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TECHNICAL MANUSCRIPT 374

FLUORESCEIN-LABELED
 β -GLUCOSIDASE AS A BACTERIAL STAIN

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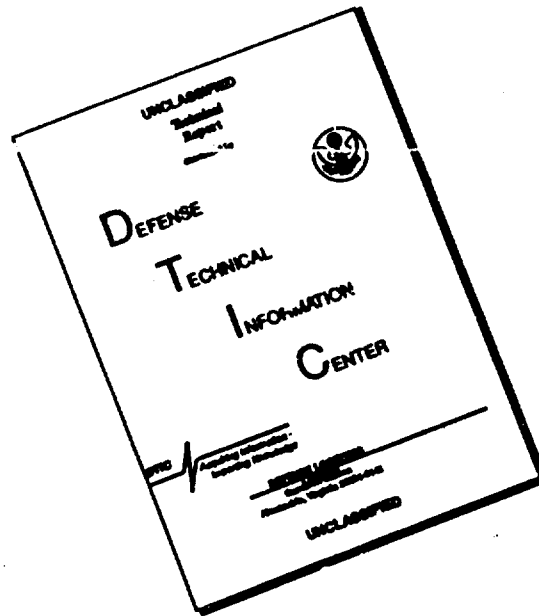
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FLUORESCHEIN-LABELED β -GLUCOSIDASE
AS A BACTERIAL STAIN

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ABSTRACT

Beta-glucosidase labeled with fluorescein isothiocyanate was used as a simple staining reagent with selected gram-positive and gram-negative organisms. Staining appeared to be dependent on the presence of accessible glycosidic-type linkages as well as the partial inhibition of enzyme activity by high enzyme concentrations. Labeled β -glucosidase separated into three major fractions upon Sephadex column passage to remove excess dye. These fractions differed in protein content and enzyme activity, but produced similar staining reactions. Extensive wall damage or lysis did not occur when stained cells were suspended in washing and mounting solutions. The apparent specificity of labeled enzyme for wall substance was determined by one-step and Alcian Blue blocking reactions as well as by formation of cell wall lesions on prolonged staining with the enzyme. Spores and a majority of the gram-negative organisms tested were effectively stained after prior exposure to thioglycollic acid at 70 C. The present studies suggest that disulfide bonds may play an important role in maintaining the cellular integrity of some gram-negative species. Fluorescein-labeled β -glucosidase appears to be a potentially useful staining reagent for demonstrating in situ glycosidic-type linkages in the bacterial cell wall. A potential for staining tissues and cell lines may also exist.

I. INTRODUCTION

The use of fluorescein-labeled enzymes as biological staining reagents has received increasing attention during recent years. A limited number of enzymes has been labeled and used primarily for staining several bacterial species. Other applications have included studies on the interaction of rhodamine-labeled lipase with normal and tumor kidney cells,¹ the effect of fluorescent labels on enzyme activity,^{2,3} and the use of rhodamine-labeled papain as a counterstain in frozen tissue sections.⁴ Benjaminson et al.⁵ have recently stained hyphae of Aspergillus niger and chitin with ferritin and fluorescein isothiocyanate (FITC) - labeled chitinase, respectively. Detailed studies on bacterial staining have been mainly confined to the use of fluorescein- or rhodamine-labeled lysozyme. Specific staining reactions with this enzyme were dependent on the presence of accessible glycosidic-type linkages in the bacterial wall.

Leonard and Thorne⁶ used egg white lysozyme labeled with FITC to stain encapsulated cells of Bacillus anthracis and Bacillus subtilis. Gould, Georgala, and Hitchins⁷ demonstrated the presence of lysozyme-sensitive glycopeptide in bacterial cell walls with rhodamine-labeled enzyme. The successful use of lysozyme was attributed by the latter investigators to autoinhibition of enzyme activity by high concentrations of the enzyme. Furthermore, they postulated that other enzymes could be employed in a similar manner provided that enzyme affinity for substrate was not destroyed by the labeling procedure. A major disadvantage of labeled lysozyme appeared to be the possible lysis of stained cells when they were diluted with wash solutions of water or buffered saline. Consequently, washing and mounting had to be conducted with high concentrations of unlabeled lysozyme. Observations on the inhibition of enzyme activity by high concentrations of enzyme have also been reported for pepsin⁸ and unlabeled lysozyme.⁹ Metzgar and Kopec¹⁰ have shown that FITC-labeled lysozyme could be employed under certain experimental conditions to stain a number of gram-positive and gram-negative organisms. In general, gram-negative species could not be stained with labeled lysozyme unless they were previously treated with reagents such as acetone or EDTA (ethylenediaminetetraacetate). More recently, Eagon and Carson¹¹ stained Pseudomonas aeruginosa with rhodamine-labeled lysozyme, but the conjugate was easily removed from the cell surface by washing.

Preliminary experiments with various enzymes revealed that β -glucosidase could be readily labeled with FITC and used as a simple staining reagent. The use of β -glucosidase in the analysis of products from enzymatic digests of bacterial walls has been described by Salcon and Ghuyssen.¹² Mergenhagen and Martin¹³ have reported on the activity of β -glucosidase in dissociating C¹⁴-labeled endotoxin with O antigen specificity from Escherichia coli. A similar activity with β -galactosidase could not be demonstrated by these investigators.

This report describes the preparation and use of FITC-labeled β -glucosidase as a staining reagent for demonstrating in situ β -glycosidic-type linkages in the bacterial cell wall. Preliminary studies on staining of chick fibroblasts are also reported.

II. MATERIALS AND METHODS

A. ORGANISMS

The following test organisms were used in staining experiments: Bacillus anthracis CD-3S; Bacillus megaterium FD-1800; Bacillus subtilis var. niger 1221a (spores); Brucella abortus CD-476; Escherichia coli 3710-211; Listeria monocytogenes ATCC-7644; Pasteurella pestis (KJMA); Pasteurella tularensis SCHU S5 and 38A; Salmonella typhosa ATCC-9992-V; Sarcina lutea ATCC-553; Staphylococcus aureus ATCC-9801; and Vibrio comma 8365-249. All organisms except L. monocytogenes and P. tularensis were grown on heart infusion agar slants (Difco) for 18 hours at 37 C; L. monocytogenes was grown on tryptose agar (Difco) and P. tularensis on glucose cysteine blood agar. Live, washed spore suspensions of B. subtilis var. niger were initially obtained by growth on a sporulating medium.¹⁴

B. FITC LABELING OF β -GLUCOSIDASE

All experiments were conducted with the same lot of β -glucosidase.* Labeling and removal of excess dye from the conjugate was conducted at 4 C. A stock enzyme solution was prepared by dissolving 278 mg of β -glucosidase in 10 ml of 0.85% physiological saline. This preparation contained 27.6 mg of protein per ml as determined by the Gornall biuret method¹⁵ and was acidic (pH 5.0). A solution of crystalline FITC** was prepared in 0.5 M carbonate-bicarbonate buffer of pH 9.6 (CBB) and contained 10 mg of FITC per ml. One ml of FITC solution was combined with 9 ml of stock enzyme solution (final pH, 8.7). This represented an initial concentration of approximately 0.05 mg FITC per mg of protein. The dye-enzyme mixture was slowly stirred for 30 minutes and then cleared of excess dye in a coarse, G-25 Sephadex column (28 by 2.5 cm) with an adjusted flow rate of 1.5 ml per minute. The column was initially washed with 150 ml of 0.01 M acetate buffer of pH 5.0 and equilibrated with the same buffer. Acetate buffer was also used for column elution. The passage of FITC-labeled β -glucosidase over a Sephadex column resulted in the separation of three labeled fractions (I, middle, and II). The volume of each eluted fraction was adjusted to 15 ml with acetate buffer. Labeled preparations were stored in the dark at 4 C for 1 week.

* Almond, Lot 8736, Nutritional Biochemicals Corp., Cleveland, Ohio.

** Baltimore Biological Laboratory, Inc., 2201 Aisquith St., Baltimore 18, Md.

C. DETERMINATION OF FITC-PROTEIN RATIOS

An FITC standard reference curve was prepared with various concentrations of FITC in 0.1 N NaOH. FITC absorption was measured at 490 μ in the Beckman DU spectrophotometer. A protein reference curve was prepared with a standard protein solution (Versatol)* and biuret reagent.¹⁵ Protein absorption was measured at 560 μ . Ratios of fluorescein to protein were determined from these curves.

D. ASSAY OF ENZYME ACTIVITY

Unlabeled whole enzyme as well as labeled fractions of β -glucosidase were assayed for enzyme activity by the method of Duerksen and Halvorson.¹⁸ Activity was measured by the continuous release of p-nitrophenol from p-nitrophenyl- β -D-glucopyranoside (NPG).** A unit of enzyme was defined as the amount of enzyme required to produce an optical density change of 0.001 (400 μ) per minute in 6.0 ml of reaction mixture. Enzyme and substrate controls were employed for each assay. Enzyme units per ml were calculated on the basis of optical density changes after a 15-minute reaction period. All readings were corrected for the proper dilution factor of each labeled fraction.

E. GENERAL STAINING PROCEDURE WITH LABELED β -GLUCOSIDASE

Initially, smear preparations were used with all test organisms for determining general staining reactions. Organisms that failed to stain or showed poor staining were then treated by special methods that are described in other paragraphs of this section. Air-dried and gently heat-fixed smears on glass slides were prepared from distilled water suspensions adjusted to contain approximately 10^8 cells per ml. Smears of chick fibroblasts were air-dried, fixed in 10% formalin for 10 minutes, and rinsed in several changes of 0.01 M phosphate-buffered saline of pH 7.2 (PBS). All labeled enzyme solutions were filtered through membrane*** filters (0.45 μ) prior to staining. Organisms and chick fibroblasts were stained with 0.1 ml of individual fractions I and II as well as with a preparation containing a combination of three fractions. Staining with the middle fraction was subsequently omitted, since its reactions were identical with those of fraction II. Combined staining solutions were mainly confined to mixtures of equal volumes of fractions I and II. Staining was allowed to proceed for 5 minutes in a moist chamber at 37 C. Chick fibroblasts were stained for 15 minutes. Stained bacterial smears were gently rinsed for 10 minutes in CBB. Chick fibroblasts were rinsed in PBS. After rinsing, the back and sides of the slide were wiped dry with blotting tissue and smears were immediately mounted in glycerol adjusted to pH 9.6 with 2% CBB. Smears were examined by fluorescence microscopy as described in a previous publication.¹⁷

* Warner-Lambert Pharmaceutical Co., Morris Plains, N.J.

** CalBiochem, Inc., Los Angeles, Calif.

*** Millipore.

F. SPORE STAINING PROCEDURE

Spores were stained by the method of Gould and Hitchins.¹⁸ According to those investigators, it was necessary to rupture disulfide bonds in the coat fraction of the spore to expose protected lysozyme substrate. Essentially, the method consisted of adding 1 ml of a concentrated and washed spore suspension to 5 ml of 25% thioglycollic acid (TGA) and incubating for 25 minutes in the 70 C water bath. After incubation, the spores were centrifuged for 15 minutes at 1,100 x g, washed in cold distilled water, and resuspended in distilled water to obtain a density of approximately 5×10^8 spores per ml. Equal volumes of spore suspension and labeled fraction were combined and incubated for 25 minutes in the 37 C water bath. Stained spores were centrifuged and washed in distilled water, and smears were prepared and given a final wash in CBB. Controls consisted of spores suspended in distilled water at 70 C for the same period of time.

Smear preparations of spores were heated at 260 C and also treated with 12 N HNO₃ according to the method of Lechtman et al.¹⁹ for rapid staining of spores with crystal violet. Washed spores were also incubated with acetone for 30 minutes at 37 C.

G. PRETREATMENT OF GRAM-NEGATIVE ORGANISMS

It has been shown that pretreatment of gram-negative organisms with reagents such as acetone, alkali, acid, or EDTA resulted in the sensitization of these species to lysozyme action.^{7,10,20-23} An attempt was made to sensitize gram-negative species to β -glucosidase by pretreatment with acetone. Washed cells of B. abortus, E. coli, P. pestis, P. tularensis, S. typhosa, and V. comma were suspended in 5 ml of acetone and gently mixed for 30 minutes at 37 C. The suspensions were centrifuged, washed, and resuspended to obtain a density of approximately 5×10^8 cells per ml in PBS. Five-tenths ml of cell suspension was combined with 2 ml of labeled enzyme fraction and incubated for 5 minutes at 37 C. After incubation, the cells were centrifuged, washed in CBB, and smears were prepared. P. tularensis was also treated with EDTA and tris buffer [tris(hydroxymethyl)aminomethane] according to the method of Repaske.²²

Schaechter and Santomassino²⁴ suggested that disulfide bonds may be involved in maintaining the structural integrity of growing cells of E. coli. It was of interest to explore the possibility of effectively exposing the protected glycopeptide layer in gram-negative organisms by rupture of disulfide bonds. The spore treatment method of Gould and Hitchins¹⁸ utilizing 25% TGA and 70 C was employed for this purpose. The latter procedure as well as a modification was used with B. abortus, P. tularensis, and P. pestis. The modification consisted of incubating cells with TGA for 5 minutes in the 37 C water bath. After incubation, the cells were centrifuged and washed in cold distilled water, and smears were prepared. Preparations were stained with labeled fractions for 5 minutes and then washed in CBB.

H. DETERMINATION OF LABELED β -GLUCOSIDASE STAINING SPECIFICITY

The apparent specificity of the staining reaction between bacterial cell walls and FITC-labeled β -glucosidase was determined by five methods: (i) Alcian Blue blocking of wall substrate in gram-positive organisms; (ii) one-step blocking with labeled and unlabeled enzyme; (iii) electron micrography of *B. megaterium* treated with labeled enzyme as well as a heat-inactivated preparation; (iv) treatment of stained cells with acetone and 1 M NaCl solution; and (v) attempted staining of substances known to be relatively free of β -glycosidic-type linkages.

Alcian Blue is known to form complexes with glycopeptide²⁰ and should partially or completely block staining with labeled β -glucosidase. A 1% stock solution of Alcian Blue 8GX* was prepared in distilled water. One ml of stock solution was combined with 9 ml of distilled water. A loopful of the test organism was added and the entire suspension was mixed on a Vortex mixer for 30 minutes. After mixing, the cells were centrifuged and washed five times in distilled water, and smears were prepared. Controls consisted of distilled water suspensions of cells mixed for 30 minutes without Alcian Blue. The Alcian Blue control was used primarily with gram-positive organisms, because blocking appeared to be ineffective with TGA-treated cells stained in enzyme solution.

The one-step blocking procedure was analogous to that employed for fluorescent antibody.²⁵ The unlabeled component partially or completely blocks the action of the labeled component. Equal volumes of undiluted, unlabeled enzyme solution were combined with labeled fractions and used as staining reagents.

To obtain electron micrographs, a washed cell suspension of *B. megaterium* was adjusted to contain approximately 5×10^8 cells per ml. Two ml of cell suspension were combined with an equal volume of labeled enzyme fraction I. Controls consisted of cell suspensions combined with 2 ml of acetate buffer of pH 5.0 as well as a combination with inactivated labeled fraction I (boiled for 15 minutes). Boiled enzyme was tested for any possible activity in the NPG assay system. Preparations were incubated with gentle agitation for 3 hours in the 37 C water bath. After incubation, the suspensions were centrifuged and washed several times in distilled water. The cells were resuspended in 2 ml of distilled water.

The firmness of FITC binding to enzyme protein was determined by washing stained smears in acetone for 10 minutes. The smears were thoroughly rinsed in distilled water and given a final wash for 10 minutes in CBB. The affinity of FITC-labeled enzyme for the cell wall was tested by washing stained smears in 1 M NaCl solution for 15 minutes. It has been shown that nonspecific reactions of egg white lysozyme as well as rabbit sera with glutamyl polypeptide from *B. anthracis* were markedly reduced in the presence of increasing concentrations of NaCl.⁶ Smears were washed and quickly rinsed in distilled water before a final 10-minute wash in CBB.

* Allied Chemical Corp., 61 Broadway, New York, N.Y.

Purified test substances that were relatively free of β -glycosidic-type linkages were obtained in a powdered or small-particulate form. Concentrated suspensions of each material were made in distilled water droplets on a slide. The suspensions were air-dried and heat-fixed. Smears were stained with labeled fractions and treated in the same manner as were bacterial preparations. Test substances were purified agar (Difco), bovine albumin fraction V (Armour), DNA (CalBiochem), human gamma globulin (Lederle), and inulin (NBC).

III. RESULTS

The protein content, enzyme activity, and concentration of FITC label for each β -glucosidase fraction are presented in Table 1. The highest protein content and associated enzyme activity was found in fraction I. The activities of unlabeled enzyme, labeled fractions, and combined fractions, as measured in the NPG assay system, are presented in Figure 1. Initial staining reactions and blocking controls of gram-positive and gram-negative organisms as well as of chick fibroblasts are shown in Table 2. In general, gram-positive organisms were brightly stained with the exception of B. subtilis var. niger spores. All gram-negative species failed to stain with labeled fractions. Staining reactions and blocking controls of B. anthracis are illustrated in Figure 2. Stained chick fibroblasts are shown in Figure 3. L. monocytogenes did not stain with fraction II, but showed bright staining with fraction I. Exposure of spores to 12 N HNO₃ and 260 C failed to produce staining with β -glucosidase.

The staining reactions of spores and gram-negative organisms after acetone and TGA treatment are presented in Table 3. Acetone failed to sensitize spores to staining with β -glucosidase, but exposure to TGA at 70 C produced bright staining reactions. However, Alcian Blue controls were rendered ineffective with TGA-treated spores. The majority of acetone-treated gram-negative organisms were poorly stained with the exception of E. coli and S. typhosa. Marginal staining of gram-negative species precluded the use of blocking controls. All gram-negative organisms, with the exception of S. typhosa, were brightly stained after TGA treatment at 70 C. Heating alone at 70 C was effective in sensitizing P. tularensis and P. pestis to staining with fraction I, but not with fraction II. S. typhosa was adversely affected by TGA, but showed bright staining with heat alone (Table 3). The gram-positive control (S. lutea) exhibited the same staining reactions with or without prior TGA treatment (Tables 2 and 3). However, exposure to TGA followed by staining with fraction I resulted in marked cell enlargement of S. lutea as well as of other gram-negative organisms.

TABLE 1. FITC-PROTEIN RATIOS AND ENZYME ACTIVITY OF LABELED β -GLUCOSIDASE FRACTIONS

Enzyme Preparation ^a /	FITC, μ g per ml	Protein, mg per ml	μ g FITC per mg Protein	Enzyme Activity, units per ml
Fraction I	53.80	7.58	7.09	16,650
Middle Fraction	14.20	1.24	11.45	2,330
Fraction II	22.20	1.18	18.81	500
Whole (unlabeled) ^b /	-	22.6	-	39,300

a. Each fraction adjusted to 15 ml with acetate buffer after Sephadex column passage; unlabeled stock enzyme solution initially prepared in a final volume of 10 ml.

b. FITC labeling and Sephadex column passage produced an approximate protein loss of 34% with a 26% reduction in enzyme activity.

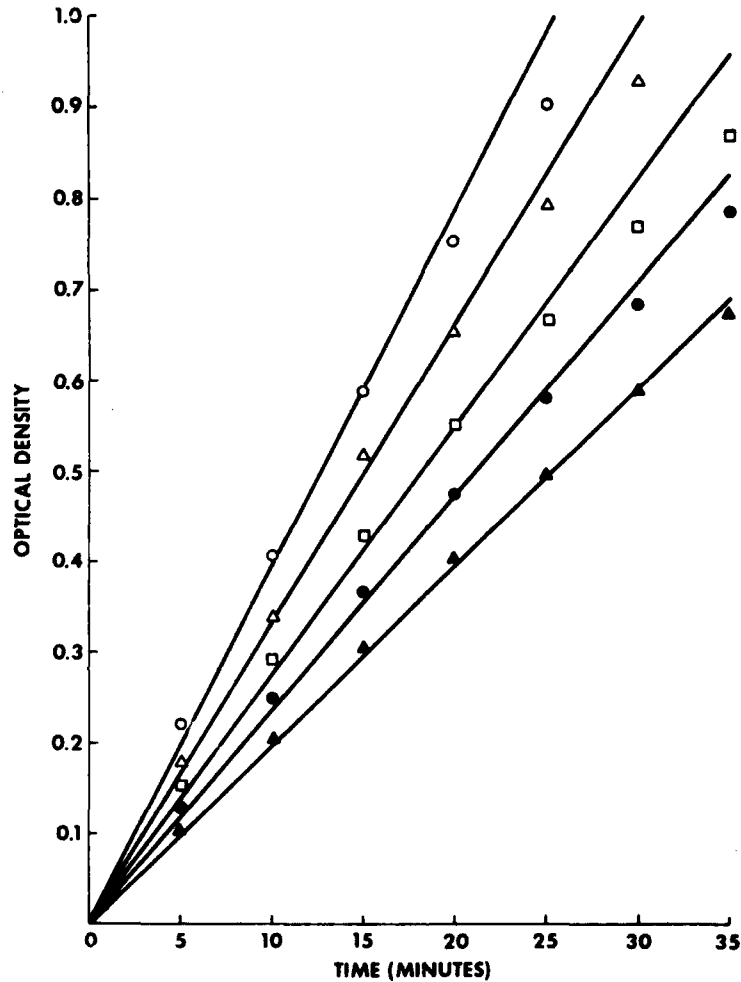


Figure 1. Enzyme Activity of FITC-Labeled Fractions of β -Glucosidase Measured at 400 $m\mu$. The reaction mixture contained in 6.0 ml: 0.2 ml of properly diluted enzyme solution (diluted in 0.85% physiological saline); 5.8 ml pre-incubated (10 min, 30 C) reaction mixture containing 5.0 ml of 0.067 M potassium phosphate buffer, pH 6.8; 0.6 ml of 10^{-2} M NPG; and 0.2 ml of reduced glutathione. Symbols: ○, unlabeled whole enzyme (1:200); Δ , labeled fraction I (1:100); \square , a combination of equal volumes of fractions I, middle, and II (diluted 1:50); ●, labeled middle fraction (1:20); and \blacktriangle , labeled fraction II (1:5). Enzyme and substrate controls contained 0.2 ml and 0.6 ml of physiological saline respectively, and showed no measurable activity.

TABLE 2. INITIAL FLUORESCENT STAINING REACTIONS
WITH LABELED FRACTIONS OF β -GLUCOSIDASE

Organism	Staining Intensity of Fractions ^{a/}								
	Experimental			One-Step Block			Alcian Blue		
	I	II	C	I	II	C	I	II	C
<u>Gram-positive</u>									
<u>B. anthracis</u>	4+	3+	4+	0	0	1+	1+	0	0
<u>B. megaterium</u>	4+	4+	4+	1+	0	1+	0	0	0
<u>B. subtilis</u> var. <u>niger</u> spores	0	0	0	-	-	-	-	-	-
<u>L. monocytogenes</u>	4+	0	4+	1+	0	1+	0	0	0
<u>S. aureus</u>	3+	3+	3+	0	0	0	0	0	0
<u>S. lutea</u>	4+	4+	4+	1+	0	0	0	0	0
<u>Gram-negative</u> ^{b/}	0	0	0	-	-	-	-	-	-
<u>Chick Fibroblasts</u> ^{c/}	4+	1+	3+	1+	1+	1+	0	0	0

- a. Symbols: C, combined fractions I and II; 0, no staining; estimated brightness based on minimal to maximal fluorescent intensity (1+ to 4+); -, no one-step or Alcian Blue blocking controls.
- b. No staining with the following test organisms: B. abortus, E. coli, P. pestis, P. tularensis, S. typhosa, and V. comma; treatment of P. tularensis with EDTA did not produce staining.
- c. Alcian Blue - treated cells showed a tendency to form fragments.

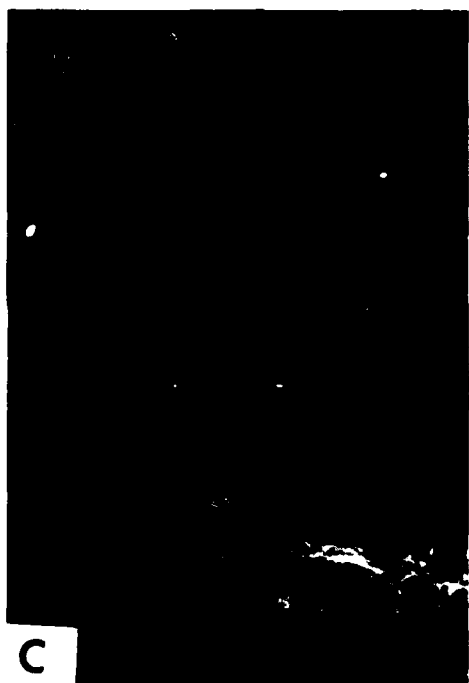


Figure 2. Staining Reactions of B. anthracis. (A) Cells stained with FITC-labeled β -glucosidase (combined fractions). (B) Blocking reaction with Alcian Blue. (C) Partial one-step blocking with a combined preparation of unlabeled and labeled enzyme.

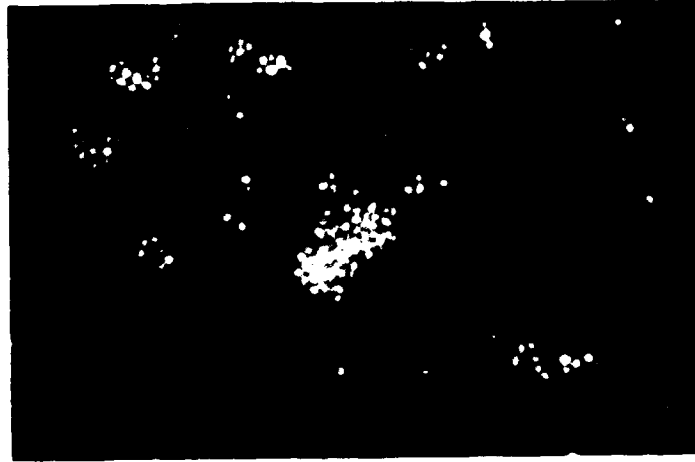


Figure 3. Chick Fibroblasts Stained with FITC-Labeled β -Glucosidase (Combined Fractions).

TABLE 3. STAINING REACTIONS OF SPORES AND GRAM-NEGATIVE ORGANISMS WITH β -GLUCOSIDASE AFTER THIOGLYCOLLIC ACID (TGA) AND ACETONE TREATMENT

Organism Treatment ^{b/}	Staining Intensity of Fractions ^{a/}								
	Experimental			One-Step Block			Heat Control(70 C) ^{c/}		
	I	II	C	I	II	C	I	II	C
<u>TGA</u>									
<u>B. subtilis</u> var. <u>niger</u> spores	4+	2+	3+	1+	2+	2+	1+	0	0
<u>B. abortus</u>	4+	3+	3+	2+	0	0	0	0	0
<u>E. coli</u>	4+	3+	3+	0	2+	0	1+	0	0
<u>P. pestis</u>	4+	4+	4+	1+	1+	1+	3+	0	0
<u>P. tularensis</u>	3+	3+	2+	0	0	0	3+	0	2+
<u>S. typhosa</u>	1+	2+	2+	1+	1+	0	4+	3+	2+
<u>V. comma</u>	4+	4+	4+	0	1+	0	0	0	0
Gram-positive Control(<u>S. lutea</u>)	4+	4+	4+	0	0	0	4+	3+	3+
<u>Acetone</u>									
<u>B. subtilis</u> var. <u>niger</u> spores	0	0	0	-	-	-	-	-	-
<u>E. coli</u>	3+	1+	3+	-	-	-	-	-	-
<u>S. typhosa</u>	3+	2+	2+	0	0	0	-	-	-

- a. Symbols: C, combined fractions I and II; 0, no staining; estimated brightness based on minimal to maximal fluorescent intensity (1+ to 4+); -, no one-step block or heat controls.
- b. Gram-negative organisms stained poorly (1+) with the exception of E. coli and S. typhosa after prior exposure to acetone; staining with fraction I after TGA treatment produced marked cell enlargement of B. abortus, E. coli, S. lutea, and P. pestis (minimal to no enlargement with fraction II).
- c. Cell suspensions incubated in distilled water for 25 minutes, approximately 90% of spores did not stain, but 10% showed variable staining with fraction I.

The incubation of *P. pestis* in TGA for 5 minutes at 37 C was sufficient to sensitizing smear preparations to bright staining with fractions I and II. One-step blocking and heat (37 C) controls showed minimal staining (0 to 1+). The same treatment was ineffective for *B. abortus* and *P. tularensis*.

Prolonged staining with fraction I produced extensive ballooning and severe cell wall lesions in *B. megaterium*, as revealed by electron micrographs (Fig. 4). Staining with boiled fraction I still caused some ballooning of cells, but extensive wall lesions comparable with those produced by unboiled enzyme were not evident.

Firm binding of FITC to enzyme protein was indicated by failure to reduce the fluorescent intensity of stained cells by extensive washing in acetone. The affinity of labeled enzyme for the cell wall was not affected by prolonged washing in 1 M NaCl solution. The latter treatment did not affect the staining intensity of either gram-positive or gram-negative organisms.

Substances known to be relatively free of β -glycosidic-type linkages failed to stain with labeled β -glucosidase fractions.

IV. DISCUSSION

Staining with labeled β -glucosidase may to a large degree depend upon the presence of accessible β -glycosidic linkages in the intact cell wall. These linkages are intimately associated with such substances as glycopeptides, mucopolysaccharides, lipopolysaccharides, and teichoic acids. The qualitative and quantitative variations in content of these substances in microorganisms would account for the observed staining reactions in this study. The staining of chick fibroblasts and possibly other cell lines may result from the presence of substances such as sialoglycopeptides on the cell surface.²⁶ The use of FITC-labeled β -glucosidase as a bacterial stain was dependent upon the following three test conditions: (i) the combining activity of the enzyme must not be impaired by the labeling procedure, (ii) enzyme specificity must be demonstrated with appropriate controls, and (iii) the use of high enzyme concentrations should produce complete or partial autoinhibition of enzyme activity during staining and washing of cell preparations.

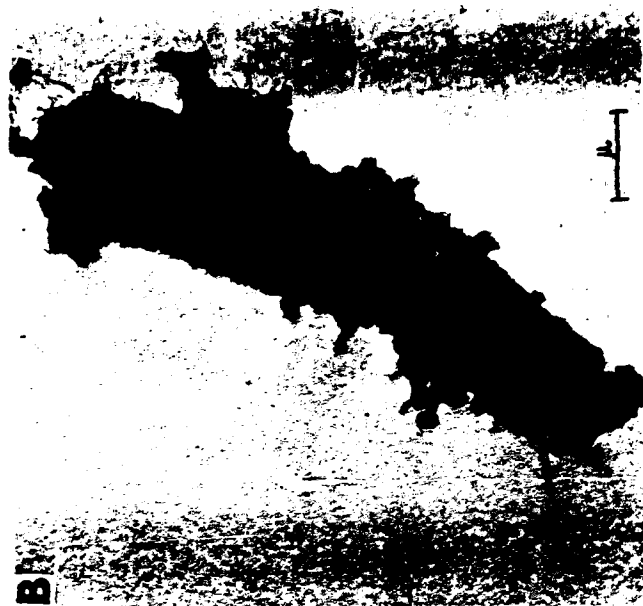




Figure 4. Electron Micrographs of *B. megaterium* Stained with FITC-Labeled β -Glucosidase (Fraction I) for 3 Hours at 37 C. (A) Untreated Control. (B) and (C) Organisms stained with labeled enzyme. (D) Organisms stained with a boiled preparation of labeled enzyme.

Labeling with FITC and column passage produced an approximate 26% loss in enzyme activity as measured in the NPG assay system. However, this loss did not materially affect the combination of labeled enzyme with wall components. Specificity for wall substance was demonstrated by appropriate blocking with Alcian Blue dye and unlabeled enzyme. A firm combination between wall substrate and enzyme appeared to be established and could not be reversed by subjecting stained cells to washing in 1 M NaCl solution. Further evidence for specificity was the production of extensive cell wall lesions after prolonged staining and failure to stain substances lacking accessible β -glycosidic linkages.

According to Metzger,¹⁰ the clearing of a cell suspension with lysozyme appears to involve the following steps: adsorption, diffusion, substrate degradation, and solution. Each step may be affected by various factors. The essential step is the combination of enzyme with substrate, and if other phenomena do not follow, cell lysis will not occur. The use of high enzyme concentrations apparently allows for combination of enzyme with substrate, but after this initial phase, the usual sequence of events does not occur. Gould, Georgala, and Hitchins⁷ have postulated that autoinhibition of lysozyme action at high enzyme concentrations probably occurs because the enzyme molecules sterically hinder their own action.

In this study, inactivated boiled enzyme still combined with wall components and produced some ballooning of the cells (Fig. 4), but extensive wall lesions were not evident. These phenomena suggest that factors affecting enzyme activity may not necessarily interfere with the combination of enzyme and substrate. This in turn may indicate the structural configuration of a particular enzyme with respect to specific chemical substances involved in either substrate attachment or enzyme action. In Veibel's review on β -glucosidase,²⁷ a carbohydrate moiety has been implicated as part of the protein structure of β -glucosidase and may function as the holding group responsible for adsorption of the glucoside molecule to the enzyme. Furthermore, according to Veibel, inactivation of the enzyme by treatment with ozone, ultraviolet radiation, or heat does not render the carbohydrate dialyzable.

A major advantage of use of β -glucosidase instead of lysozyme is the ease with which stained preparations can be washed and mounted. Dilution with washing and mounting solutions did not produce lysis or extensive wall damage during this period. The use of labeled β -glucosidase should increase the number of available enzyme stains that may be conveniently utilized for determining structural components and their specific linkages in the cell wall. For example, Salton and Ghuyssen^{12,28} showed that lysozyme acted on specific linkages to liberate disaccharides and tetrasaccharides from cell walls of Micrococcus lysodeikticus. Further treatment with β -glucosidase produced free N-acetyl-glucosamine and N-acetylmuramic acid.

Other investigators have shown that the underlying glycopeptide layer in gram-negative organisms can be stained with fluorescent lysozyme if the cells were previously exposed to various reagents. In the present study, only E. coli and S. typhosa were brightly stained with fraction I after previous exposure to acetone. The latter reagent may be effective in sensitizing gram-negative organisms to lysozyme but not necessarily to β -glucosidase. The hypothesis that disulfide bonds are involved in maintaining the structural integrity of E. coli²⁴ also appears to be applicable to other gram-negative species. The staining results obtained with TGA-treated cells (Table 3) suggest that complex layering of some gram-negative species^{29,30} may also be closely associated with disulfide bond linkages, but the harshness of the treatment precludes any definitive conclusions. However, staining of S. typhosa with β -glucosidase fractions was adversely affected by prior contact with TGA. It appears that acetone and heating at 70 C were more effective than TGA in sensitizing S. typhosa to β -glucosidase. On the other hand, mild heat alone was effective in sensitizing P. pestis and P. tularensis to staining with fraction I (Table 3). The latter phenomena probably reflect differences in wall composition and varying susceptibility to specific chemical and physical treatments. Staining of P. pestis and P. tularensis may also be related to the higher enzyme content of fraction I (Table 1). Approximately 10% of the heat-control (70 C) spores were variably stained with fraction I (Table 3). This might be attributed to the initiation of a pregermination stage (activation) in some dormant spores by mild heating.³¹

It is possible that the number and concentration of β -glucosidase fractions may vary with the source and purity of a particular enzyme preparation as well as with the efficiency of the method chosen for resolution of the labeled product. This may also apply to other enzymes that are eventually selected. However, standard methods of preparation should yield the proper components necessary for producing a reliable staining reagent.

Fluorescein-labeled β -glucosidase appears to be a potentially useful staining reagent for demonstrating in situ β -glycosidic-type linkages in the bacterial cell wall. A potential may also exist for staining tissues and various cell lines. Furthermore, a combination of different enzymes might be employed to obtain a structural profile of the cell wall. Enzyme stains might also be useful for determining the effects of certain antibiotics on normal cell wall synthesis. Finally, it may be of interest to study cell wall replication by combining differential staining of walls with fluorescent antibody^{32,33} and enzyme stains.

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13. ABSTRACT Beta-glucosidase labeled with fluorescein isothiocyanate was used as a simple staining reagent with selected gram-positive and gram-negative organisms. Staining appeared to be dependent on the presence of accessible glycosidic-type linkages as well as the partial inhibition of enzyme activity by high enzyme concentrations. Labeled β -glucosidase separated into three major fractions upon Sephadex column passage to remove excess dye. These fractions differed in protein content and enzyme activity, but produced similar staining reactions. Extensive wall damage or lysis did not occur when stained cells were suspended in washing and mounting solutions. The apparent specificity of labeled enzyme for wall substance was determined by one-step and Alican Blue blocking reactions as well as by formation of cell wall lesions on prolonged staining with the enzyme. Spores and a majority of the gram-negative organisms tested were effectively stained after prior exposure to thioglycollic acid at 70 C. The present studies suggest that disulfide bonds may play an important role in maintaining the cellular integrity of some gram-negative species. Fluorescein-labeled β -glucosidase appears to be a potentially useful staining reagent for demonstrating in situ glycosidic-type linkages in the bacterial cell wall. A potential for staining tissues and cell lines may also exist.		
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