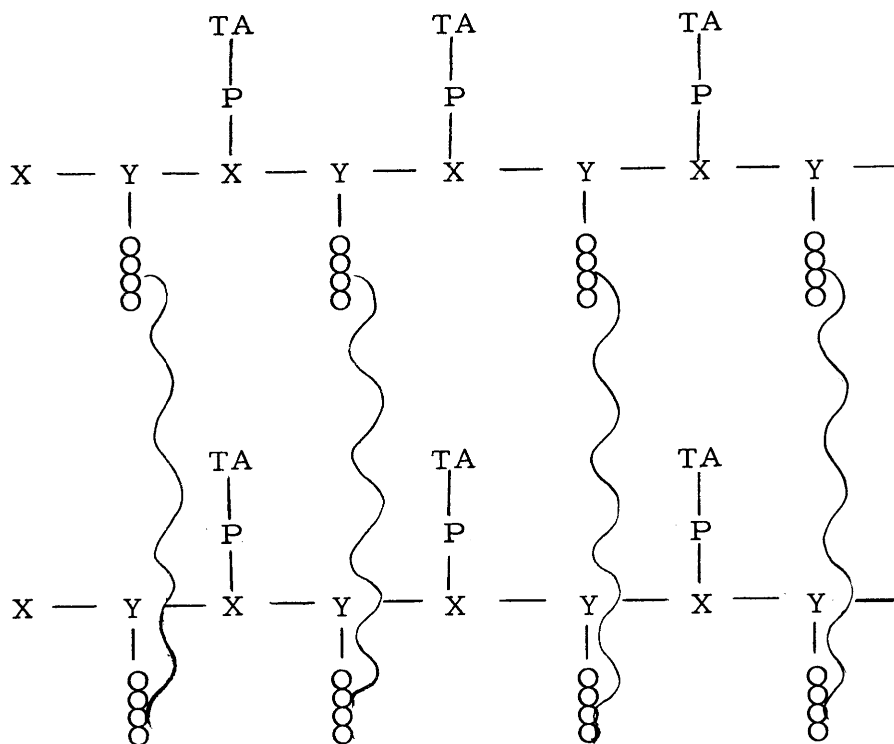
(reviewed by Archibald and Baddiley, 1966).

Teichoic acids are linear polymers of either glycerol or ribitol phosphate subunits connected in 1, 3- or 1, 5- phosphodiester linkage, respectively. The hydroxyl groups of the polyol monomers may be substituted with D-alanine and with different glycosidically linked mono- or oligosaccharides. It has been suggested that the teichoic acid of B. subtilis 168 belongs to the glycerol type (Young, 1966b). In addition to the polyol phosphate moeity, this polymer contains D-alanine, glucose, and different amounts of amino sugars such as N-acylmuramic acid, N-acylglucosamine, and N-acylgalactosamine.

The mode of linkage between teichoic acid and "murein" has been a subject of controversy for a long time (Archibald, et al., 1961; Mandelstram, Hecht and Strominger, 1961; Shockman, 1963). Recently, however, a glycopeptide-teichoic acid complex was isolated from cell walls of S. aureus which could not be dissociated by electrophoresis, ultracentrifugation, nor chromatography on ECTEOLA-cellulose; whereas artificial mixtures of acid extracted teichoic acid and glycopeptide were readily separated by either of these techniques. The covalent linkage of these components was demonstrated (Tipper, Ghuysen and Strominger, 1964; Ghuysen, et al., 1965). Thus, it appears that the teichoic acid is linked to other components of the cell wall by covalent bonds.

From this brief description emerges a possible interpretation of the bacterial cell wall as diagrammed in Figure 1. On the basis of this proposed structure, the disruption of the glycopeptide network, causing solubilization of cell walls or lysis of bacterial cells, can be effected by three classes of autolytic enzymes: glycosidases which hydrolyze the polysaccharide chains, endopeptidases which split the peptide cross-links, or acetylmuramyl -L-alanine amidases which hydrolyzes the amide bond between the carbonyl group of N-acetyl-muramic acid and L-alanine. The absolute characterization of these enzymes as to class requires the isolation and identification of the products of autolysis. One example will illustrate this point. Autolysis of *B. subtilis* 168 was accompanied by the release of N-terminal alanine without a concurrent release of C-terminal amino acids, reducing groups, or phosphomonoester groups (Young, Tipperand Strominger, 1964b; Young, 1966b). The autolysate was further analyzed by a combination of ion-exchange chromatography on diethylaminoethyl cellulose and gel filtration on Sephadex G-25. The major products were copolymers of N-acylmuramic acid and N-acylglucosamine, mucopeptides, cross-linked peptides, and teichoic acid (Young, 1966b). These findings elucidated the mechanism of autolysis and established the enzyme as an N-acylmuramyl-L-alanine amidase.

The physiological role of autolytic enzymes has not been



X = N-Acetylglucosamine

Y = N-Acetylmuramic acid

O = Amino acid

〜 = A cross bridge

TA = Teichoic acid

P = Phosphorus

Figure 1. An Interpretation of Cell Wall Stucture (From Ghuysen, *et al.*, 1965).

defined; nevertheless, two major hypotheses have been promoted to account for their behavior. One possibility is that during growth of the cell wall, the opening of the "murein" by autolytic enzymes could permit the addition of new subunits resulting in enlargement of the structure (Mitchell and Moyle, 1957; Weidel and Martin, 1960; Shockman et al., 1967). Another explanation is that by making gaps the enzyme facilitates permeation of the wall by large molecules such as proteins and deoxyribonucleic acid (DNA). The transformation system of B. subtilis provides evidence for this hypothesis. The autolytic N-acylmuramyl -L-alanine amidase is found maximally in competent strains. This enzyme could conceivably provide the necessary openings for penetration by transformable DNA (Young and Spizizen, 1963; Young, et al., 1964b). Both hypotheses require a mechanism for repair of the gaps introduced in this way, but so far, a suitable mechanism has not been described.

In discussing the role of autolytic enzymes, it must be realized that present knowledge of the autolytic process is primarily descriptive. Many systems have been reported but the various steps involved in this event remain obscure. The enzymes released during autolysis usually occur in a complex mixture which has not been further refined in a majority of cases. It is believed that description of methods for the purification and characterization of

autolytic enzymes will greatly facilitate these studies. The work presented in this thesis was undertaken with this objective in mind. A description of this research which involved the isolation, purification and partial characterization of the N-acylmuramyl-L-alanine amidase from transformable *B. subtilis* 168 is presented.

MATERIALS AND METHODS

General Procedures

Bacterial Culture

The strain used throughout these studies was *B. subtilis* 168 $I^- C^+$ which was obtained from Dr. Frank Young, Scripps Clinic and Research Foundation, La Jolla, California. The I^- refers to the auxotrophic character of the strain, either indole or tryptophan being necessary for growth on minimal medium. The C^+ refers to the ability to bind DNA irreversibly (Young and Spizizen, 1963). This culture was maintained as spores on potato agar (Spizizen, 1958).

Media

Two media were used routinely for growth of organisms. The first was tryptose blood agar base (TBAB, Difco) and its broth counterpart (TB broth), the second was a supplemented minimal medium (MS) containing (g/l): $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 14.0; KH_2PO_4, 6.0; $Na_3C_6H_5O_6 \cdot 2H_2O$, 1.0; $MgSO_4 \cdot 7H_2O$, 1.23; vitamin-free casein hydrolysate, 0.2; glucose, 5.0; L-tryptophan, 0.05. The last four constituents were sterilized separately and added after cooling. The pH was 6.8.

Buffers

Tris (hydroxymethyl -) -aminomethane (tris) was a product of Sigma (Trizma Base). Ammonium carbonate (AC) was obtained from Matheson, Coleman and Bell (MCB).

The buffer solutions were prepared by dissolving the appropriate weight of salt in distilled water to give the desired concentration. The pH was adjusted where necessary. All solutions were stored at $4^{o}C$.

Growth Conditions

The following procedure was employed for growing cells with high autolytic activity. An aqueous suspension of spores was streaked onto a petri dish containing TBAB and allowed to incubate for 24 hours. One-third of the growth from this plate was scraped off and added to 10 ml TBB. This suspension was incubated on a reciprocal shaker for three hours at 37 C. The entire 10 ml were added to a 2 liter flask containing 800 ml MS medium prewarmed to 37 C. This culture was incubated for 10 hours at 37 C on a rotary shaker. Longer periods resulted in significant lysis of the culture. The larger inoculum was added to 35 liters of sterilized MS medium in a Fermacell vessel (New Brunswick Scientific). The culture was incubated with vigorous aeration for five hours at 37 C. Cells were

harvested with a refrigerated Sharples centrifuge and frozen until ready for use.

When cells were required for other purposes, spores were streaked onto TBAB or added to TBB and incubated for the desired period at 37 C.

Protein Assays

The concentration of protein was estimated by the Lowry method (Lowry, *et al.*, 1951) using bovine serum albumin (Sigma, Inc.) as a standard. When rapid determinations were necessary, protein concentration was estimated from its absorption at 260 and 280 mμ using a nomograph distributed by CalBiochem based on data of Warburg and Christian (1942).

Determination of Phosphorus

Total phosphorus and inorganic phosphorus determinations were made by a modification of the procedure described by Lowry, *et al.* (1954).

Total Phosphorus. Estimation of total phosphorus consisted of mixing the sample, 0.1 ml, and 0.1 ml digestion mixture (30.6 ml concentrated H_2SO_4 and 6.7 ml 70% $HClO_4$ diluted to 100 ml with water) in acid cleaned three ml pyrex test tubes. The tubes were placed in an oven and digested two hours at 165°C. After

cooling, 1.0 ml development reagent I (1 ml M Na OAc, 1 ml 2.5% NH_4 molybdate, 8.0 ml distilled water, and 100 mg ascorbic acid) was added to each tube. Following incubation for 1.5 hours at 37°C, the optical density at 820 mμ was read in a Zeiss spectrophotometer. Appropriate blanks and standard samples were treated in the same manner. The amount of phosphorus was estimated by comparing the O.D. of unknown and standard samples (10, 20 and 40 mμ moles/ml KH_2PO_4).

Inorganic Phosphorus. Inorganic phosphorus was determined by mixing 0.1 ml of sample and 0.9 ml of development reagent II (0.7 ml 10N H_2SO_4, 0.6 ml 2.5% NH_4 molybdate, 7.7 ml water, and 300 mg ascorbic acid) in a three ml test tube. The tubes were incubated for 1.5 hours at 37°C and then read as described above.

Organic Phosphorus. Organic phosphorus (P_o) is a value represented by the difference between total and inorganic phosphorus.

Enzyme Activity

During the purification enzyme activity was measured by observing the decrease in turbidity of a buffered suspension of heat-inactivated (HI) cell walls with a Klett-Summerson photoelectric colorimeter (filter #66).

Typically the incubation mixture contained 2.0 mg of HI-cell walls, 0.005 M $MgSO_4$, and 0.1 ml of sample in a 2.0 ml volume. Incubation was for 30 minutes at 42°C. During this period the reaction was linear.

One unit of enzyme activity is defined as that amount which causes a decrease of one klett unit in 30 minutes.

Purification Procedures

Preparation of Cell Walls for Enzyme

Cell walls were prepared by a method which is described as follows (Young and Spizizen, 1963):

Eight to fourteen gram aliquots of cells were thawed slowly and transferred to a 75 ml Duran 50 flask containing an amount of acid-washed, 0.1 mm glass beads (Minnesota Mining and Manufacturing Co.) that equaled three times the wet weight of the cells. Sufficient cold water was added to produce a 50% suspension of bacteria (w/v). The mixture was shaken for two 1-minute intervals in the Braun cell homogenizer (Braun Co., Melsungen, Germany) and cooled with intermittent jets of CO_2. The slurry was distributed equally to four 50 ml centrifuge cups, diluted with cold water and centrifuged at 1,000 x g at 4°C to remove glass beads and intact bacteria. Complete disruption of the remaining bacteria was achieved by subjecting the residue to two more treatments in the homogenizer.

The 1000 x g supernatant fractions were centrifuged two more times at 1,000 x g for 10 minutes at 4°C and then at 10,000 x g for

10 minutes. The high speed sediments were pooled, suspended in cold distilled water and centrifuged again at 10,000 x g for 10 minutes. Following 12 washings in this manner, the cell walls were lyophilized.

Preparation of Cell Walls for Substrate

Cell walls used for substrate were prepared as described above; however, the following exceptions are noted. Cells for making cell walls were grown in TB broth for 12 hours. Cell walls prepared from these cells had a much lower level of autolytic enzyme.

Following the twelfth wash the cell walls were heat-inactivated by adding boiling distilled water to the thick suspension. Boiling was continued for five minutes. The suspension was cooled, centrifuged at 10,000 x g and lyophilized.

Autolysis of Cell Walls

The autolytic enzyme could be isolated from intact cell walls by autolysis in buffer. In a typical experiment, 200 mg of cell walls were suspended in 40 ml of 0.02M $(NH_4)_2CO_3$ buffer, pH 9.2. The suspension was incubated for 100 minutes at 37^oC. This was followed by centrifugation at 10,000 x g for 20 minutes to remove intact cell walls. The supernate (crude autolysate) was stored at

$-20^{o}C$ until ready for use.

Ammonium Sulfate Fractionation

Generally, the procedures for salt fractionation of the autolysate conformed to the guidelines recommended by Dixon and Webb (1961; 1964).

Method a. Nine and five-tenths ml of crude autolysate were dialyzed overnight against three changes (100 volumes each) of distilled water. The solution was placed in a beaker and stirred magnetically in the cold room. For the first fractionation (45% salt), 2.7 g of $(NH_4)_2SO_4$ was added slowly; the solution was stirred until all the salt was dissolved. After standing for 30 minutes, the precipitate which formed was collected by centrifugation at 15,000 x g for 30 minutes. The supernatant fluid was retained and further fractionated by adding 1.3 g of $(NH_4)_2SO_4$ (65% salt). The precipitate was again collected by centrifugation. The supernatant liquid was finally fractionated by adding 1.54 g of ammonium sulfate (85% salt). All precipitates were resuspended in a small volume of distilled water and dialyzed overnight against several changes of distilled water. All samples were assayed for protein and enzyme activity.

Method b. In this method the dialyzed enzyme solution was brought to 30% saturation and the precipitate was recovered by centrifugation. The remaining solution was brought to 100%

saturation and the precipitate recovered.

Gel Filtration

Sephadex G-100 and G-200. Dextran gels (Sephadex) were obtained from Pharmacia, Inc. The required amount of powdered gel was added to an excess of buffer. This suspension was allowed to swell with intermittent stirring, settling, and decantation for three days. The hydrated gel was diluted to 10 times its volume with buffer and packed into columns as follows.

Glass columns (Kontes) 1.2 x 25 cm were half-filled with buffer. The thin slurry was added until the column was filled. A glass funnel was then added to the top of the column and filled with the gel. After about two cm of gel had settled on the bottom, the stopcock was opened carefully and the liquid allowed to flow slowly. The gel in the funnel was agitated constantly by a mechanical stirrer. Care was taken to ensure that the column was kept vertical throughout the packing. When the gel was within three cm of the top of the column, the stopcock was closed and the funnel disconnected. A one cm filter paper disc (Whatman no. 1) was placed on top of the bed to prevent disturbances. The column was connected to a buffer reservoir and allowed to equilibrate overnight.

Blue dextran (MW $= 2 \times 10^6$) was used to determine the void volume (V_o) and to check the column packing for irregularities.

The sample was added to the top of the bed immediately after the level of eluent dropped below the filter disc. After the sample had drained into the bed it was chased with 1.0 ml buffer. Three ml of buffer were added and the column connected to the buffer reservoir. Fractions were collected using a Radi Rac LKB 3400 assembly. Fractions were analyzed for protein and enzyme activity as described previously. All operations were carried out in the cold room at 0-4°C.

Agarose. Sepharose 4B (exclusion limit 2×10^6) was obtained from Pharmacia, Inc. Bio Gel A50m (exclusion limit 50×10^6) was obtained from Bio Rad Laboratories. Both products were in the hydrated form containing 2% sodium azide as a preservative. The gel was washed twice, by suspension and decantation, with 0.01 M tris pH 7.1 to remove azide. All subsequent operations were carried out as described above.

Ethanol Fractionation and Gel Filtration

A second purification scheme was devised employing fractionation with ethanol followed by chomatography on Bio Gel A50m. A detailed description of this procedure is diagrammed in Figure 2. Fractions were assayed for enzyme activity, protein and phosphorus at each stage of the purification.

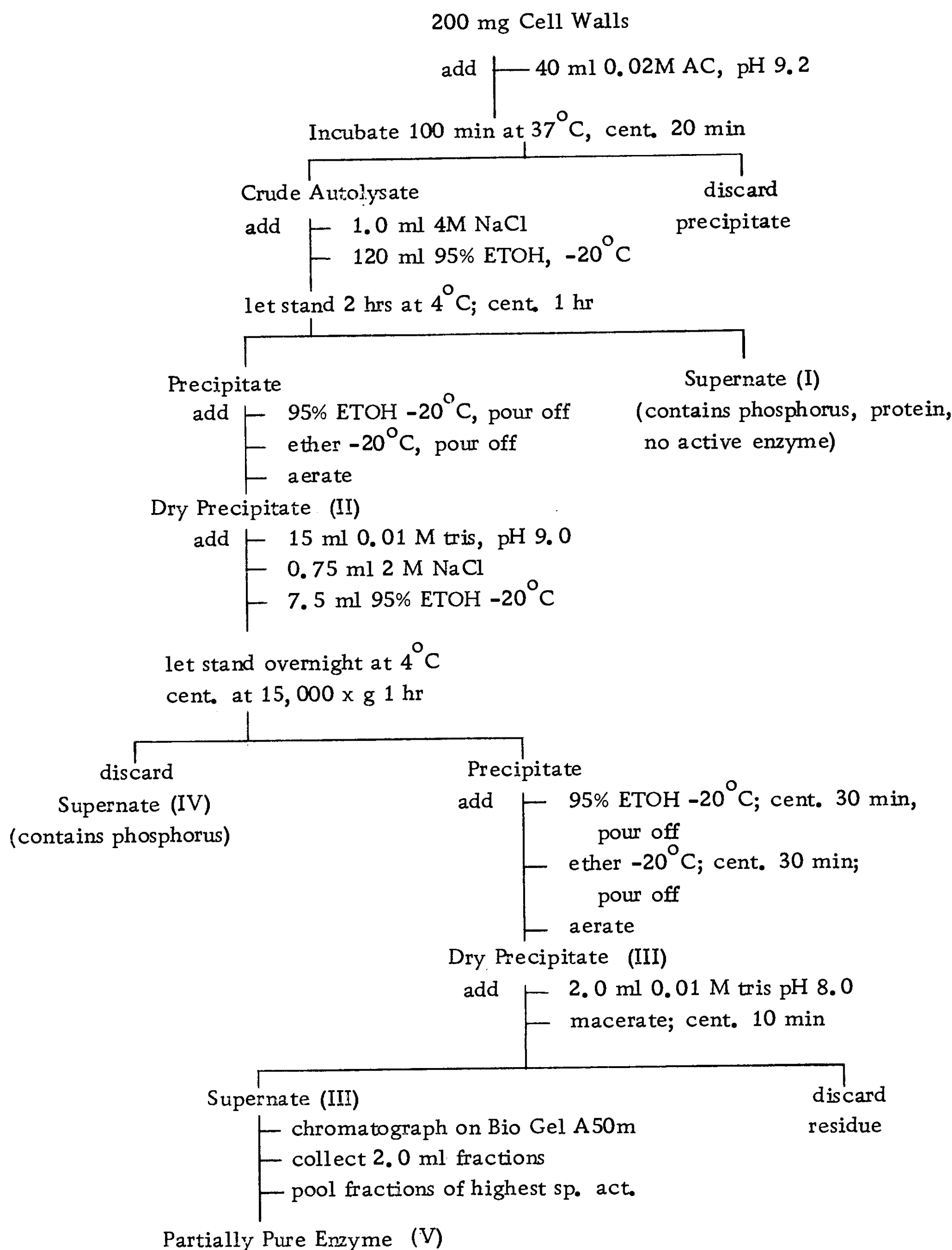


Figure 2. Description of Ethanol-Agarose Purification Procedure.

Characterization Procedures

Kinetic Assays

A Zeiss PMQ II spectrophotometer was used for following the kinetics of hydrolysis of heat-inactivated cell walls by the partially purified enzyme. The reaction mixture consisted of HI-cell walls, 0.01 M tris buffer, and enzyme. The total volume was 2.5 ml. Incubation was carried out in a 37°C water bath for 30 minutes, except that during temperature studies a polystat was used. Concentrations of enzyme, cell walls and the pH were varied as required by the experiment. All readings were made at 660 mμ.

Test for Proteolytic Activity

The proteolytic activity of both crude and partially purified enzyme was determined using Azocoll (CalBiochem) as the substrate. Solutions containing 12.5 μg of enzyme or trypsin in 5.0 ml of 0.1 M phosphate buffer pH 7.5 were mixed with 25 mg of Azocoll and incubated at 37°C for 15 minutes. The suspension was centrifuged for five minutes at 3,000 x g. A blank containing buffer and Azocoll was treated in the same manner. The supernatant solutions were read at 580 mμ in the Zeiss PMQ II spectrophotometer. The relative proteolytic activity was determined using a nomograph distributed by CalBiochem.

RESULTS

Enzyme Purification

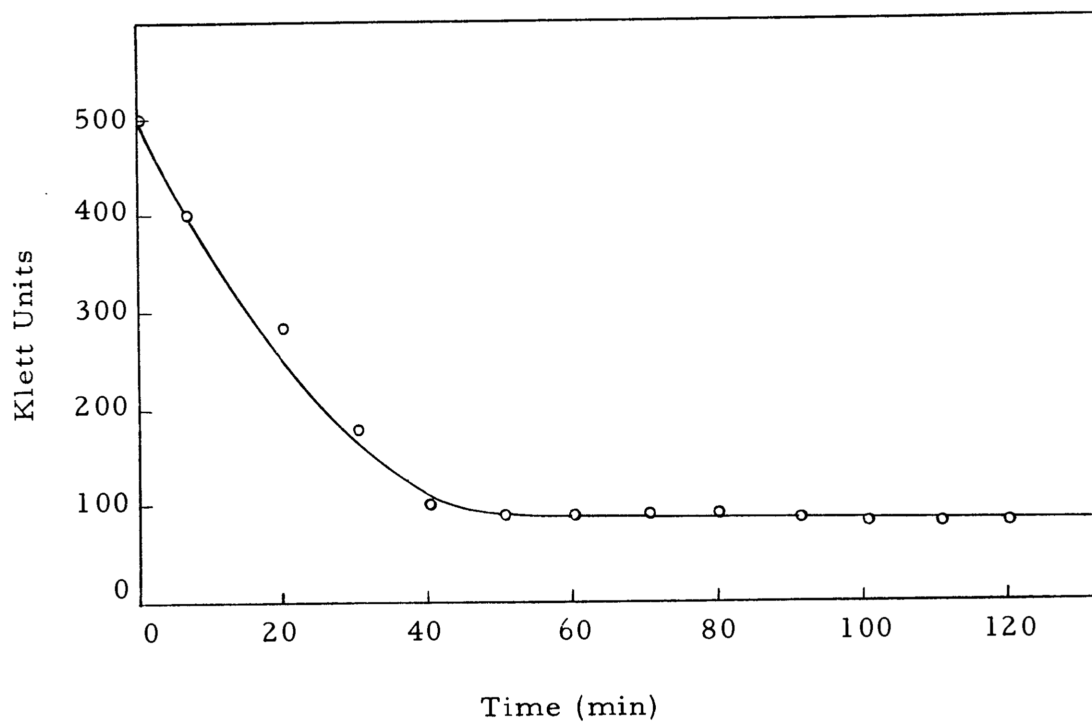
Autolysis of Cell Walls

Extensively washed cell walls were suspended in buffer and allowed to autolyse. The time course of autolysis is shown in Figure 3. Autolysis was rapid and essentially complete in 40 minutes. This procedure usually resulted in the solubilization of 75-95% of the total mass of the cell walls.

Ammonium Sulfate Fractionation and Gel Filtration

Method a. Fractional precipitation with ammonium sulfate allowed a small degree of purification of the autolytic enzyme. The majority of the activity was found in the 65-85% fraction. This fraction contained 50% of the original activity and 50% of the starting protein representing an overall purification of $\simeq$ 4 fold. It may be seen in Figure 4, however, that the enzyme activity was not restricted to this fraction. Fractions of lower yield, 45-65% and 25-45% (though not shown here), were of sufficient specific activity to suggest that the enzyme was soluble over a wide range of ammonium sulfate concentrations.

The 65-85% fraction was further subjected to chromatography



Assay conditions:
100 mg cell walls suspended in 20 ml
0.02 M tris pH 9.2; Incubated at 37°C.

Figure 3. Autolysis of B. Subtilis 168 $I^{-}C^{+}$ Cell Walls.

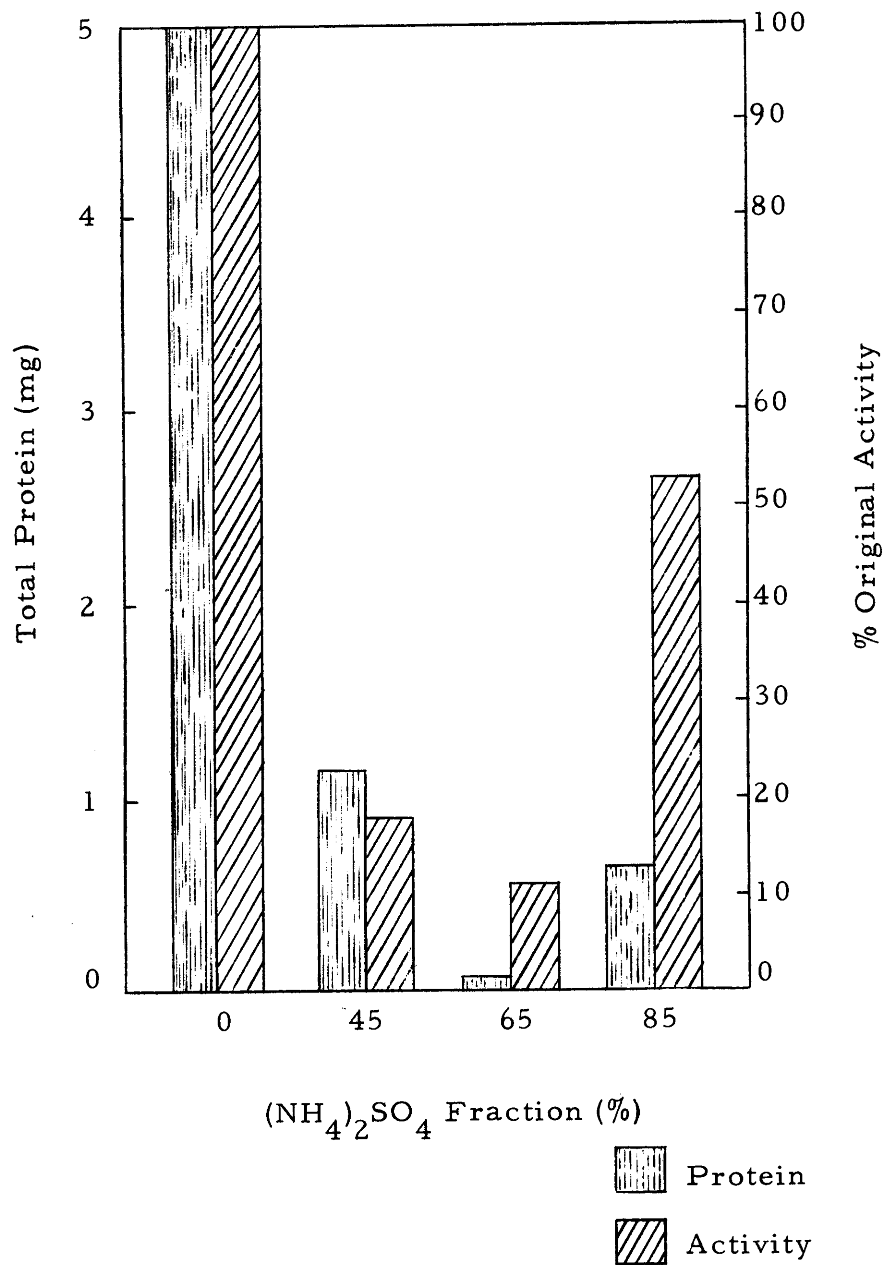
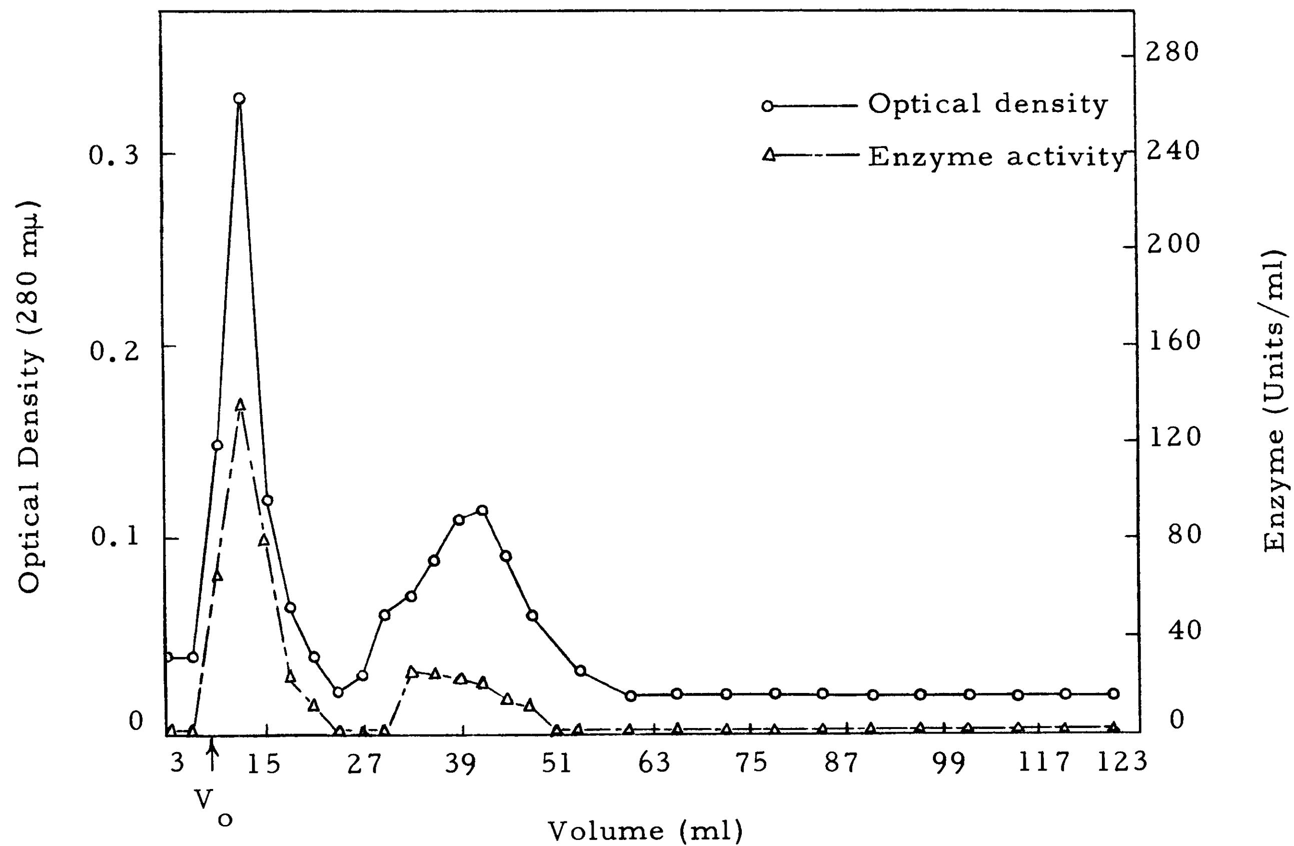


Figure 4. Fractionation of Autolytic Enzyme with Ammonium Sulfate.

on Sephadex G-100 (Figure 5). The enzyme activity was located in two regions. The bulk of the activity emerged close to the void volume (V_o) of the column (peak A), but another region, representing about one-third the activity of peak A was seen at V_e (elution volume) $\simeq 3V_o$. The final yield by this method was about 33%. Attempts to achieve more retention of the enzyme on the column by chromatography on Sephadex G-200 were unsuccessful.

Method b. From the results described above, it appeared that little could be gained by a multistep fractionation procedure. It was noticed earlier, however, that a large amount of inactive protein was precipitated at values below 30% saturation. Consequently, the fractionation was reduced to two steps with hopes of improving recovery and possibly achieving a higher purification factor. The results of this method are shown in Figure 6. Approximately 90% of the protein was removed by this process. The bulk of the activity was found in the 30-85% fraction. This material was subsequently subjected to chromatography on Sepharose 4B. It was anticipated that this gel, with an exclusion limit of 2×10^6, would cause sufficient retention of the enzyme so that greater purification could be achieved. Hopefully, this step would also eliminate the organic phosphorus, presumably in teichoic acid present as a contaminant, which had been detected at all levels of purification. The results of this experiment are shown in Figure 7. All of the



Assay conditions:
column dimensions 1.2 x 25 cm;
eluted in 0.02 M AC buffer pH 9.0

Figure 5. Chromatography of Autolytic Enzyme on Sephadex G-100.

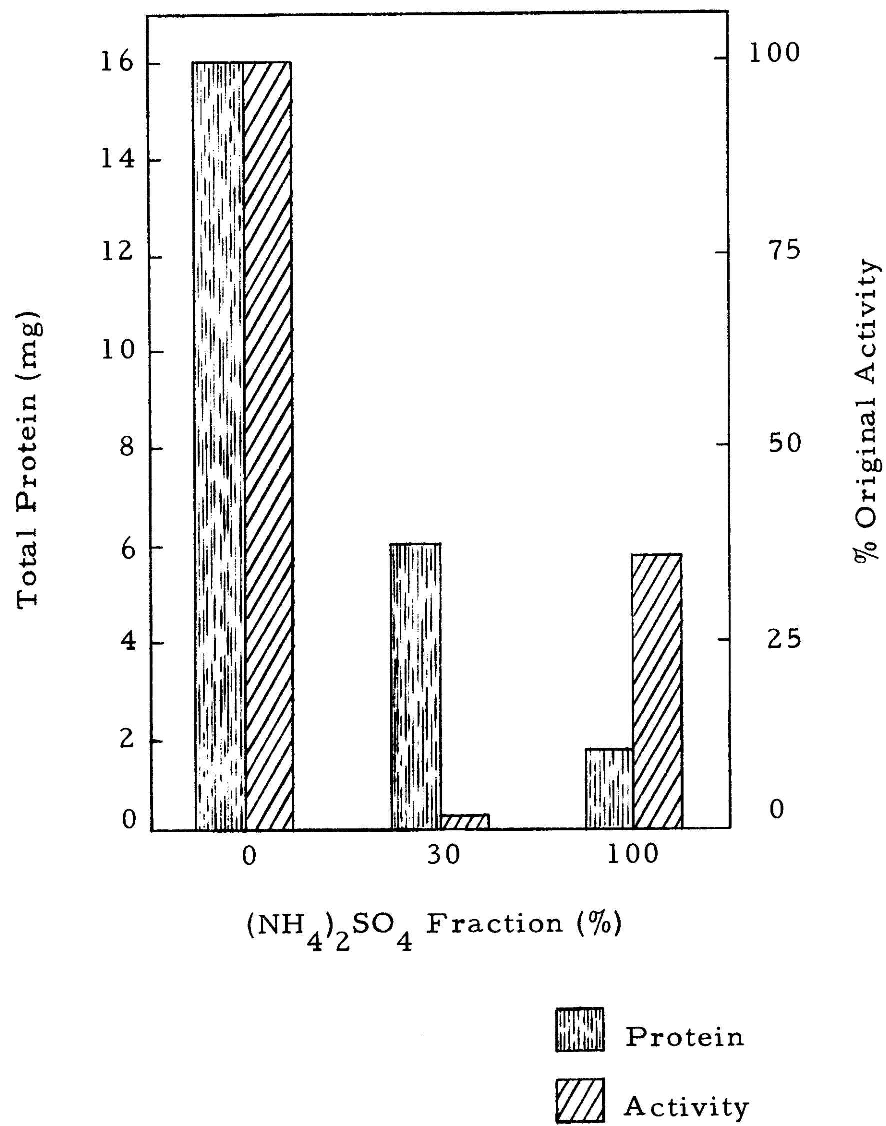
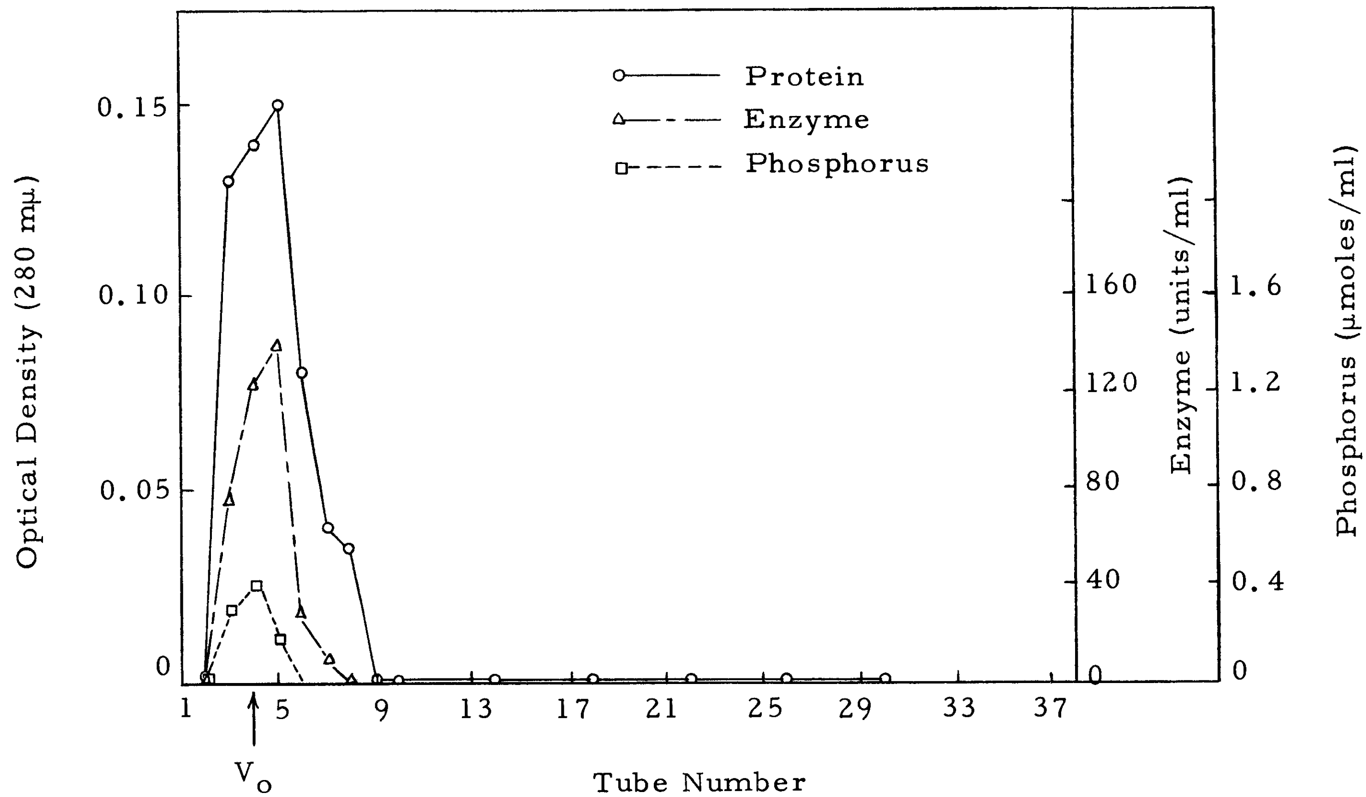


Figure 6. Fractionation of Autolytic Enzyme with Ammonium Sulfate.



Assay conditions:
column dimensions 1.2 x 25 cm;
eluted in 0.01 M tris buffer pH 7.1;
collected 2 ml fractions.

Figure 7. Chromatography of Autolytic Enzyme on Sepharose 4B.

protein and enzyme activity were again found in the fast migrating peak observed previously. No other peaks were found. These results show that chromatography on Sepharose 4B was ineffective in achieving greater purification. In addition, this step failed to reduce the organic phosphorus to insignificant levels; therefore, it is eliminated from the purification scheme which follows.

A summary of this simplified purification scheme is shown in Table I. The purification and recovery obtained by this procedure are not better than that obtained by the previous method; however, the outstanding advantage is that this method is simple and less time consuming.

Ethanol Fractionation and Gel Filtration

The lack of impressive results with purification by ammonium sulfate and gel filtration prompted the survey of other methods. The one which proved to be most successful was a scheme involving fractional precipitation by ethanol and subsequent chromatography on agarose.

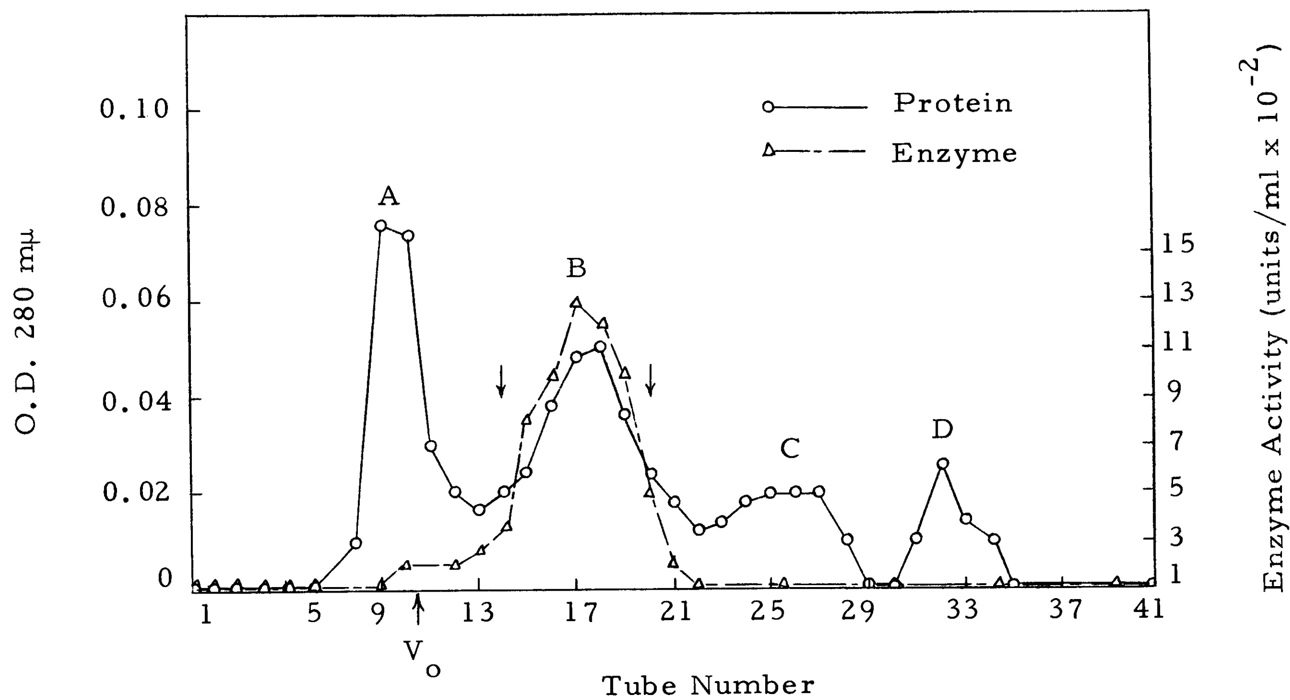
One of the first observations made during the ethanol precipitation was that the procedure was highly dependent on ionic strength. At concentrations below $\simeq$ 0.1 M salt no precipitation occurred. Precipitation at concentrations greater than 0.1 M salt were not attempted.

Table I. Summary of Autolytic Enzyme Purification by Ammonium Sulfate Fractionation.

	Volume (ml)	Activity (units/ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity (units/mg)	Yield (%)	Purification (fold)
Crude autolysate	28	630	17, 640	0.585	1, 077	100	--
After dialysis	27	460	12, 420	0.380	1, 210	71	1.1
Ammonium sulfate fraction, 30-100%	2	3, 050	6, 100	0.87	3, 506	35	3.2

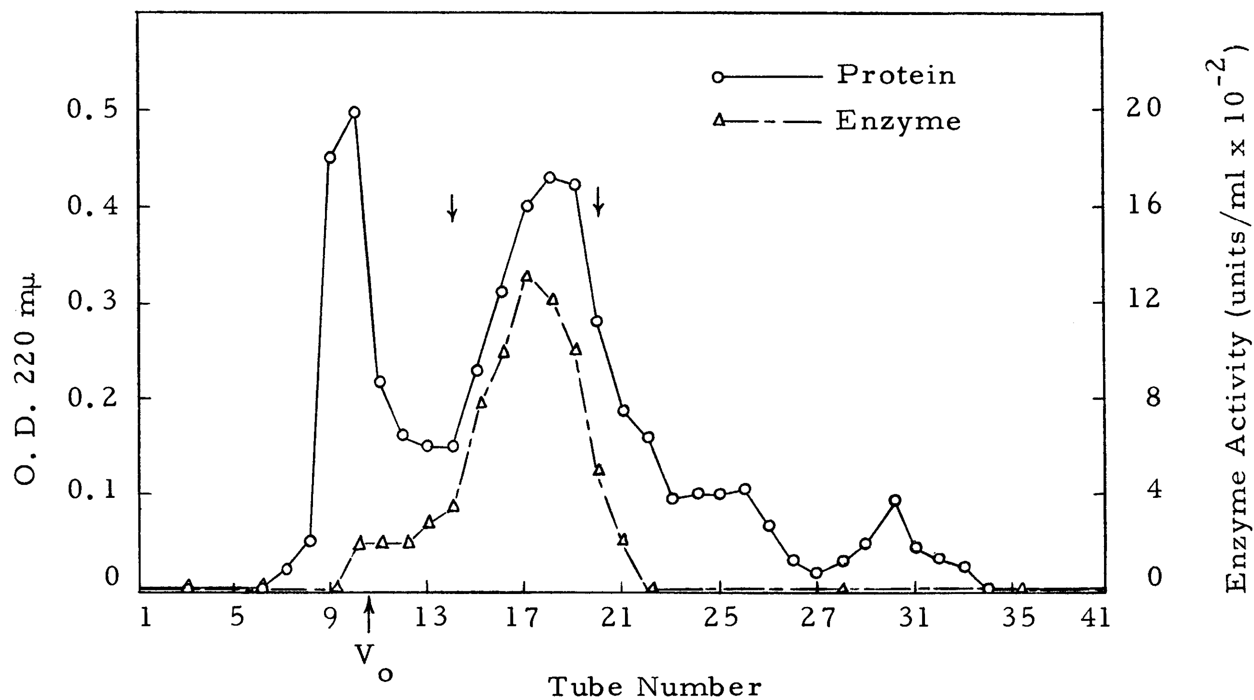
One problem encountered during the purification was the presence of a massive insoluble residue which occurred following the first precipitation and to a lesser extent with each precipitation thereafter. The enzyme was apparently physically trapped by this residue and was cosedimented when centrifuged. This association was easily disrupted in the following manner. The ethanol precipitate was suspended by maceration in a small amount of buffer. More buffer was added and the mixture was centrifuged at 10,000 x g. The precipitate was treated again in this manner and this supernate added to the first. Usually two such treatments were necessary to solubilize all of the enzyme.

Following precipitation with ethanol, attempts were made to increase purification by chromatography on Sepharose 4B. The enzyme was eluted as a shoulder on a large peak which emerged in the void volume. It was discovered later that these components could be separated by using a longer column (1.2 x 50 cm) and a more porous gel (Bio Gel A50m). The results of this experiment are shown in Figure 8. Four distinct peaks were identified: A, $V_e \simeq V_o$; B, $V_e \simeq 1.6\ V_o$; C, $V_e \simeq 2.5\ V_o$; and D, $V_e \simeq 3.0\ V_o$. Only one peak (B) contained significant enzymatic activity. The same elution pattern was observed when the results were plotted at 220 mμ (Figure 9). This confirmation was necessary since the autolytic enzyme has very low absorption at 280 mμ. About 75% of



Assay conditions:
column dimensions 1.2 x 50 cm;
eluted in 0.01 M tris buffer pH 8.0;
collected 2 ml fractions;
arrows refer to samples pooled.

Figure 8. Chromatography of Autolytic Enzyme on Bio Gel A50m.



Assay conditions:
column dimensions 1.2 x 50 cm;
eluted in 0.01 M tris buffer pH 8.0;
collected 2 ml fractions;
arrows refer to samples pooled

Figure 9. Chromatography of Autolytic Enzyme on Bio Gel A50m.

the activity added to the column could be recovered although the pooled fractions represented only 62% of the added units.

Of the purification methods surveyed in this study, a combination of ethanol fractionation and gel filtration proved to be the method of choice. A summary of the results obtained by this procedure are shown in Table II. The overall yield was 31.3%, while the purification was 14.3 fold.

The Phosphorus Problem

The amount of organic phosphorus was determined on crude samples and at each stage of the purification. The results are shown in Table III.

Table III. Organic Phosphorus Content of Autolytic Enzyme at Various Stages of Purification.

Fraction	Total P_o (μmoles)	μmoles/mg Protein
crude	5.7	0.325
II	4.8	1.07
III	0.17	0.30
V	0.21	0.57

Although greater than 96% of the phosphorus is removed by the purification steps it is still present in significant amounts at

Table II. Summary of Autolytic Enzyme Purification by Ethanol Fractionation and Chromatography on Bio Gel A50m.

Procedure	Volume (ml)	Activity (units/ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity (units/mg)	Yield (%)	Purification (fold)
Crude autolysate	40	360	14,400	0.43	837	100	--
75% Ethanol (II)	15	860	12,900	0.37	2356	89.6	2.8
33% Ethanol (III)	2	3,600	7,200	0.29	12,203	50.0	14.6
Bio Gel A50m; pooled (V)	15	300*	4,500	0.025*	12,000	31.3	14.3

*These are average values since a pool has been used.

the final stage. Attempts to remove this organic phosphorus by fractionation with ammonium sulfate, ethanol, acetone, or gel filtration, or by electrophoresis were unsuccessful. This phosphorus, representing 3% by weight of fraction V, was believed to be bound to the enzyme since repeated ethanol precipitations did not change the phosphorus/protein ratio.

It is well known that teichoic acid is present in large amounts in cell wall extracts. Additionally, cell walls of the organism used in this study contain 1.7 μmoles P_o/mg protein (Young, 1966c). If all the P_o in fraction V were present in teichoic acid, then the partially pure enzyme would contain 25% teichoic acid by weight.

Miscellaneous

Several attempts were made to effect further purification of ethanol precipitates by small scale chromatography on ECTEOLA-cellulose. Samples were eluted with concentrations of NaCl (in tris, pH 8.0) extending from 0-4 M. A fraction was eluted which represented 20% of the starting optical density at 280 mμ. This fraction was devoid of enzyme activity and contained relatively insignificant amounts of phorphorus; also, samples concentrated 10 fold failed to give a positive reaction with Lowry protein reagents (Lowry, et al., 1951). It was concluded that the enzyme and phosphorus remained on the column, suggesting that they behave as a single entity during

ion-exchange chromatography.

Another limited attempt to purify the enzyme was made using density gradient-isoelectric point electrophoresis (Svensson, 1961, 1962). The active enzyme recovered after equilibration still contained a high level of phosphorus--a factor which supported the findings described above. This method offered great promise as a purification procedure but the equipment was not available in this laboratory for extended use.

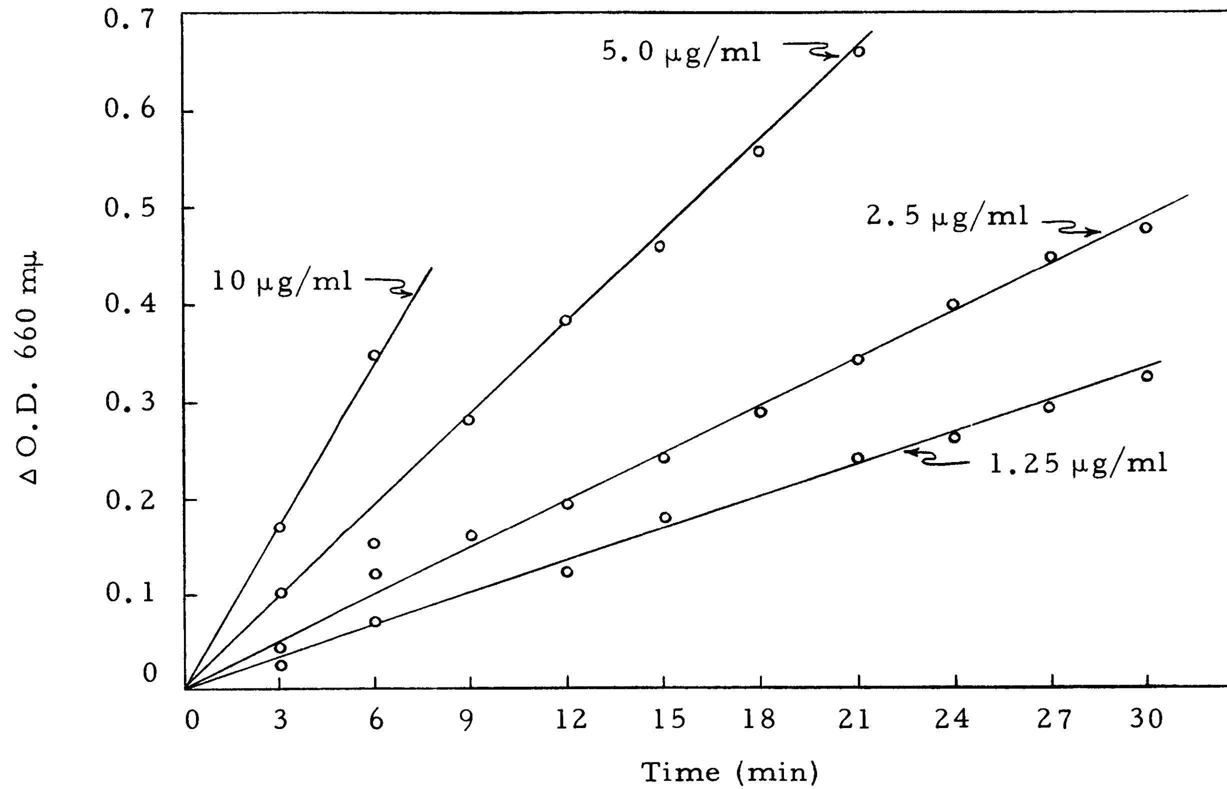
Enzyme Characterization

Effect of Enzyme Concentration

Under defined concentrations of substrate, the lysis of heat-inactivated cell walls was found to be a linear function of enzyme concentration (Figure 10). This linearity persisted until 50% of the available substrate was decomposed.

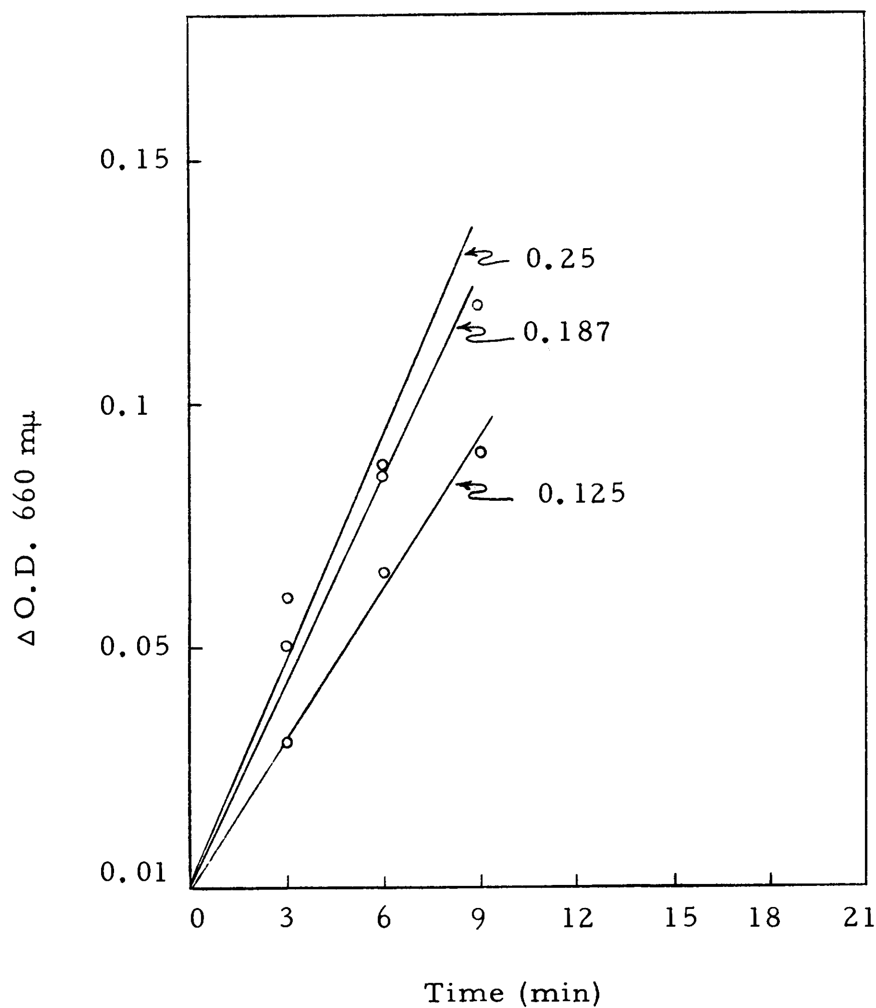
Effect of Substrate Concentration

In general, the lysis of HI-cell walls was a linear function of substrate concentration. However, these results tended to fluctuate greatly between experiments. A representation of the best data obtained is shown in Figures 11 and 12. In view of these irregularities, calculation of a Michaelis constant (Km) from these data was not possible.



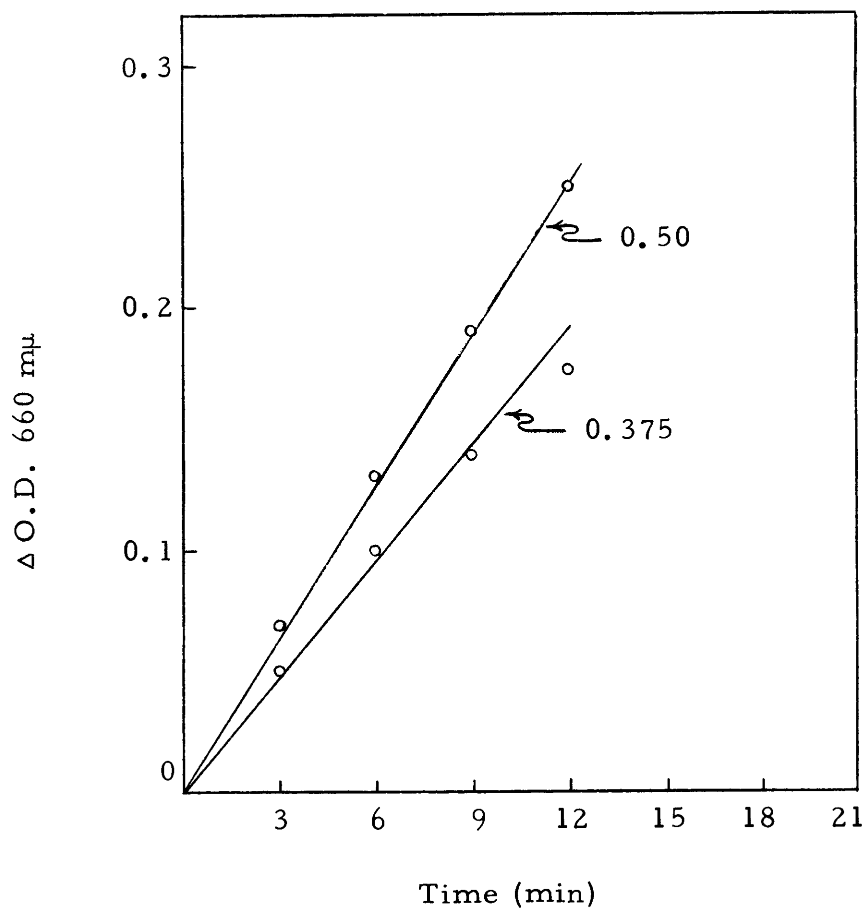
Assay conditions:
0.5 mg/ml HI cell walls in 0.01 M tris buffer pH 9.0; 37°C, total volume 2 ml; numbers refer to enzyme concentration.

Figure 10. Effect of Enzyme Concentration on Lysis of Heat-Inactivated Cell-Walls.



Assay conditions:
5.6 μg/ml enzyme; 0.01 M tris buffer
pH 9.0; 37°C; total volume 2.5 ml;
numbers refer to substrate concentration
(mg/ml).

Figure 11. Effect of Substrate Concentration on Reaction Velocity.



Assay conditions:
5.6 μg/ml enzyme; 0.01 M tris buffer pH 9.0; 37°C; total volume 2.5 ml. numbers refer to substrate concentration (mg/ml).

Figure 12. Effect of Substrate Concentration on Reaction Velocity.

Effect of Temperature

The rate of lysis of HI-cell walls was studied from 30°C to 62°C. The enzyme displayed a broad optimum with maximum activity at 54°C (Figure 13).

Effect of pH

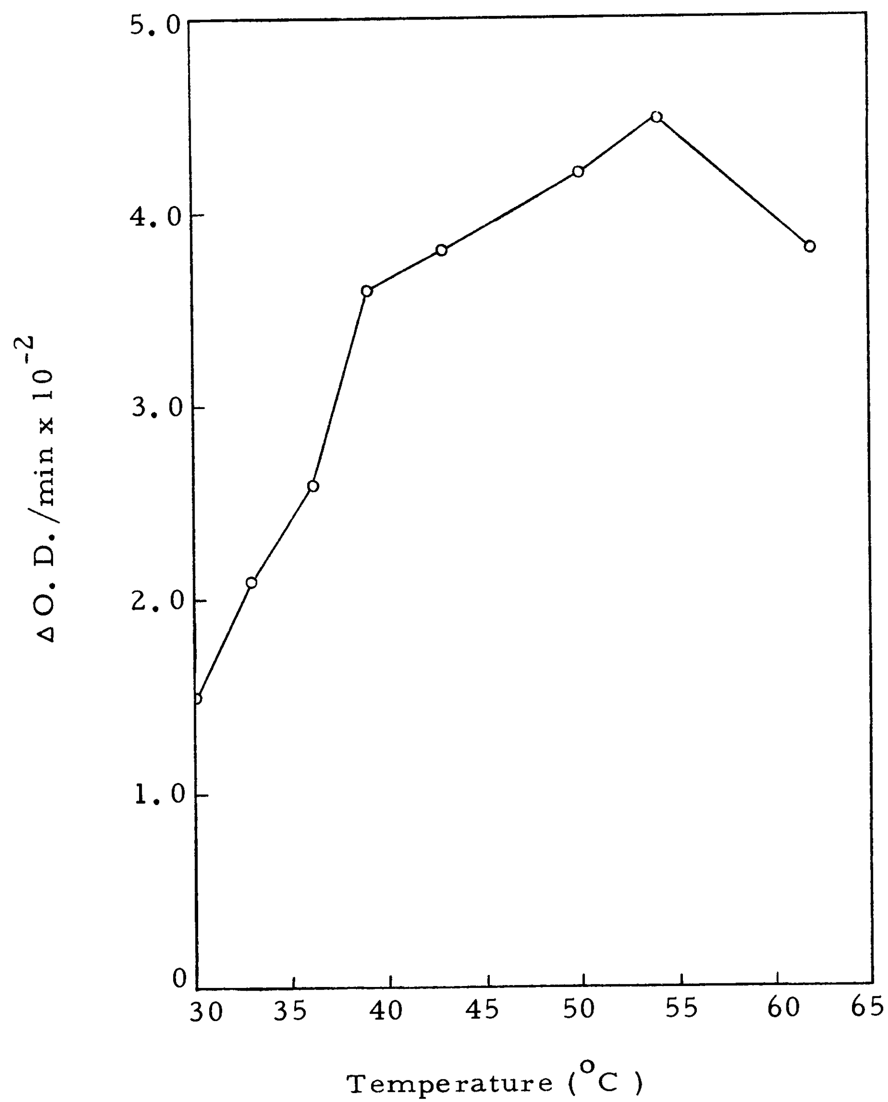
A study of the reaction rate as a function of pH revealed a broad pH optimum. Maximum activity occurred in the range pH 9-9.5 (Figure 14).

Effect of Divalent Cations

Lysis of HI-cell walls by autolytic enzyme appeared to require divalent cations. The enzyme was markedly activated by Ba^{++}, Mg^{++}, Ca^{++}, and Mn^{++}, but was inhibited by Fe^{++} and Cu^{++} (Table IV).

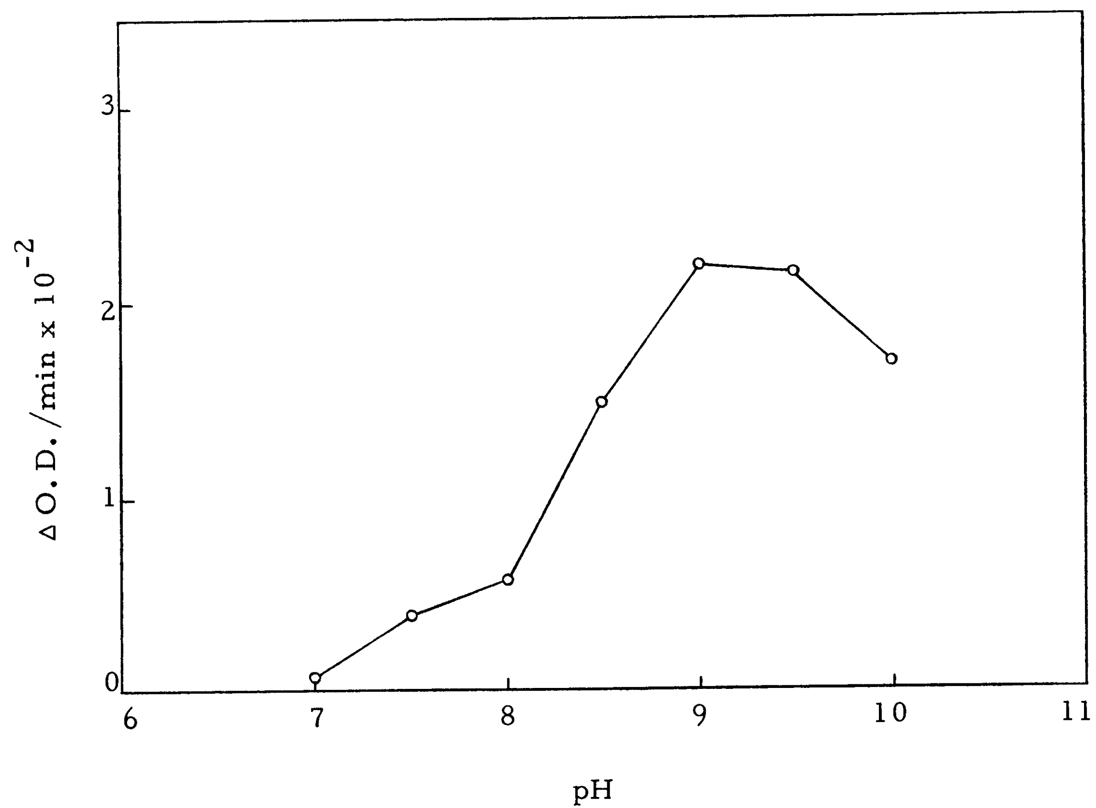
Enzyme Stability

Crude enzyme preparations were stable for several months at -20°C. Both crude and partially pure enzymes could undergo numerous freezings and thawings without noticeable effect. Preparations were also stable for several hours at room temperature. On the other hand, lyophilization caused a 50% reduction in activity.



Assay conditions:
2.5 μg/ml enzyme protein;
0.5 mg/ml HI cell walls;
0.01 M tris buffer pH 9.0;
total volume 2.0 ml.

Figure 13. Effect of Temperature on Lysis of Heat-Inactivated Cell Walls.



Assay conditions:
2.5 μg/ml enzyme protein;
0.01 M tris buffer;
0.5 mg/ml HI cell walls;
37°C;
total volume 2.0 ml.

Figure 14. Effect of pH on Lysis of Heat-Inactivated Cell Walls.

Table IV. Effect of Divalent Cations on Reaction Velocity.

Additions	O. D./min x 10^3
None	1.3
$MgSO_4$	12.6
$MnSO_4$	8.7
$CaCl_2$	12.6
$BaCl_2$	14.0
$MgSO_4$ + $FeSO_4$	0.0
$MgSO_4$ + $CuSO_4$	8.7

Assay conditions:

HI-cell walls (0.5 mg/ml) suspended in 0.01 M tris buffer,

pH 9.0;

2.4 μg/ml enzyme, specific ion added at 0.001 M;

final volume 2.5 ml;

reaction was stopped after 15 min at 37°C.

Apparently, there was no specific buffer requirement. Activity was equally effective in tris, phosphate, or ammonium carbonate buffers.

Proteolytic Activity

Assays for proteolytic activity were carried out on samples of crude and partially purified autolytic enzyme. No evidence of proteolytic activity was found. These data together with the earlier findings that crude autolysates were devoid of commonly occurring enzymes (Young, 1966b) argue strongly for the presence of a single lytic enzyme in this preparation.

DISCUSSION

The early studies in this investigation were devoted almost completely to a survey of methods that would separate the enzyme from teichoic acid. A significant development occurred when accumulated evidence suggested that these components were associated by other than ionic bonding. The properties disclosed during the purification provided a basis for tentatively characterizing the enzyme as a glycoprotein. The justification and the significance of this hypothesis will form the major facets of the discussion. Finally, additional experiments will be suggested which are essential for evaluating these problems.

Purification

Ammonium Sulfate Fractionation

The presence of organic phosphate was first detected during salt fractionation studies. It was tacitly assumed that this material, being an integral component of teichoic acid, was a contaminant, and thus was not relevant to the enzyme. The possible presence of teichoic acid might provide an explanation for the anomalous behavior of the enzyme during ammonium sulfate fractionation. The effect could be similar to that pointed out by Heppel (1955) that large amounts of polyanions impede salt fractionation of enzymes. For

example, a series of fractions may be obtained which differ very little in enzyme specific activity. Probably, in these mixtures the prominent, widespread intermolecular interactions prevent a given enzyme from being salted out discretely. With the crude autolysate, a wide range of ammonium sulfate concentrations (25-100%) salted out the enzyme with only a modest yield and low purity. Later, after the ethanol procedure was devised, a step which removed loosely associated polyanion, it might have been possible to precipitate the enzyme in a much narrower salt range.

Association of teichoic acid with other cell wall components as suggested from these data is not unprecedented. Other workers (Tipper, *et al.*, 1964; Ghuysen, *et al.*, 1965) have studied a glycopeptide-teichoic acid complex from *Staphylococcus aureus* which could not be dissociated by several physical methods such as chromatography on ECTEOLA-cellulose, electrophoresis or ultracentrifugation. It was demonstrated further that these components, as predicted, were linked covalently (Tipper, *et al.*, 1964). It is possible that a similar relationship exists between components of the presumed glycoprotein isolated in these studies. There is also some evidence that the "autolysin" of *Streptococcus faecalis* may be a glycoprotein (Shockman, *et al.*, 1967).

Gel Filtration

The glycoprotein-like character of the enzyme was illuminated by its aberrant behavior during gel filtration studies. The enzyme elution data in Figure 7 suggested that there were two forms of the same enzyme, differing in molecular weight or size. One explanation for such a pattern is the physical dissociation of a multiple form of the enzyme into constituent subunits. On the other hand, this bimodal distribution of activity during chromatography is typical of glycoprotein enzymes (Gibbons, 1966). In addition, the inability of the enzyme to extensively penetrate chromatographic gels of any pore size can also be accounted for on the basis of its glycoprotein nature. Some glycoproteins such as those isolated from epithelial cells have so large an effective molecular size that they are completely excluded from the widest pore size gels available (Gibbons, 1966). The atypical behavior of glycoproteins was also observed by Andrews (1965) 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 may be due to a greater hydration in solution of carbohydrate chains as contrasted to polypeptide chains.

Another factor emphasized during the gel filtration studies

was the apparent lack of aromatic amino acid residues in the enzyme protein. The low level of these residues in cell wall autolysates was specified earlier by Young (1963); therefore, weak absorption by the enzyme at 280 mμ was expected. A similar observation was made with the "autolysin" of S. faecalis (Shockman, et al., 1967). It is well known that many glycoproteins, especially those of epithelial origin, contain almost no aromatic or sulfur amino acids. Sensitive chemical tests for these substances, if negative, are often interpreted as indicating homogeneity (Aminoff, Morgan and Watkins, 1950; Annison and Morgan, 1952).

Final Purification Scheme

The dependence of ethanol precipitation on ionic strength was not surprising. The importance of this factor in such precipitations has been stressed by Pennel (1960).

Initially a one-step precipitation was attempted. The crude autolysate was precipitated directly with 33% ethanol. The physical characteristics of this precipitate made it too difficult to handle and chromatograph. On the other hand, samples prepared in the scheme finally selected were very easy to handle (once the unidentified insoluble residue was removed) and were uniformly reproducible during gel filtration.

The application of ethanol precipitation greatly aided

concentration of the enzyme. Previously, the autolytic enzyme could not be concentrated, with high yield, by such common methods as lyophilization, salt precipitation, evaporation or ultra-filtration. During the latter phases of this research, precipitation with ethanol was used routinely.

The purification obtained by this method appears modest. It should be considered, however, that starting with cell walls rather than whole cells eliminates large amounts of contaminants. Moreover, this method itself results in the removal of 98% of the protein found in the crude autolysate.

It is anticipated that more sensitive assay procedures might show that the recovery is actually higher than the present figures indicate.

One problem which pervaded the purification studies was the availability of starting material. An inspection of Figure 2 and Table II will bear this out. It may be seen that starting with 200 mg of cell walls (obtained from about 10 g of cells) 0.37 mg of partially purified enzyme was recovered. It is apparent that further purification and characterization requires the accumulation of several milligrams of the enzyme for satisfactory physical studies. Also in this regard, a more sensitive assay system may provide the answer. One such system would involve the use of ^{14}C-labeled cell walls. Minute quantities of enzyme could be determined by

measuring the amount of ^{14}C released into the supernatant liquid. There are two outstanding advantages of such a system: (1) much smaller quantities of enzyme could be detected, and (2) smaller substrate concentrations would be necessary. The latter would minimize settling of the cell walls.

It is emphasized that tentative characterization of the enzyme as a glycoprotein is made with caution. The findings that the enzyme and teichoic acid remained associated through several physical methods and that repeated precipitations and washings of the partially purified enzyme did not alter the phosphorus/protein ratio argue strongly for this hypothesis. On the other hand, it is still possible that this small amount of phosphorus represents fortuitous contamination resulting from deficiencies in the purification procedure. Hence, the precise characterization must await further developments in purification and analysis.

Characterization As Related to Synthesis and Function

The kinetic data for the partially purified enzyme conformed generally to those determined for the crude preparation (Young, 1966a), but differed in some respects from the results obtained for the "autolysin" of *S. faecalis* (Shockman, *et al.*, 1967). The properties of "autolysin" indicated that it is a muramidase while the *B. subtilis* autolytic enzyme is an amidase.

It is evident from these results that additional characterization studies are necessary. These should include experiments in electrophoresis, ultracentrifugation, and chemical analysis of the purified enzyme molecule. The results of this broader investigation should lead to a precise definition of the autolytic enzyme and its physiological role in the cell. This point will be made clear in the discussion which follows.

The activity of the autolytic enzyme increases rapidly during logarithmic growth, levels off and then decreases after maximum stationary growth. It has been shown that this effect is not due to an alteration of the substrate but is a function of the enzyme itself (Young, 1966a). It would be interesting to determine if the differences in rate are due to the quantity of enzyme or to its activity. A chemical analysis of the purified enzyme would be helpful in this regard. If the analysis revealed the presence of compounds associated with the enzyme that were absent in the rest of the cell wall or present in smaller molar ratios, these compounds would indicate the amount of enzyme present at various stages of growth. On the other hand, one could envision a control mechanism whereby the enzyme active sites were masked periodically thereby resulting in lower activity. In this case, an analysis would reveal the presence of foreign constituents in enzyme digests. It is possible that the activity of stationary phase autolytic systems could be restored by

the addition of trypsin or other proteolytic enzymes. Activation by proteolytic enzymes is a regular feature of the _S. faecalis_ autolytic system even during the logarithmic phase (Shockman, _et al._, 1967).

There has been much speculation as to the site of synthesis of the enzyme and its location in the cell. Based on its large size, as suggested by gel filtration, it is unlikely that the enzyme is synthesized at a remote location and then transported to its site of action. It is more likely that synthesis occurs at least close to the site finally occupied. The autolytic enzyme is found associated with cell walls after extensive washing with water. It is not known whether this association occurs by entrapment or by ionic or covalent linkage. The fact that the enzyme is associated even after extensive washing with 2M NaCl suggests that the relationship is not a loose one. Recent studies involving the osmotic release of certain enzymes from bacteria may help substantiate this point (discussed by Heppel, 1967). If the enzyme is loosely situated in the space between the cell wall and cell membrane, then "osmotic shock" would release it to the supernatant liquid. On the contrary, a strong relationship would enable the enzyme to remain associated with the cell wall fraction throughout this treatment. The observations made during this investigation support the viewpoint that the relationship between enzyme and cell walls is a strong one brought about by the covalent linkage of the enzyme with teichoic acid. This compound has been

found associated with both cell walls and cell membranes (reviewed by Archibald and Baddiley, 1966). One could assume that the enzyme is synthesized on the membranes and held in place by a linkage with teichoic acid from both wall and membrane. If it were assumed that the bonding between membrane teichoic acid and enzyme were weaker (such as hydrophobic or hydrogen bonding) than that between enzyme and cell wall teichoic acid, then it could be that the weaker forces are severed during mechanical disruption procedures thereby releasing the enzyme to the cell wall fraction.

The whole concept of structure and function could best be elucidated if it were possible to isolate mutants deficient in autolytic enzyme. Restoration of the deficient character could probably be made by transformation or transduction. An analysis of the genetic transfer process could provide useful information concerning mode of synthesis, location of synthesis, control mechanisms involved, and physiological function. One approach to such a problem would be to study the chemical and physical properties of enzyme from asporogenic and phage resistant mutants. These strains are reported to have low levels of autolytic enzyme (Young and Spizizen, 1963; Young, 1966c). It would be interesting to determine if these genetic alterations in the cell have brought about changes in the enzyme structure resulting in lower activity.

Another point to mention here concerns the role of the

autolytic enzyme. It was postulated (as discussed earlier) that the enzyme was necessary for cell wall synthesis in that it provided breaks in the cell wall for the insertion of new material by a trans-peptidase. If future studies reveal that cell wall synthesis occurs normally in mutant strains, then a new physiological role must be implicated for the enzyme.

SUMMARY

Autolysis of cell walls from _B. subtilus_ 168 I^- C^+ yielded an enzyme capable of lysing heat-inactivated cell walls of the same strain. This enzyme could be partially purified by two methods.

Ammonium sulfate fractionation resulted in a purification of 3.2 fold and a yield of 35%. Attempts to further purify this fraction by gel filtration were unsuccessful.

Fractional precipitation of crude autolysates with ethanol was a very effective purification procedure, and in addition provided a means for enzyme concentration. Chromatography of ethanol fractions on Bio Gel A50m yielded four peaks; only one contained significant enzyme activity. This ethanol fractionation-gel filtration procedure resulted in a purification of 14 fold and a recovery of 31%.

The partially purified enzyme was optimally active between 30°C and 62°C with maximum activity occurring at 54°C. A broad pH optimum (7-10) was observed. Maximum activity was found at pH 9-9.5.

Divalent cations were required for autolytic enzyme activity. The enzyme was activated by Ba^{++}, Ca^{++}, Mg^{++}, and Mn^{++}, but was inhibited by Fe^{++} and Cu^{++}.

Solutions of enzyme were stable for several hours at room

temperature and could undergo unlimited freezing and thawing. Lyophilization resulted in a 50% reduction in activity.

No evidence of proteolytic activity was demonstrated.

The enzyme and teichoic acid were inseparable by several physical methods such as electrophoresis, ion-exchange chromatography and gel filtration. Additionally, the enzyme behaved abnormally during ammonium sulfate precipitation and gel filtration. These findings suggested that the cell wall autolytic enzyme of *B. subtilis* 168 is an acidic glycoprotein.

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