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INDUCIBLE ENZYME DEGRADING SEROLOGICALLY ACTIVE POLYSACCHARIDES FROM MYCOBACTERIAL AND CORYNEBACTERIAL CELLS[1]

SHOZO KOTANI, TAKAMASA KATO, TOSHIRO MATSUBARA, MASAHIKO SAKAGOSHI and YOSHIYUKI HIRACHI

Department of Microbiology, Osaka University Dental School, Joan-cho, Kita-ku, Osaka

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SUMMARY A gram-positive coccobacillus was isolated from soil enrichment cultures. It could grow on an antigenic polysaccharide from *Corynebacterium diphtheriae* cell walls, containing arabinose, galactose and mannose, as a sole source of carbon, and caused loss of reactivity of the polysaccharide in the precipitin reaction. In the presence of suitable inducer, the organism produced an enzyme(s) that degraded a variety of neutral polysaccharides, whose constituent sugars were D-arabinose, D-galactose, D-mannose, or D-glucose, from mycobacterial and corynebacterial cells and from other sources, and deprived some of them of ability to give a precipitin reaction with the corresponding rabbit antisera.

The preparation, properties, and method of assay of the enzyme (designated as M-2) are described. Analyses of the digest of an arabinogalactan from BCG cell walls with the M-2 enzyme revealed that this enzyme hydrolyzed arabinosidic linkages by an endo-attack mechanism, the products being high- and low-molecular-weight fractions, rich in galactose and in arabinose, respectively. The latter was shown to be a very effective inhibitor of the precipitin reaction between arabinogalactan and anti-BCG serum.

INTRODUCTION

Enzymes which hydrolyze antigenic polysaccharides have proved very useful in investigating the immunological significanses and immunochemical structures of polysaccharides from bacteria and other sources. For instance, they have been used in studies on enzymatic hydrolysis of the capsular polysaccharides of pneumococci (Dubos and Avery, 1931; Dubos, 1940; Torriani and Pappenheimer, 1962), of the group specific C-carbohydrate from group A hemolytic streptococci (Krause, 1963), and of the blood group substances (Stack and Morgan, 1949; Iseki and his collaborators, 1951, 1952, 1959; Furukawa, Fujikawa and Iseki, 1962; Watkins, 1953). Enzymes which depolymerize polysaccharides into constituent

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oligosaccharides by an endo-attack mechanism are especially useful in conjunction with chemical methods, for studying the chemical structures of these polysaccharides, since chemical attack often results in excessive hydrolysis so that fragments giving information on the structure of the polysaccharides cannot be isolated (Bartell, Lam and Orr, 1968).

This paper reports studies on an enzyme depolymerizing antigenic polysaccharides from *Mycobacteria* and related bacteria. Preliminary reports of this work have been presented (Kotani, Matsubara and Sakagoshi, 1966; Kotani et al., 1968). The enzyme was used to investigate the chemical structure of a cell wall arabinogalactan of *Mycobacterium phlei*, and the chemical nature of antigenic determinants of a cell wall polysaccharide of *Mycobacterium tuberculosis* strain H37Rv, as already reported (Misaki et al., 1970; Kotani et al., 1971).

MATERIALS AND METHODS

1. *Basal medium for enrichment culture*

The prescription of Baker and Whiteside (1965) was followed with slight modification. The medium consisted of 0.1% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.0005% $MgSO_4 \cdot 7H_2O$, and 0.0005% $FeCl_3 \cdot 6H_2O$, and was neutralized to pH 7.0.

2. *Casamino acid (CA-) solution for induction of the enzyme*

One gram of Bact-casamino acid (technical grade), 250 mg of $MgSO_4 \cdot 7H_2O$ and 250 mg of K_2HPO_4 were dissolved in one liter of water and the solution was adjusted to pH 7.2.

3. *Cell wall preparations*

Cell wall specimens of BCG (strain Takeo) were prepared from cells grown as a surface pellicle on Sauton's medium for 9 to 12 days at 37 C. Cell walls of *C. diphtheriae* (strain Park-Williams No. 8) were prepared from cells grown in shaking cultures in modified Taylor's medium for two days at 34 to 35 C. They were obtained by sonic disruption, differential centrifugation and digestion with trypsin essentially as described previously (Kotani et al., 1959). Preparation of the cell walls of *M. tuberculosis* strain H37Rv was also described previously (Kotani et al., 1971).

4. *Non-dialyzable portion of the cell walls of C. diphtheriae solubilized enzymatically (abbreviated as CDCL)*

A specimen of *C. diphtheriae* cell walls was solubilized by digestion with *Streptomyces* L-3 enzyme as reported by Kato, Strominger and Kotani (1968). The digest was thoroughly dialyzed against water and the non-dialyzable portion, composed of complexes of antigenic polysaccharide and glycopeptide was lyophilized.

5. *Specimens of polysaccharides*

Antigenic cell wall polysaccharides from BCG, *M. tuberculosis* strain H37Rv and *C. diphtheriae* strain Park-Williams No. 8 (abbreviated hereafter as BCGPs, H37RvPs and DPs, respectively) were prepared by repeated extractions of the cell wall preparations with a solution of 5 g/dl of trichloroacetic acid in the cold and by fractional precipitation of the extracts with ethanol, as described elsewhere (Kotani et al., 1971). The constituent sugars of BCGPs were arabinose and galactose, and those of H37RvPs and DPs were arabinose, galactose and mannose.

Preparations of arabinogalactan from BCG and of mannan from *M. tuberculosis* H37Ra were generously supplied by Dr. A. Misaki (Laboratory of Applied Biochemistry, Institute of Scientific and Industrial Research, Osaka University), and those of arabinogalactan, arabinomannan and glucan from *M. tuberculosis* strain Aoyama B, glucan and galactomannan from *Aspergillus fumigatus*, arabinogalactan from *Larix occidentalis*, and a rabbit antiserum specimen against *A. fumigatus* were generous gifts from Dr. I. Azuma (the Third Department of Internal Medicine, Osaka University Medical School). Specimens of arabinomannan and glucan from BCG, and a water-soluble polysaccharide from wax D of *M. tuberculosis* strain Aoyama B were obtained through the courtesy of Dr. T. Tsumita (Research Institute of Medical Science, Tokyo University) and Dr. A. Tanaka (Research Institute for Diseases of the Chest, Kyushu University), respectively. Dr. A. Kaji (Faculty of Agriculture, Kagawa University) kindly supplied specimens of araban from beet and α-D-galactosidase from *Corticum rolfsii*.

6. *Analytical methods*

Identification of sugars and sugar alcohols: Test specimens were hydrolyzed in 0.1 N hydrochloric

acid at 100 C for 15 hr, and hydrolyzates were dried over conc. sulfuric acid and sodium hydroxide under reduced pressure to remove hydrochloric acid. Hydrolyzed specimens were spotted on Toyo Roshi No. 514 paper, and developed by one-way descending paper chromatography, using the upper layer of *n*-butanol-acetic acid-water (4: 1: 5, v/v/v) as solvent at room temperature. Paper electrophoresis was carried out by application of 20 volt/cm, in 0.1 M sodium molybdate adjusted to pH 5.0 with sulfuric acid, at 4 C. Spots of sugars and sugar alcohols developed on the paper were detected by spraying with ammoniacal silver nitrate reagent (Trevelyan, Procter and Harrison, 1950).

Determination of a sugar residue at the reducing end: Aliquots of test specimens (800 μg of BCGPs or 300 μg of its degradation products in 300 μl of water) were reduced with 20 μl of unbuffered 2.5% sodium borohydride solution at room temperature in the dark for four hr, as described by Schiffman, Kabat and Leskowitz (1960). Then 80 μl of 5 N hydrochloric acid were added to stop the reaction, and the mixture was dried. The dried residue was mixed with 200 μl of methanol, and borates produced during the reaction were removed by repeated evaporation with methanol. After this, the reduced specimens were hydrolyzed with 0.1 N hydrochloric acid at 100 C for 15 hr, and a sugar alcohol in the hydrolyzates, derived from the reducing end, was identified as described in the preceding paragraph.

Quantitative determinations: Pentose and hexose were estimated by the orcinol and anthrone methods, as described by Ashwell (1957), using arabinose and galactose, respectively, for reference. Reducing groups (as glucose) were measured by the method of Park and Johnson (1949). Protein contents of enzyme preparations were determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as a reference. The average chain lengths of BCGPs and its degradation products were given by the molar ratios of total sugar residues to reducing groups.

7. *Immunological methods*

Preparation of rabbit antiserum to whole cells of BCG or *C. diphtheriae*: The method described previously (Kotani et al., 1971) was followed except that untreated cells of BCG (strain Takeo) or heat-killed (at 60 C for one hr) cells of *C. diphtheriae* (strain Park-Williams No. 8) were used as an immunizing antigen.

Precipitin and precipitin inhibition tests: Ouchterlony double diffusion in agar gel and the quantitative precipitin inhibition test were performed as described by Kabat and Mayer (1961). The ring precipitin test was carried out using test tubes of 4 mm internal diameter and 70 mm length.

TABLE 1. *Assay of loss of serological reactivity of DPs in culture supernatants from soil enrichment cultures*

No. of soil specimen	Precipitin reaction with anti-*C. diphtheriae* serum[a]								Bacteria degrading DPs isolated as a pure culture
	2nd subculture in media containing 0.006% DPs	3rd subculture in media containing 0.015% DPs							
	Undiluted	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	
1	−	+	−	−	−	−	−	−	M-2, M-3
2	##								
3	##								
4	##								
5	##								
6	−	+	+	+	−	−	−	−	None
7	##								
8	##								
Not inoculated	##	##	##	##	##	##	##	−	

a By ring test.

RESULTS

1. *Isolation from soil of organism producing the enzyme degrading polysaccharide*

Specimens (0.5 g each) of soil from various areas were inoculated into 5 ml portions of the basal medium supplemented with 0.006% DPs as a sole source of carbon, and incubated at 30 C. One week later, 0.2 ml aliquots of the cultures were transferred to 2 ml of the medium described above. After one week, one ml aliquots of the subcultures were centrifuged at 9,500 $\times g$ for 20 min, and the supernatant fluids were examined for the presence of serologically reactive DPs by the ring precipitin test with anti-*C. diphtheriae* rabbit serum. Aliquots of 0.2 ml of subcultures in which the supernatant no longer gave a positive precipitin reaction were transferred to 2 ml of fresh medium supplemented with 0.015% DPs, and incubated for one week. The supernatant fluids from these subcultures were serially diluted two-fold with physiological saline, and at each dilution the serological reactivity with anti-*C. diphtheriae* serum was assayed by ring precipitin method (Table 1). The subcultures (Nos. 1 and 6), in which DPs had markedly less activity to precipitate with the antiserum than in an uninoculated control, were streaked onto nutrient agar plates and cultured at 30 C for a few days. Of the resulting colonies those with different appearances were transferred to basal media supplemented with 0.006% DPs. After appropriate periods of incubation at 30 C, the cultures were assayed for their reactivities with anti-*C. diphtheriae* rabbit serum. The colonies giving cultures showing a definite descrease in serological reactivity were replated onto nutrient agar media. Thus, pure cultures were obtained of two strains which could grow on DPs as the sole source of carbon and destroy the ability of DPs to give a precipitin reaction with antiserum. One of them, a gram-positive coccobacillus (designated as M-2 bacterium), was used in the following study.

2. *Production of enzyme by the M-2 bacterium*

Preliminary experiments were made on the effects of the amount of inducer and incubation period on production of the enzyme degrading DPs. The conditions used in the representative experiment described below gave a satisfactory yield of enzyme.

Nutrient broth (100 ml) was inoculated with a loopful of M-2 bacterium which had been grown on a 0.1% Bacto-peptone agar slant overnight at 30 C. The culture was incubated with shaking at 30 C for two days. Then 10 ml portions were transferred to six, long-neck shaking flasks each containing 200 ml of nutrient broth. The bottles were incubated with shaking at 30 C for 24 hr. Their contents were then transferred to 16 liter of nutrient broth in a 20 liter stainless steel vessel. After cultivation at 30 C for 24 hr under vigorous aeration, cells at half maximum growth were harvested with a refrigerated centrifuge equipped with a continuous flow rotor. The cells were thoroughly washed successively with 400 ml each of deionized water, physiological saline and CA-solution by centrifugation at 9,500 $\times g$ for 30 min. The washed cells were suspended in one liter of CA-solution supplemented with CDCL (4 mg / dl) to give an optical density at 550 mμ of approximately 10. The suspension was shaken at 30 C for four hr, and was centrifuged to remove the cells. The supernatant fluid was fully saturated with ammonium sulfate and stood overnight at 4 C. A precipitate was dissolved in water, and thoroughly dialyzed. The enzyme solution thus obtained was concentrated to about 25 ml by ultrafiltration through cellulose tubing (M-2 enzyme preparation).

3. *Factors affecting the activity of the M-2 enzyme*

Effect of pH: To test the effect of pH 0.02 M buffers, of pH 2.5 to 11.7, were used containing one mg of CDCL and an appropriate amout of enzyme. The total volume of the reaction mixture was 200 μl. After incuba-

tion for one hr at 37 C, aliquots were withdrawn for determination of released reducing groups. As shown in Fig. 1, the optimum pH for the hydrolytic activity of the M-2 enzyme was found to be around 5.9.

Effect of ionic strength: Specimens (2 mg) of CDCL were incubated at 37 C for four hr with an appropriate amount of enzyme in a total volume of 200 μl of 0.025 M Tris-maleate buffer (pH 5.9) containing various concentrations of sodium chloride. The presence of final concentrations of up to 1.0 M sodium chloride in the reaction mixture had some, but not very much effect on liberation of reducing groups from CDCL by the M-2 enzyme, as shown in Fig. 2.

Effects of divalent cations: Salts of divalent cations (Cu^{++}, Mg^{++}, Ca^{++}, Ba^{++}, Zn^{++}, Cd^{++}, Mn^{++}, Fe^{++}, Co^{++}, and Ni^{++}) were added at final concentrations of 0.001 M to reaction mixture (160 μl) containing 2 mg of CDCL and an appropriate amount of the M-2 enzyme. After incubation for three hr at 37 C, the amounts of reducing groups liberated in these mixtures were compared with that in a control mixture without added divalent cations. Results showed that the divalent cations tested had no significant effects upon enzyme activity.

Stability of the M-2 enzyme: Aliquots of a specimen of the M-2 enzyme dissolved in water were heated at 56($\pm$1.5) C for 15, 30, 60, 90 and 120 min. The hydrolytic actions of the heated enzyme specimens on DPs (200 μg) were compared with that of an unheated control specimen in 200 μl of 0.025 M Tris-maleate buffer, pH 5.9. Fig. 3 shows that the activity of the M-2 enzyme was heat-labile and half the activity was lost on heating at 56 C for

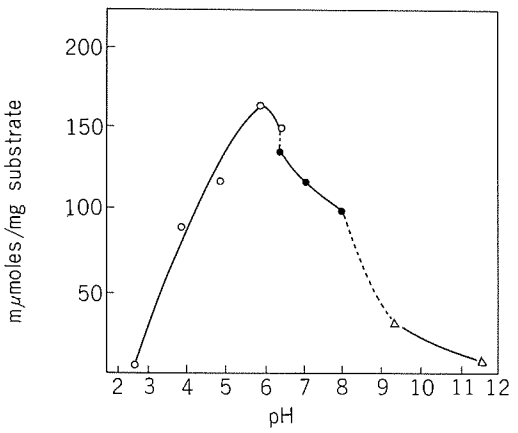

FIGURE 1. *pH-optimum for hydrolysis of CDCL by the M-2 enzyme.*
Buffer:
○ *Citrate-phosphate*
● *Tris-maleate*
△ *Borax-sodium hydroxide*

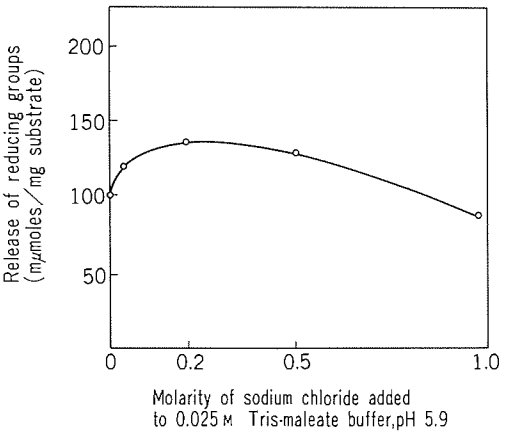

FIGURE 2. *Effect of ionic strength on hydrolytic activity of the M-2 enzyme against CDCL.*

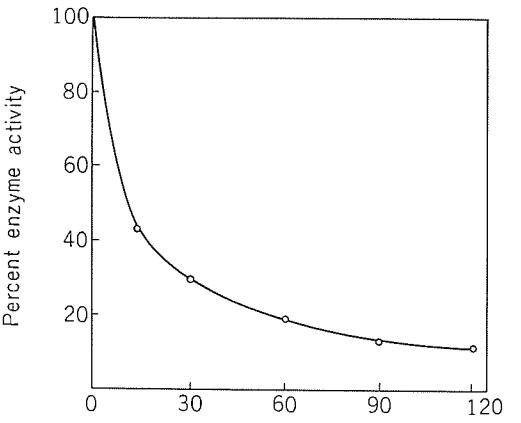

FIGURE 3. *Heat inactivation of the M-2 enzyme.*

15 min. Storage of the M-2 enzyme preparation at −20 C for several months, caused no significant decrease in the activity if care was taken to avoid repeated freezing and thawing.

4. *Activity ranges of the M-2 enzyme*

The susceptibilities to the M-2 enzyme of a variety of neutral polysaccharides of microbial and plant origin, containing arabinose, galactose, mannose or glucose were examined. Mixtures in 0.02 M Tris-maleate buffer, pH 5.9, 400 μl were incubated at 37 C for 24 hr, and then the liberation of reducing groups and serological reactivities of the test polysaccharides were examined. The latters were semi-quantitatively estimated by the serial two-fold antigen dilution method using the ring precipitin technique. The results are summarized in Table 2. All the polysaccharides tested except mannan from *M. tuberculosis* strain H37Ra, araban from beet and arabinogalactan from *Larix occidentalis* were significantly hydrolyzed by the M-2 enzyme, liberating a considerable amount of reducing groups. It is interesting that while the susceptible polysaccharides of microbial origin are known to be composed of the D-isomers of sugars (Misaki and Yukawa, 1966; Azuma et al., 1969; Ohashi, 1970; etc.), the constituent arabinose residues of araban from beet and those of arabinogalactan from *Larix occidentalis*, both of which were resistant to the M-2 enzyme, have been shown to be L-isomers (Tagawa and Kaji, 1963, and Bouveng and Lindberg, 1958, respectively). Among the antigenic polysaccharides which were susceptible to the M-2 enzyme, as judged by liberation of reducing groups, arabinogalactan (BCG), arabinomannan (BCG), BCGPs, H37RvPs, DPs and a water-soluble polysaccharide from the wax D preparation of *M.*

TABLE 2. *Degradation of various polysaccharides of microbial and plant origin by treatment with the M-2 enzyme, with or without loss of their abilities to give a precipitin reaction with the corresponding antisera*

Substrate	Reducing groups liberated mμ moles/mg	Precipitin reaction with[f]			
		anti-BCG serum		anti-*C. diphtheriae* serum	
		Untreated	Treated	Untreated	Treated
DPs	550	1.6	>100	3.1	>100
BCGPs	585	0.4	>100		
H37RvPs	560	3.2	>100		
Arabinogalactan (BCG)[a]	630	0.4	>100	3.1	>100
Arabinomannan (BCG)[b]	295	1.6	>100	>100	
Glucan (BCG)[b]	500	1.6	6.3	>100	
Mannan (*M. tuberculosis*, H37Ra)[a]	25	>200			
A polysaccharide from wax D (*M. tuberculosis*, Aoyama B)[c]	100	3.2	>200		
		anti-*A. fumigatus* serum			
Glucan (*A. fumigatus*)[d]	210	156	156		
Galactomannan (*A. fumigatus*)[d]	140	3.2	3.2		
Araban (beet)[e]	tr				
Arabinogalactan (*L. occidentalis*)[d]	tr				

a,b,c,d,e Generously supplied by Drs. A. Misaki, T. Tsumita, A. Tanaka, I. Azuma and A. Kaji, respectively.
f Minimum concentration (μg/ml) giving a positive reaction in the ring test.

tuberculosis strain Aoyama B were shown to lose their abilities to give a precipitin reaction with rabbit antisera against whole cells of BCG and/or *C. diphtheriae* after treatment with the M-2 enzyme. On the other hand, glucan from BCG and glucan and galactomannan from *A. fumigatus* (Azuma et al., 1969), retained their abilities to precipitate with anti-BCG and anti-*A. fumigatus* rabbit serum, respectively, even after liberation of a significant amount of reducing groups, though BCG glucan showed some decrease in reactivity. Mannan from *M. tuberculosis* strain H37Ra, which released very few if any reducing goups during incubation with the M-2 enzyme, did not react with anti-BCG serum even before digestion with the M-2 enzyme.

The antigenic relationships of the test polysaccharides were studied by the Ouchterlony double diffusion method. As shown in Fig. 4, all the polysaccharides except BCG glucan gave one broad precipitation line on reaction with anti-BCG rabbit serum. These lines all fused with each other, though spur formation extending toward the antigen well containing BCG arabinogalactan was recognized between the precipitation line of arabinogalactan and that of either BCG arabinomannan or BCGPs. BCG glucan gave one sharp and one broad precipitation arc, and these lines crossed, but did not fuse with the lines of other test polysaccharides. These findings indicate that the test polysaccharides which contained the arabinose residue as an essential component possess common antigenic determinants and BCG glucan is not related antigenically to the polysaccharides containing arabinose*.

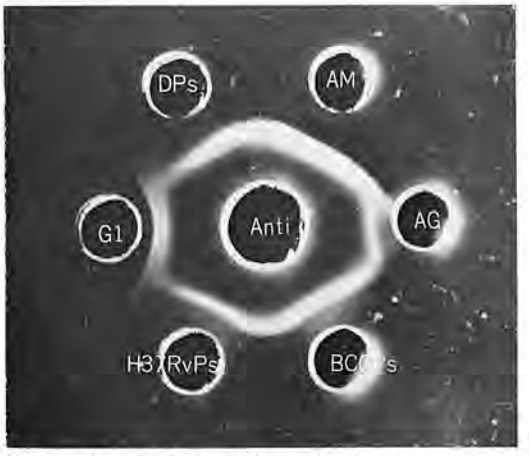

FIGURE 4. *Analysis of the antigenic relationships of test neutral polysaccharides by double immunodiffusion in agar gel.*

Anti: anti-BCG rabbit serum (70 μl), AG: BCG arabinogalactan (40), AM: BCG arabinomannan (16), Gl: BCG glucan (80), DPs (140), BCGPs (20) and H37RvPs (8). Figures in parenthesis represent the amount (μg/40 μl) of test fractions in the antigen well.

Table 3 shows the susceptibilities of a variety of commercially available oligosaccharides and polysaccharides to the M-2 enzyme. In this study, 40 μg of test specimens were incubated with an appropriate amount of the M-2 enzyme in 200 μl of 0.02 M Tris-maleate buffer pH 5.9, at 37 C for 24 hr. Of the test substrates, dextrin, glycogen and soluble starch were found to be suceptible to the M-2

TABLE 3. *Susceptibilities of commercially available oligosaccharides and polysaccharides to the M-2 enzyme*

Unsusceptible	
Disaccharides:	Maltose, cellobiose, α,α′-trehalose, gentiobiose, lactose, melibiose, turanose
Trisaccharides:	Melezitose, raffinose
Tetrasaccharides:	Stachyose
Polysaccharides:	Dextran, inulin, pectin
Susceptible	
Polysaccharides:	Glycogen, dextrin, soluble starch

* Later rabbits were hyperimmunized by administration of the particle-, cell wall- and soluble cytoplasmic fractions obtained from sonically disrupted BCG cells with the Freund's incomplete adjuvant (Difco). It was found that their antiserum specimens sometimes gave a weak or faint precipitation line or lines besides the line described in the text in the immunodiffusion reaction with BCGPs, H37RvPs, DPs and BCG arabinogalactan. However, they did not give these weak lines with BCG arabinomannan.

enzyme, liberating as much as 1,000 to 2,000 mμmoles of reducing groups per mg. However, other polysaccharides and the oligosaccharides tested were not degraded by the M-2 enzyme.

5. *Relationship between enzyme concentration and the amount of reducing groups liberated from representative polysaccharides*

Aliquots of test substrates (100 μg each of BCGPs H37RvPs and DPs, and 10 μg of dextrin) were incubated with various amounts of the M-2 enzyme in 50 μl of 0.02 M Tris-maleate buffer, pH 5.9. After incubation for 15 min, the reaction mixtures were immediately heated at 70 C for 15 min to stop the enzyme action, and assayed for liberated reducing groups. Fig. 5 shows that there was a linear relationship between the concentration of enzyme and the extent of liberation of reducing groups, up to 70 mμmoles/mg of BCGPs, H37RvPs or DPs, and up to 600 mμmoles/mg of dextrin. From these findings, one unit of M-2 enzyme activity to hydrolyze BCGPs, H37RvPs or DPs was defined as the amount of enzyme required for liberation of 30 mμmoles of reducing groups from one mg of the corresponding polysaccharides under the standard conditions described above. Similarly one unit for hydrolysis of dextrin was defined as the amount of enzyme which liberated 300 mμmoles of reducing groups per mg dextrin.

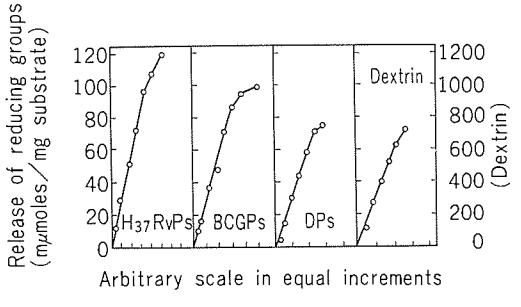

FIGURE 5. *Dose-response curves for the hydrolysis of various polysaccharides by the M-2 enzyme.*

6. *Attempt to induce the M-2 enzyme with dextrin*

Use of CDCL as an inducer is unsatisfactory, since large amounts of CDCL are not available so a large quantity of the M-2 enzyme cannot be prepared. Therefore, the effect of dextrin as an inducer of the M-2 enzyme was tested.

Washed cells of the M-2 bacterium at the stage of half maximum growth in nutrient broth were divided into two portions. One portion was suspended in CA-solution containing CDCL (4 mg / dl) and the other was put into CA-solution supplemented with dextrin (4 mg / dl). The suspensions were incubated with shaking at 37 C for four hr, and then centrifuged. Then the supernatant fluids were fully saturated with ammonium sulfate to precipitate the enzyme induced by CDCL or dextrin [designated as the M-2(CDCL) or M-2(dex) enzyme, respectively].

The hydrolytic actions of the two enzyme preparations against several test polysaccarides were compared, as shown in Fig. 6. Both

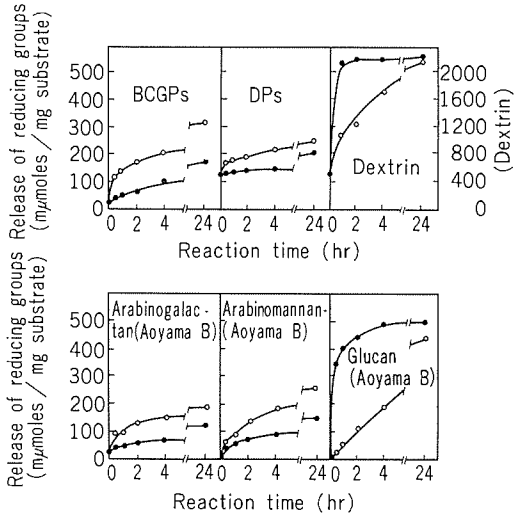

FIGURE 6. *Degradation of polysaccharides from BCG, M. tuberculosis, C. diphtheriae and dextrin by the enzymes induced by CDCL and dextrin.*
○——○ *M-2(CDCL) enzyme.*
●——● *M-2(dex) enzyme.*

the M-2(dex) enzyme and the M-2(CDCL) enzyme hydrolyzed not only dextrin and glucan from *M. tuberculosis* strain Aoyama B but also BCGPs, DPs, Aoyama B arabinogalactan and Aoyama B arabinomannan. Immunodiffusion in agar gel showed that the serological reactivities of the latter polysaccharides containing arabinose residues to precipitate with anti-BCG serum were abolished by treatment with either the M-2(CDCL) or M-2(dex) enzyme.

However, there seemed to be distinct differences between the M-2(CDCL) and M-2 (dex) enzymes. Fig. 6 shows that the M-2 (dex) enzyme was more active than the M-2 (CDCL) enzyme against dextrin and Aoyama B glucan, but less active against BCGPs, DPs, Aoyama B arabinogalactan and Aoyama B arabinomannan, in terms of the rate and/or extent of liberation of reducing groups. A similar conclusion can be drawn from Table 4. The relative hydrolytic potencies against BCGPs and dextrin of three sets of M-2 (CDCL) and M-2(dex) enzymes were compared. It was found that the ratios of BCGPs units to dextrin units ranged from 1.8 to 4.4 for preparations of the M-2(CDCL) enzyme and from 0.06 to 0.24 for those of the M-2 (dex) enzyme.

TABLE 4. *Activities of enzymes induced by CDCL and dextrin against BCGPs and dextrin*

Preparation No.	Inducer	Protein mg/ml	Specific activity (units/mg protein) against		Ratio of specific activities BCGPs/dextrin
			BCGPs	Dextrin	
680A	CDCL	3.46	84	19	4.4
680B	Dextrin	3.00	8	126	0.06
722A	CDCL	3.20	25	14	1.8
722B	Dextrin	3.62	12	223	0.05
725A	CDCL	3.71	54	14	3.9
725B	Dextrin	3.90	29	121	0.24

7. Evidence for the presence of separate enzyme entities responsible for degradation of DPs and BCGPs and of dextrin

Different pH optima: Specimens of DPs (200 μg) or dextrin (20 μg) were incubated with the M-2(CDCL) or M-2(dex) enzyme in 0.02 M Briton-Robinson's wide pH range buffers, at pH values of 3 to 11 in a total volume of 100 μl. After appropriate periods of incubation, liberation of reducing groups in the mixtures of different pH values was determined. Fig. 7 shows that the optimum pH for degradation of DPs by the M-2(CDCL) was about 5.9 (see Fig. 1), but the optimum pH for hydrolysis of dextrin by either the M-2(CDCL) or M-2 (dex) enzyme was around 7. No definite pH optimum was recognized for degradation of DPs with the M-2(dex) enzyme, under the present conditions.

Separation of the enzyme activities against BCGPs and dextrin: The M-2(CDCL) and M-2(dex) enzyme preparations were fractionated by acetone precipitation at −5 C. The

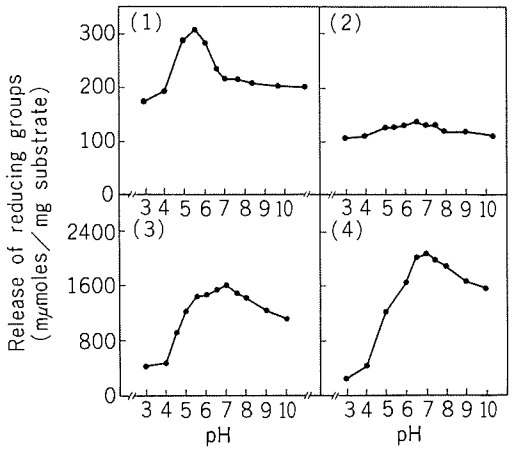

FIGURE 7. *pH-optima for hydrolysis of DPs and dextrin by the M-2(CDCL) and M-2(dex) enzymes.*
(1) DPs—M-2(CDCL) enzyme (30 min incubation).
(2) DPs—M-2(dex) enzyme (30 min incubation).
(3) Dextrin—M-2(CDCL) enzyme (60 min incubation).
(4) Dextrin—M-2(dex) enzyme (15 min incubation).

TABLE 5. *Degradation of DPs and dextrin by acetone fractions of the M-2(CDCL) and M-2 (dex) enzymes*

	Fraction No.	Final concentration of acetone (%, v/v)	Specific activity (units/mg protein) against		Recovery of activity (%) against		Ratio of specific activities DPs/dextrin
			DPs	Dextrin	DPs	Dextrin	
M-2(CDCL)	Starting material		45	13	100	100	3.4
	1	0–50	39	6	8	5	6.8
	2	50–60	68	7	42	15	9.6
	3	60–67	42	20	6	9	2.1
	4	67–80	96	36	17	21	2.7
	5	80–90	2	7	1	13	0.29
M-2(dex)	Starting material		22	121	100	100	0.18
	1	0–50	30	193	9	10	0.15
	2	50–60	23	151	17	20	0.15
	3	60–67	0	122	0	13	0
	4	67–80	49	195	38	28	0.24

activities against DPs and dextrin were not clearly separated, but the results in Table 5 indicate that the ratio of DPs units to dextrin units varied widely in different fractions of the M-2(CDCL) and M-2(dex) enzymes separated by acetone precipitation.

These findings suggest that the hydrolytic activities to BCGPs and DPs and to dextrin, of the M-2(CDCL) and M-2(dex) enzyme preparations, are due to separate enzyme proteins.

8 *Degradation of BCGPs by hydrolysis with the M-2(CDCL) and M-2(dex) enzymes*

Chemical properties of the degradation products: A specimen (90 mg) of BCGPs was subjected to gel filtration on a column (2× 97 cm) of Sephadex G-50 (coarse, bead form, Pharmacia, Uppsala, Sweden) connected in series with another column (2.5×97 cm) of Sephadex G-25 (coarse, bead form). Material with an effluent volume of 200 to 380 ml (Fig. 8) was concentrated. The concentrated solution was incubated for 48 hr at 37 C with 164 BCGPs units of the M-2(CDCL) enzyme (1.8 units/mg substrate). The enzyme was added in two portions; first 106 units and then 24 hr later 58 units. The total volume of the reaction mixture was 5 ml initially and 5.5 ml after 24 hr incubation. The digest, containing 550 mμmoles of liberated reducing groups per mg, was applied to a similar series of columns to those described above. The gel filtration patterns in Fig. 8 show that BCGPs which gave essentially one peak, as judged by elution

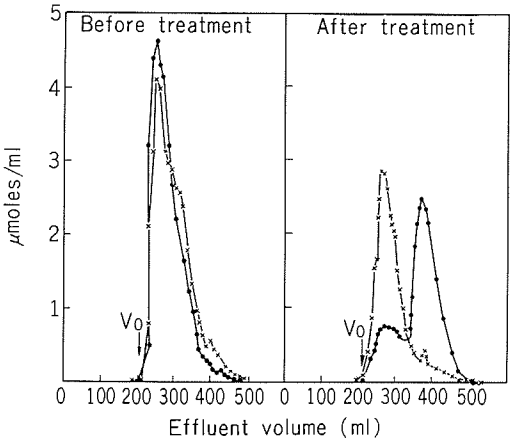

FIGURE 8. *Gel filtration patterns of BCGPs before and after treatment with the M-2(CDCL) enzyme.* ●——● *Pentose.* ×——× *Hexose.*

TABLE 6. *Chemical and serological properties of BCGPs and its degradation products by the M-2(CDCL) and M-2(dex) enzymes*

Test material		Yield (%)	Sugar composition (molar ratio)		Sugar residue at reducing end	Average chain length[a]	Serological reactivity (μg/ml)[b]
			Arabinose	Galactose			
M-2(CDCL) enzyme digest	Unhydrolyzed	100	1.1	1.0	Arabinose	31	1.6
	F-1	39	1.0	3.2	Arabinose	16	>200
	F-2	41	5.8	1.0	Arabinose	6	>200
M-2(dex) enzyme digest	Unhydrolyzed	100	1.2	1.0		25	3.2
	F-1′	61	1.0	1.4		10	>200
	F-2′	10	2.6	1.0		7	>200

a Molar ratio of total sugar residues to reducing groups.

b Minimum concentration giving a positive precipitin reaction with anti-BCG serum in the ring test.

of pentose and hexose, was degraded by digestion with the M-2(CDCL) enzyme to a high-molecular-weight portion (F-1; elution volume, 200 to 330 ml) rich in hexose and a low-molecular-weight portion (F-2; elution volume, 340 to 490 ml) rich in pentose. The analytical data in Table 6 show that BCGPs consisting of arabinose and galactose in a molar ratio of 1.1 to 1.0 and having an average chain length of approximately 31 sugar residues, was cleaved at internal arabinosidic linkages. The products were an F-1 fraction, with an average chain length of about 16 sugar residues, containing 3.2 moles of galactose per mole of arabinose, and an F-2 fraction, composed of about 6 moles of arabinose and one mole of galactose and containing an average of six sugar residues. The sugar residue at the reducing end of both degradation products as well as the original BCGPs was essentially all identified as arabinose. Treatment of the F-2 fraction with α-D-galactosidase in 0.067 M McIlvaine's buffer, pH 2.2, for as long as five days at 30 C resulted in no significant liberation of reducing groups.

A similar, but small-scale experiment was carried out on a separate preparation of BCGPs incubated with the M-2(dex) enzyme (1.9 BCGPs units/mg substrate). On incubation for 44 hr at 37 C, 351 mμmoles of reducing groups were liberated. Fig. 9 shows the gel

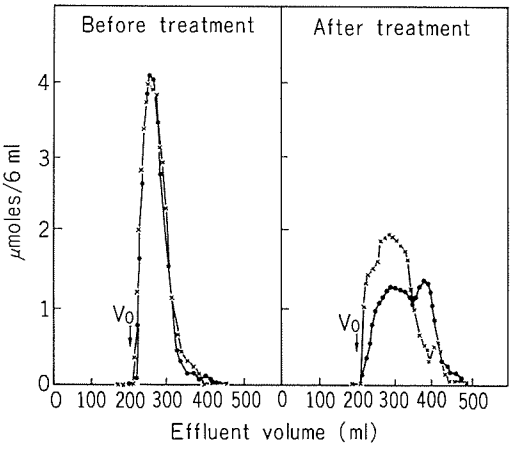

FIGURE 9. *Gel filtration patterns of BCGPs before and after treatment with the M-2(dex) enzyme.*
●——● *Pentose.* ×——× *Hexose.*

filtration patterns of BCGPs before and after treatment with the M-2(dex) enzyme, indicating that BCGPs was degraded to high-molecular-weight and low-molecular-weight fragments by enzymatic hydrolysis. The cleavage products (fractions F-1′ and F-2′) obtained by digestion with the M-2(dex) enzyme showed distinct differences from the corresponding fractions (F-1 and F-2) of the digest with the M-2(CDCL) enzyme, both on gel filtration and on chemical analysis (Table 6). The molar ratios of galactose to arabinose in F-1′ and of

arabinose to galactose in F-2′ were much lower than those in F-1 and F-2, respectively. Furthermore, the recovery of the low-molecular-weight F-2′ fraction was much lower than that of the F-2 fraction.

Immunological properties of degradation products: All the products of digestion of BCGPs by the M-2(CDCL) and M-2(dex) enzymes gave negative reactions in the ring precipitin test with anti-BCG rabbit serum even at concentrations as high as 200 μg/ml. This was in sharp contrast to the original BCGPs which gave a positive precipitin reaction at a concentration of 1.6 to 3.2 μg/ml (see Table 6).

However, the quantitative precipitin inhibition test showed that the F-1 and F-2 fractions, and especially F-2, strongly inhibited the precipitin reaction between BCGPs and anti-BCG serum (Fig. 10). Five moles of the F-1 and F-2 fractions per mole of BCGPs (tentatively calculated on the basis of the average chain lengths of the cleavage products and BCGPs) caused approximately 20% and 75% inhibition, respectively. Nearly 90% inhibition was observed with about 10 moles of the F-2 fraction per mole of BCGPs.

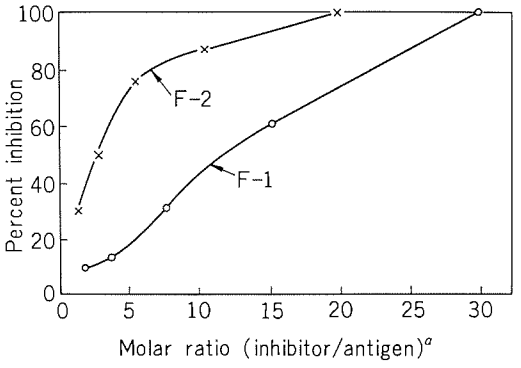

FIGURE 10. *Inhibition of the precipitin reaction between BCGPs and anti-BCG serum by the cleavage products of BCGPs obtained by digestion with the M-2(CDCL) enzyme.*

a Tentatively calculated on the basis of the average chain length.

DISCUSSION

Since the classical work of Dubos and Avery (1931) on the decomposition of the capsular polysaccharide of pneumococcus type 3 by an enzyme from a soil bacterium, there have been many reports on depolymerizing enzymes acting on bacterial polysaccharides. Enzymatic hydrolysis of pneumococcal capsular polysaccharides was studied extensively by Torriani and Pappenheimer (1962). They used a strain of *Bacillus palustris* isolated by Sickles and Shaw (1950) which was morphologically similar to the bacillus of Dubos and Avery. They investigated the formation and properties of inducible depolymerases produced by this organism and analyzed the hydrolysis products of the capsular polysaccharides of types 3 and 8 with specifically induced enzymes. Baker and Whiteside (1965) isolated a bacillus, identified as *Bacillus sphericus*, from soil. This produced an inducible enzyme which depolymerized the Vi-antigen of *Salmonella typhi* that was extremely resistant to acid hydrolysis, and caused rapid loss of serological reactivity of the antigen. The most recent study along these lines appears to be that reported by Kuroda et al. (1971) on an enzyme degrading the K antigen from *Vibrio parahaemolyticus* K-15 and produced by a new species of the genus *Bacillus*.

There have also been many reports on phage-associated enzymes that are known to cause hydrolysis of various structural components of bacterial cells, such as the capsule and cell wall. Among them, Sutherland and Wilkinson (Sutherland and Wilkinson, 1965; Sutherland, 1967; Sutherland and Wilkinson, 1968) followed the pioneering work of Adams and Park (1956) on *Klebsiella pneumoniae* strain B and its phage system, and extensively investigated the phage-induced depolymerases active against expolysaccharides of *Escherichia coli* K12, *Klebsiella pneumoniae* A3(Sl) (Type 54) and other similar polysaccharides. On the other hand, Eklund and Wyss (1962) described a phage-associated enzyme which hydrolyzed the polysaccharide capsule of *Azotobacter vinelandii*,

and Bartell and others (Bartell, Orr and Lam, 1966; Bartell, Lam and Orr, 1968) reported on a polysaccharide depolymerase associated with phage-infected *Pseudomonas aeruginosa*.

However, the present work seems to be the first specifically designed to obtain an enzyme depolymerizing antigenic neutral polysaccharides from mycobacterial and corynebacterial cells. The enzyme(s) produced by the M-2 bacterium isolated in this study actively hydrolyzed a variety of polysaccharides in which the constituent sugars were arabinose, galactose, mannose or glucose. Only a little enzyme preparation could be obtained because shortage of inducer, so a concentrated enzyme specimen was used without further purification throughout the present study. Consequently, it is uncertain whether the activities of the present crude enzyme preparations to liberate reducing groups from various polysaccharides are due to a single enzyme protein. The data available indicate that degradation of BCGPs (arabinogalactan) by the M-2(CDCL) enzyme was caused by hydrolysis of arabinosidic linkages by an endo-attack mechanism. However, it is unknown how polysaccharides which did not contain arabinose were hydrolyzed by the M-2(CDCL) enzyme, or how the M-2(dex) enzyme induced with dextrin degraded BCGPs which does not contain a glucose residue. The reducing sugar residue in the cleavage products obtained with the M-2 (dex) enzyme was not identified but the significant differences recognized between the gel filtration patterns and chemical properties of digests of BCGPs with the M-2(CDCL) and M-2(dex) enzymes suggest that these enzymes attack BCGPs differently.

An oligosaccharide fraction was isolated from the digestion products of BCGPs with the M-2 (CDCL) enzyme. It was mainly composed of arabinose residues, and almost completely inhibited the precipitin reaction between undegraded BCGPs and anti-BCG serum. No definite conclusions can yet be drawn on the chemical nature of the antigenic determinants of BCGPs, but the above finding suggest that the M-2 enzyme will be extremely useful in elucidation of the chemical entity of the antigenic determinants of polysaccharides which are susceptible to it (see a separate paper, Kotani et al., 1971).

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