ENZYMATIC DEGRADATION OF ALPHA AND BETA CYCLODEXTRINS BY *BACTEROIDES* FROM THE HUMAN COLON

by

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INTRODUCTION

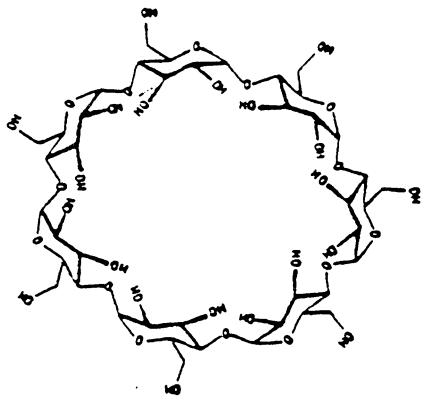
Cyclodextrins (CDs) are cyclic olgiosaccharides composed of 6 or more α-1,4 linked glucose units (Radely, 1964). CDs readily form inclusion complexes with various chemicals, often significantly increasing the stability and/or water solubility of the complexed compounds (Saenger, 1981). This complexation is the basis for recent publications and patents which propose the use of CDs as nutritionally inert stabilizers in various food and pharmaceutical products. For example, CDs stabilize anthocyanin pigments; increase water solubility of vitamins A, D, E, and K; stabilize food flavors, unsatturated fatty acids and vitamin A, as well as stabilizing a variety of foods including rice, cheese, and noodles (Szejtli, 1981, Pitha, 1981).

In contrast to the considerable information concerning the use and role of CDs in stabilizing various foods and food ingredients, the fate of ingested CDs or CD complexes is not clear. Evidence suggests that CD are only slowly hydrolyzed, if at all, by salivary or intestinal amylases (French, 1950). This has led to suggestions that CDs are essentially indigestible and non-caloric (Radely, 1964). Thus, CDs ingested by humans could pass virtually intact into the colon, where they may either be hydrolyzed by colon anaerobes or eliminated from the body in the feces.

The purpose of this research was to investigate the *in vitro* degradation of CDs by human colon anaerobes, isolate and examine some properties of the enzymes causing degradation, identify the products

of CD hydrolysis by these enzymes, and attempt to purify the enzyme(s) responsible for cleaving the cyclic ring of CDs.

CDs are a series of homologous oligosaccharides composed of 6 or more α-D-glucopyranose units linked by α-1,4 bonds to form cyclic structures (Radely, 1968). The most common CDs are 6 unit cyclohexaamylose (α-CD), 7 unit cycloheptaamylose (β-CD), and 8 unit cyclooctaamylose (γ-CD). CDs are torus shaped circular molecules with an inner diameter of about 4.5 Å for α-CD, 7.0 Å for β-CD, and about 8.5 Å for γ-CD (Yonezawa et al, 1981). One of the primary characteristics of CDs is that the outer ring tends to be hydrophilic due to primary and secondary hydroxyl groups occupying both rims of the cyclic ring, whereas the inner cavity tends to be hydrophobic (Saenger, 1981). As a result of their structure, CDs have neither reducing nor non-reducing ends (Radely, 1968). The chemical structure of β-CD is shown below.



The chemical structure of β-cyclodextrin.

CDs are formed during the hydrolysis of starch by certain bacterial strains including *Klebsiella pneumoniae*, *Bacillus megaterium*, and *Bacillus macerans* (Radely, 1968, Bender, 1981). These organisms produce a specific CD-glucosyl transferase enzyme which catalyzes the cyclization of linear glucose chains (Bender, 1981). Maximum cyclization rates occur with substrate chain lengths of 16 - 18 glucose units, signifying a preference for the helical structure of amylose and amylopectin (Bender, 1981). α-CD is formed preferentially, with β and γ-CD being formed at lower rates.

Once formed, CDs are completely resistant to hot alkali, yeast fermentation, and β-amylase; and fairly resistant to acid hydrolysis, α-amylases, and salivary amylases (French, 1950). However, CDs were found to be suseptible to degradation by certain bacterial and mold amylases. α and β-CDs are hydrolyzed by α-amylases of *Bacillus polymyxa* to yield glucose, maltose, and maltotriose (Robyt and French, 1963). *Aspergillus oryzae* α-amylase also hydrolyzed CD, with β-CD being hydrolyzed 5 times faster than α-CD (Hanrahan et al, 1952). Takadiastase appears to contain two distinct enzymes, one cleaving α-CD with inversion of configuration, the other attacking β-CD with retention of configuration (Thoma et al, 1960).

The unique structure of CD allow reaction with various compounds through formation of inclusion complexes (Szetjli, 1981). In most CD inclusion complexes, guest molecules located within the CD cavity are not engaged in covalent bonding, but are stabilized by forces

described as Van der Waals, London dispersion and dipole-dipole interaction (Saenger, 1981). The requirement for this inclusion complexation appears to be primarily spatial since guest molecules ranging from molecular to ionic in character are accepted (Saenger, 1981).

The ability of CD to form inclusion compounds has been studied for medicinal, industrial, analytical, and research applications (Radely, 1968). Labile drugs such as unsaturated fatty acids and vitamin A are stabilized by complexing with CDs (Lach et al, 1963). Pitha et al (1981) found that there was a considerable increase in the ability of CD to solubilize non-polar compounds in aqueous medium, thus enhancing the water solubility of Vitamins A, D, E and K. Inclusion complexes of β-CD with guana-zulene, an antiulcerus and antiinflamitory agent, had increased photostability and stability against acid which prolonged storage time, as well as increasing stability in acid gastric medium (Yonezawa et al, 1981). CD inclusion complexes also protect anthocyanins from decomposition in spite of their labile psuedobase form (Yamada et al, 1980).

The ability of CD to form inclusion complexes is the basis for recent publications and patents which propose the use of CD as nutritionally inert stabilizers in various food products. Texture and flavor deterioration in rice can be prevented by treating brown or polished rice with a compound containing 40% purified bran oil, 1.2% vitamin E, 32.8% H_2O, and 26% CD (Fujimoto, 1981). The shelf life of noodles was found to be markedly increased by the addition of a

mixture containing ethanol or glycine, and β-CD (Nippon, 1981). The addition of CD to cheese and cheese products increased moisture retention and storage life (Ota et al, 1981). Ogawa Company Ltd. (1981) patented a procedure in which volatile food flavorants are mixed with CD to form highly heat stable flavorants suitable for use in bakery products or thermally processed foods. The formation of white precipitate can be prevented in canned bamboo shoots by addition of CD at 0.01 - 2.0% of the bamboo shoot weight (Takeda Chemical Co., 1981).

The above are examples of the use and role of CDs in stabilizing various foods and food ingredients. However, little information is available concerning the metabolism of CD and CD inclusion complexes. The slow hydrolysis of CD by salivary and intestinal amylases has led some to conclude that CD are essentially indigestable and non-caloric (Anderson et al, 1962). Three possible pathways have been proposed for the fate of orally administered CD in the mammalian organism. One possibility is that CD are not degraded or absorbed in the gastrointestinal tract and are excreted unchanged in the feces. The second possibility is that a very small amount of CDs are adsorbed unchanged. Third, CD could be metabolized like any other α-1,4 linked polysaccharide (Gerloczy et al, 1981).

A study by French et al (1957) found that rats fed highly purified β-CD as part of the carbohydrate requirement in their diet died after one week. However later information revealed that the rats refused to eat the test diet and more likely died from insufficient food intake

rather than the toxic effects of the CD (Anderson et al, 1962). Recent toxicity studies of β-CD given orally to rats and dogs showed no side effects during long term administration (Gergely et al, 1981). The LD-50 for oral administration of β-CD in rats is greater than 5000 mg/kg (for comparison, the LD-50 for NaCl is 2,500 mg/kg) and greater than 372.9 mg/kg for intraperitoneal applications (Gergely et al, 1981). These findings are in accordance with expectations that CDs as such do not penetrate from the gastrointestinal tract into the blood stream. Thus, if CD are degraded by gastrointestinal bacteria, only glucose and short chain oligomers should be produced with no expected toxic effects (Pitha et al, 1981).

CD metabolism was investigated by Anderson et al (1963) through oral administration of ^{14}C-β-CD to rats. Results showed that peak radioactivity (48 - 65% of ^{14}C recovered) exhaled by the rats occurred between 4 and 10 h. Recently, a similar study by Gerloczy et al (1981) showed that maximum radioactivity (approximately 52 - 69% of ^{14}C recovered) appeared in the respired CO_2 from the rats within the first 2 h for ^{14}C labelled glucose as compared to 4 - 8 h for the ^{14}C labelled β-CD. These results indicate that CDs are metabolized by the rat and the time delay in release of ^{14}C suggests that the intestinal flora are responsible for CD utilization or partial degradation. When radioactivity was measured in the blood after oral administration of ^{14}C-β-CD to rats, highest radioactivity was observed between the 6th and 8th h (Szejtli, 1981). Szejtli (1981) suggested that adsorption

of CD does not occur from the small intestine, but from the colon where CD are presumably degraded. *In vivo* studies involving rats and rabbits showed that colon organisms were able to degrade CDs (Szabo et al, 1980). These results also suggest that the microflora of the colon may have an important role in the metabolism of CDs. Although information concerning human metabolism of CD is not yet available, research involving human metabolism of other complex carbohydrates provides evidence that certain colon organisms may be involved in the degradation and/or metabolism of various food carbohydrates (Salyers et al, 1977).

The most common bacterial species of the human colon are the *Bacteroides* species (Holdeman et al, 1976, 1977). *Bacteroides* comprise approximately 20% of the total colon bacterial population. *Bacteroides* are saccharolytic, non-spore forming, anaerobic species that ferment a variety of simple sugars (Salyers et al, 1977). However, since most simple sugars are adsorbed in the small intestine, a more likely substrate available to the bacteria in the human colon are the food polysaccharides (Salyers et al, 1977). *In vivo*, fractions containing carbohydrates characteristic of mucin and plant polysaccharides were found at lower concentrations in the colon than in the ileum of 4 accident victims (Vercelotti et al, 1977), suggesting that degradation of these carbohydrates takes place in the colon.

Information concerning the ability of *Bacteroides* species to utilize complex plant polysaccharides is limited. Salyers et al (1977) surveyed 188 strains of ten *Bacteroides* species from the human intes-

tinal tract for their ability to utilize polysaccharrides for growth. This study involved substrates that reflected a wide variety of carbohydrate components and linkages. Results indicated that a number of carbohydrates that were normally considered inert, indigestible substances (i.e. alginate, guar gum, laminarin, etc.) were degraded by colon bacteria. Also, certain strains showed a surprising range of glycosidase activities and degraded polysaccharides containing a wide range of glycosidic linkages (Salyers et al, 1977). An extension of this survey included 154 strains of 22 species of the genera *Bifidobacterium*, *Peptostreptococcus*, *Lactobacillus*, *Ruminococcus*, *Coprococcus*, *Eubacterium*, and *Fusobacterium*. No species of these genera, which account for 50% of the normal human flora, fermented as wide a variety of carbohydrates as those of *Bacteroides* species (Salyers et al, 1977).

Bacteroides strains also show ability to synthesize enzymes when grown in the presence of various carbohydrates. When laminarin (a linear β 1,3 glucan) was degraded by 3 species of *Bacteroides*, Salyers et al (1977 b) found that the laminarinase activity of these 3 species was induced by growth on laminarin, rather than constitutive. Balascio et al (1977) found that enzymes in 3 *Bacteroides* strains which catalyzed degradation of xylan and guar gum were also induced. *Bacteroides* were also able to degrade peanut cell wall polysaccharides, utilizing cell bound, inducible enzymes (Dekker, 1979). It may be advantageous for organisms to induce enzymes to degrade various substrates, particularily in a competitive environment (Salyers et al, 1977).

The purpose of this study was to identify *Bacteroides* strains which would grow on CDs, to investigate the *in vitro* degradation of CD by certain *Bacteroides* strains, to isolate and examine some properties of the enzymes causing degradation, to identify the products of CD hydrolysis by these enzymes, and attempt to purify the enzyme(s) responsible for cleaving the cyclic ring of CD.

MATERIALS AND METHODS

A. Bacterial Strains

Bacteroides strains were supplied by the Anaerobe Laboratory, Virginia Polytechnic Institute and State University. Stock cultures were maintained in chopped meat broth (CMB) under a CO_2 atmosphere at room temperature (Holdeman and Moore, 1976) and were transferred every 6 weeks. Working stock cultures were inoculated from the mother stock cultures when needed. Gram stains were used periodically after transfers to check for culture contamination.

B. Cyclodextrins

α and β-CD used in the screening studies and enzymatic assays were a gift from Dr. Derek Ball, U.S. Army Natick Laboratories, Natick, MA. β-CD used in growth and enzymatic assays was from Sigma Chemical Company (St. Louis, MO). All CDs were checked for purity by high performance liquid chromatography (HPLC).

C. Screening for Fermentation of CDs by *Bacteroides*

The CDs hydrolyzing ability of *Bacteroides* strains was tested using the replicator method of Wilkins and Walker (1975). This microsystem permits testing carbohydrate fermentation patterns of large numbers of anaerobic bacteria.

1. Replicator Medium

Growth medium consisted of a defined medium of Varel and Bryant (1974) for growth of *Bacteroides*, modified by Salyers et al (1977) by eliminating carbonate buffer and adding agar and phenol red (Table 1).

Table 1. Composition of the basal medium used in replicator, and growth studies

Component	Amount/liter
$(NH_4)_2SO_4$	1.0 g
Vitamin B_{12}	5.0 µg
Hemin	5.0 mg
K_2HPO_4	2.26 g
KH_2PO_4	0.9 g
$FeSO_4 \cdot 7H_2O$	4.0 mg
NaCl	0.9 g
$CaCl_2 \cdot 2H_2O$	27.0 mg
$MgCl_2 \cdot 6H_2O$	20.0 mg
$MnCl_2 \cdot 4H_2O$	10.0 mg
$CoCl_2 \cdot 6H_2O$	10.0 mg
Phenol Red[a]	0.1 g
Agar[a]	1.5 g
Cysteine HCl	0.5 g
Carbohydrate[b]	5.0 g

[a] Used only in the replicator studies.

[b] Alpha or beta CD as indicated in the text.

All chemicals were of reagent grade quality. All components of the medium, except cysteine HCl and CD, were mixed with distilled water under aerobic conditions and pH was adjusted to 7.6. The mixture was autoclaved for 15 min at 121° C. Filter sterilized cystein HCl (0.05g/100ml) and autoclaved CD solution were mixed with the medium to give a final substrate concentration of 5 mg/ml. HPLC analysis showed no breakdown of CD during autoclaving. The pH of the medium was checked aseptically and necessary adjustments were made using 8N NaOH to give a final pH of 7.6.

2. Dispensing Medium into Microtiter Plates

A sterilize glass syringe (Cornwall, Becton-Dickinson Co.) fitted with an 8 prong dispensor was flushed first with boiling water and then with medium. One depression of the syringe filled 8 wells of the microtiter plates (Librio, Mclean, VA) with a premeasured volume of medium. Medium was rapidly dispensed into the 96 wells of sterile microtiter plates. The plates were covered with plastic lids, cooled, and incubated aerobically at 37° C for 24 h to check for contamination. Plates were placed in an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, MI) for 4 days prior to inoculation to reduce O_2 tension.

3. Inoculation

All procedures were carried out in an anaerobic glove box. Stoppered tubes containing cultures of the 30 *Bacteroides* strains were opened individually, 0.5 ml of inocula was withdrawn with a sterile pipet and dispensed into designated wells of the sterilized 96 well

master plate. The 30 cultures were used to fill 60 wells, thereby seperating the plate into two halves. Inocula was dispensed in a checker-board pattern leaving 36 uninoculated wells as controls. The replicator head was depressed to change the 96 brads with inoculum from the wells of the master plate. A microtiter plate containing growth medium was then positioned under the replicator head and inoculated by depression of the head into the growth medium contained in the wells of the microtiter plates. The brads of the head could then be recharged repeatedly with inocula for inoculation of additional plates.

4. Sealing and Incubating Inoculated Plates

After inoculation, the microtiter plates were covered with non-toxic plate sealers (Cook Engineering Co., Alexandria, VA). This was done to reduce the possibility of cross contamination of wells by motile bacteria and diffusion of volatile fatty acids (Wilkins and Walker, 1975). Pin holes were punched over each well to allow the escape of gases produced during fermentation. Sealed plates were incubated anaerobically for 7 days at 37° C.

5. Determination of CD Fermentation

A reduction of pH of the inoculated medium was used as an indicator of growth in the replicator system. Inoculated plates were monitored for pH reduction on a daily basis by observing a change in color from the initial red to yellow (reaction of acid with phenol red). After 7 days, plates were removed from anaerobic incubation and the

pH of each well was measured using a pH micro-electrode. A pH reduction of 1.0, as compared to control wells, was considered as fermentation of CD by a given strain.

D. Growth Medium

The growth medium composition was the same as the replicator medium (Table 1) except that no agar or phenol red was added and all components except cysteine-HCl were mixed and boiled for 20 min to reduce oxygen tension. The medium was cooled to room temperature while sparging with O_2-free N_2; cysteine-HCl was added, and the pH was adjusted to 7.1 with 8N NaOH. The medium was anaerobically dispensed into glass anaerobic culture tubes (Bellco Glass Inc., Vineland, NJ). The tubes were stoppered, press racked and autoclaved for 15 min at 121° C. Upon cooling, the tubes were incubated at 37° C for 24 h to check for contamination. All medium preparations, pH adjustments, and medium dispensing were performed under N_2 using the VPI Anaerobic Culture System (Bellco, Inc., Vineland, NJ).

E. Bacterial Growth

Two *Bacteroides* strains were selected randomly from the replicator study for detailed examination. Both strains were transferred at 1% inoculum level into CMB and incubated for 18 h at 37° C. Cultures were then diluted 1:100 in 1% peptone blanks and 1% inoculum was transferred into CMB and incubated at 37° C for 18 h. CD growth medium was then inoculated at the 5% level and incubated for 18 h at 37° C. All transfers were made under O_2-free N_2.

F. Preparation of Crude Cyclodextrinase

After incubation, cultures were harvested by centrifugation at 17,000 x *g* for 15 min, 4° C in a model RC-5B Sorvall refrigerated centrifuge (Newton, CT). The cell free supernatent fluid was collected to represent the crude extracellular enzyme fraction (ECF). The cell pellet was washed by resuspending in an equal volume of 0.05M phosphate buffer (pH 6.8) and centrifuged (17,000 x *g*, 15 min, 4° C). The supernatent fluid was discarded and the pellet was resuspended in phosphate buffer containing 1 mM dithiothreitol.

Cells were disrupted using a sonifier cell disrupter W 185 (Ultrasonics Inc., Plainview, NY). Sonication totalled 4 min; two 2 min pulses with a 2 min cooling period in between. Cell suspensions were kept on ice during sonication. After sonication, cell suspensions were centrifuged (17,000 x *g*, 15 min, 4° C) and supernatent fluid was collected to represent the crude intracellular enzyme fraction (ICF). All enzyme fractions were stored at -20° C.

G. Enzyme Assay

Cleavage of α-1,4 bonds between glucose units comprising CDs yields new reducing ends which were quantified colormetrically by the reducing end assay.

1. Reducing End Assay

The increase in reducing ends (calculated as glucose) of an enzyme-substrate incubation mixture was measured using a modified method of Mcfeeters (1979). Reagent A was prepared by adding 1 g of

$CuSO_4 \cdot 5H_2O$ and 3.7 g of aspartic acid to H_2O giving a final volume of 1 l. Reagent B was prepared by adding 38 g of $NaCO_3$ and 2 g of Sodium 2,2' bicinchoninate (Sigma Chemical Co., St. Louis, MO) to H_2O giving a final volume of 1 l. Both reagents were stored at room temperature and protected from light.

α and β-CDs were dissolved in phosphate buffer (0.05 M, pH 6.8) to a concentration of 10 mg/ml. The CDs were dissolved by stirring at 85° C and dispensed into glass test tubes. The tubes were stoppered, press racked and autoclaved. Substrate solutions were stored at room temperature until use.

Assay mixtures were prepared by adding one volume of crude enzyme to one volume of 10 mg/ml substrate solution. Final substrate concentration was 5 mg/ml. This final substrate concentration was not a limiting concentration but was selected for routine assay because the limited solubility of CD prevented preparation of solutions with concentrations greater than 10 mg/ml. The enzyme-substrate mixtures were incubated for 4 h at 37° C, unless otherwise noted. After incubation, the mixtures were boiled for 10 min to inactivate enzymes and were then centrifuged (10 min, 5,000 x *g*). An aliquot (100 ml) of 1:10 diluted sample was mixed with distilled H_2O to give a final volume of 2 ml. Reagents A and B were mixed 1:1 just before use and 2 ml of mixed reagent was added to each sample. Samples were mixed, boiled for 2.5 min, cooled for 10 min, and absorbancies were read at 560 nm in a Perkin-Elmer Lambda 3 UV-VIS Spectrophotometer (Coleman

Instruments, Oak Brook, IL). A water blank and boiled enzyme control were tested in parallel with each set of assays. Enzyme activity was proportional to protein concentrations of crude enzyme preparations up to 500 µg protein.

H. Protein Determination

Protein concentration was determined by the method of Lowry et al, (1951). Bovine serum albumin (1 mg/ml and 5 mg/ml) served as a standard. Absorbancies were read at 750 nm.

I. High Performance Liquid Chromatography (HPLC)

1. Equipment and Columns

Products of CD hydrolysis were examined by HPLC. A model ALC 201 chromatograph was equipped with a model 6000 A solvent delivery system, a model R-401 refractive index detector, a 440 UV absorbance detector (Waters Assoc., Milford, MA), a model 2175 injector system (Rheodyne, Berkeley, CA), a Perkin-Elmer M-2 calculating integrator (Coleman Instruments, Oak Brook, IL), and a dual channel omniscribe recorder (Houston Instruments, Austin, TX).

A "µ Bondapak / carbohydrate" column (Water Associates, Milford, MA) was used for separating monosaccharides and short chain oligosaccharides (Palmer, 1975, 1976, Conrad and Palmer, 1976). Samples were eluted with 70% acetonitrile / 30% H_2O mixtures at a flow rate of 2.0 ml/min (2000-2500 psig). The elution solvents were filtered through 0.45 or 0.50 µm pore size filters (Millipore Corp., Bedford, MA) and degassed under vacuum before use.

2. HPLC Standards

Maltooligosaccharide standards of degrees of polymerization (DP) ranging from DP1 to DP7 were prepared by hydrolysis of CD with sulfuric acid according to the method of Kondo et al (1981). This involved dissolving 3 g of β-CD in 9 ml of boiling distilled H_2O, adding 2.7 ml of 1 N sulfuric acid, and holding the mixture for 1.5 h at 100° C. The volume of reaction mixture was kept approximately constant by occasionally adding distilled H_2O. The reaction was quenched by adding 2.4 g of $BaCO_3$. The reaction mixture was filtered through Whatman filter paper No. 41 and a 0.5 μm pore Millipore filter successively. The filtrate was diluted 3:2 with acetonitrile, vortexed, and filtered through 0.3 μm glass fiber filter (Gelman, Ann Arbor, MI).

Standard solutions (10 mg/ml) of α and β-CD were prepared by dissolving appropriate quantities of CD in distilled H_2O and diluting with acetonitrile and filtering as above. Standard solutions were kept frozen when not in use and were re-filtered after thawing.

3. Preparation and Identification of Sample Mixtures

Reaction mixtures were diluted 3:2 with acetonitrile prior to analysis to precipitate any insoluble materials in the eluent employed. The mixtures were deionized by adding an equivalent volume of mixed bed ion-exhange resin (AG 501-x8, 100-200 mesh, Biorad Lab., Richmond, VA) and stirred for 10 min at room temperature. The deionized samples were filtered through 0.3 μm glass fiber filters. A 4 ml aliquot of the filtered sample was placed into a graduated reactivial and heated

to 40° C in a reacti-therm heating module (Pierce Chem. Co., Rockford, IL) while a gentle stream of nitrogen gas agitated the surface of the solution. Heating was halted when the solution evaporated to 1 ml. This procedure increased sugar concentration of the sample solutions approximately 4-fold.

Between .02 and .95 ml of samples and standards were injected with a 25 or 100 µl syringe (Prescision Sampling Corp., Baton Rouge, LA) into the HPLC for separation on the "µ Bondapak / carbohydrate" column. Eluting glucose, maltose, and higher maltooligosaccharides were detected with the refractive index detector and identified from their retention times as compared to standards. However, no attempt was made to precisely quantify the individual products due to lack of appropriate standards. Relative concentrations were determined from the peak heights on the chromatograms.

J. Determining Properties for Crude Cyclodextrinase

1. Temperature Optimum

An aliquot of cyclodextrinase was mixed with an equal volume of 10 mg/ml CD substrate solution (pH 6.8), and incubated for 4 h at 4°, 25°, 37°, 42°, 55°, and 80° C. Mixtures were then analyzed for reducing sugar (as glucose) as previously described.

2. Temperature Stability

Volumes of 5 ml of cyclodextrinase were incubated at 4°, 25°, 37°, 42°, 55°, and 80° C for a total of 48 h. After incubation, cyclodextrinase activity was determined for 500 ml aliquots of enzyme by the

reducing end assay.

3. pH Optimum

A .5 ml aliquot of cyclodextrinase was dispensed into an equal volume of buffered CD solution (10 mg/ml) of pH 3, 4, 5, 6 (citrate, 0.05 M), 7, 8, 9 (Tris, 0.05 M), and 10 (carbonate, 0.05 M). Mixtures were incubated for 4 h at 37° C, and then analyzed for reducing sugar (as glucose) as previously described.

4. pH Stability

A 5 ml aliquot of cyclodextrinase was dispensed into an equal volume of buffer solution of pH 3, 4, 5, 6 (citrate, 0.05 M), 7, 8, 9 (Tris, 0.05 M), and 10 (carbonate, 0.05 M). The pH was checked, and adjustments were made when necessary. Mixtures were stored at 4° C for 48 h. Cyclodextrinase activity was determined for 500 µl aliquots of enzyme via the reducing end assay.

K. Growth Study

Test tubes containing 9.5 ml of CD medium were inoculated at a 5% level with the appropriate organism. The tubes were incubated at 37° C. At 2 h intervals up to 18 h, tubes were removed from incubation for analysis. Bacterial growth was anlyzed by optical density (turbidity) as compared to uninoculated controls at 600 nm using a Bausch and Lomb spectrophotometer (Rochester, NY). Bacterial cells were harvested to obtain ECF and ICF as previously described. The protein content of disrupted cell suspensions (intracellular fraction) was determined by the method of Lowry et al (1951). Cyclodextrinase activity was

assayed as previously described.

L. Enzyme Purification

1. Preparation of Crude Enzyme

Bacterial cells were grown and harvested essentially as described previously (section F) except that 2,400 ml of induction medium was inoculated at the 5% level. Also, the pellet was resuspended into 1/10 the original volume of phosphate buffer prior to sonication.

2. Nucleic Acid Removal and Salt Concentration of Crude Enzyme

$MnSO_4$ was added to the intracellular crude enzyme fraction (0.1 M final concentration) to remove nucleic acids (Higgins et al, 1978). The mixture was gently stirred until the salt dissolved and then centrifuged (17,000 x *g*, 15 min, 4° C) to sediment the precipitate. The supernatent fluid was collected, ammonium sulfate was added to 40% saturation, and the mixture was centrifuged as above. Supernatent fluid was collected and dialyzed (Membrane dialysis tubing, 3500 M.W. cut off, Spectrum Medical Industries, Inc., Los Angeles, CA) against several changes of distilled water for 5 days. After dialysis, the enzyme fraction was lyophilized and kept in frozen storage until use. These procedures are outlined (Fig. 1).

3. Hydrophobic Interaction Chromatography

A model 2137 chromatography column (65 cm, 2.0 cm^2, LKB Instruments, Rockville, MD) containing approximately 60 ml bed volume of phenyl sepharose CL-4B (Pharmacia Inc., Piscataway, NJ) was equilibrated with 500 ml of 40% ammonium sulfate in phosphate buffer (0.05 M, pH 6.8).

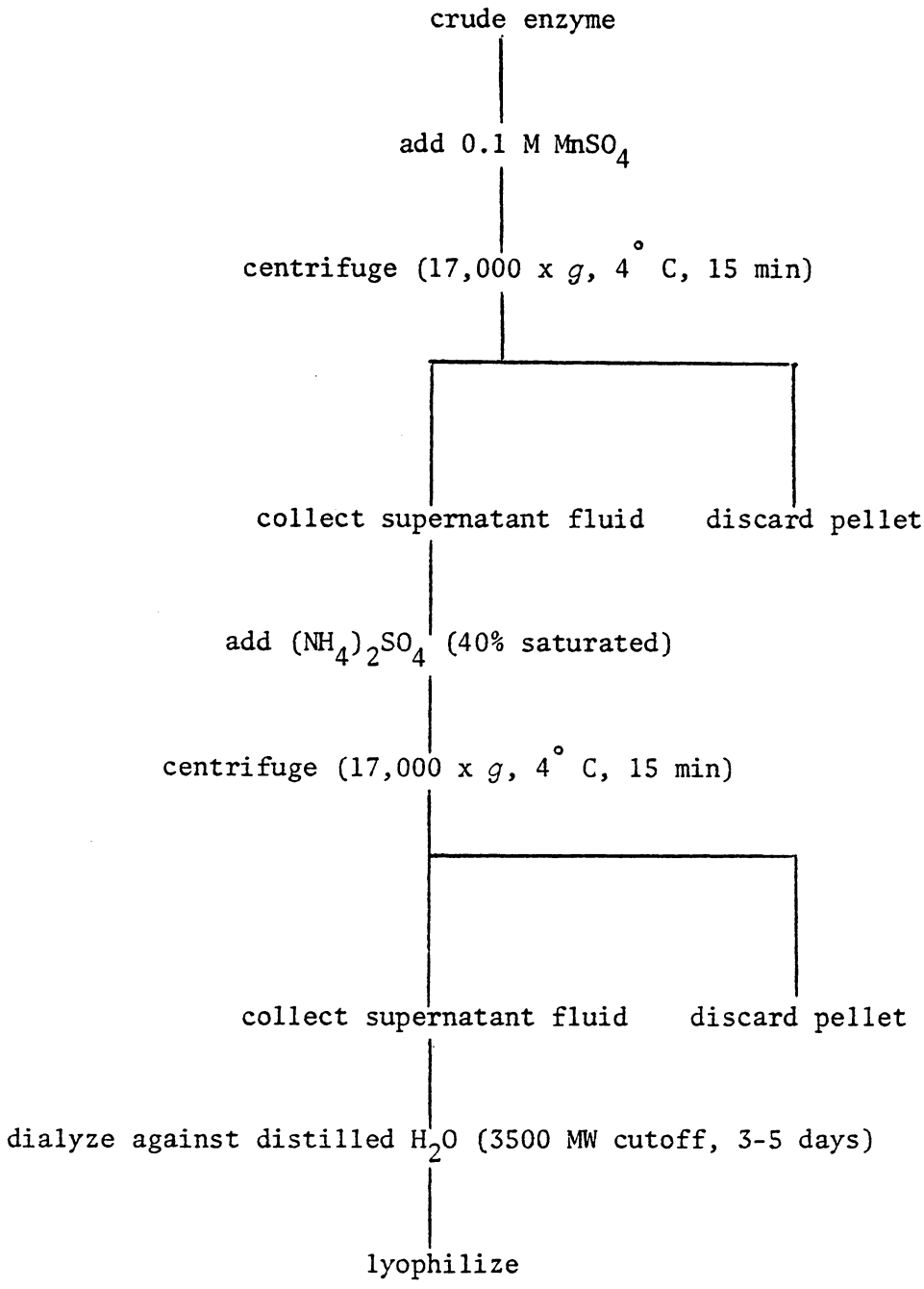


Figure 1. Flow diagram for nucleic acid removal and salt concentration of enzyme.

Lyophilized enzyme (50 mg) was rehydrated in 10 ml of 40% $(NH_4)_2SO_4/PO_4$ buffer, centrifuged (17,000 x *g*, 10 min, 4° C) and supernatent fluid was collected. Sample was loaded onto the column by gravitational force. Sample was eluted through the column using a reverse gradient of $(NH_4)_2SO_4$. Flow rate was 50 ml/h and 5 ml fractions were collected using a model alpha-400 fraction collector (Bachler Instruments, Fort Lee, NJ). Protein was detected by measuring absorbancies of fractions at 280 nm. All peaks were assayed for cyclodextrinase activity as previously described.

4. Electrophoresis

Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was conducted in 7.5% polyacrylamide slab gels (Fairbanks et al, 1971). Freeze dried enzyme fractions were dissolved in 0.01 M Tris-HCl, pH 8.0, containing 1 mM EDTA, and 1% SDS. Bromphenol blue and sucrose were added to samples and proteins were separated by electrophoresis at 80 v for 3 h. Polypeptides were stained with 0.05% Coomassie blue in 50% methanol (v/v) and 10% acetic acid (v/v) (Weber and Osborn, 1969). Phosphorylase b, bovine serum albumin, ovalbumin, carbonic amhydrase, soybean trypsin inhibitor, and α-lactalbumin were purchased from Pharmacia Fine Chemicals (Piscataway, NJ) and were used as molecular weight standards.

RESULTS

A. Replicator Screening for Fermentation of CDs

The *Bacteroides* strains screened in this study were selected based upon their ability to degrade certain carbohydrates (Salyers et al, 1977). The screening procedure resulted in 24 of the 30 *Bacteroides* strains tested showing ability to degrade and grow on CDs. Although the final determination was made 7 days after inoculation, most of the 24 strains showed evidence of growth after 24 h of incubation. The screening results are summarized in Table 2. Note that strains of DNA homology group 3452-A failed to grow on CDs.

B. Cyclodextrinase Activity

Ability to grow on CDs implies the presence of cyclodextrinase, defined here as the enzyme(s) which catalyze CD degradation. Cyclodextrinase activity was detected in cells of *B. ovatus* 3524 and *B. distasonis* C18-7 after 18 h growth of the organisms on CD medium. Higher enzymatic activity was detected in *B. ovatus* 3524, as compared to *B. distasonis* C18-7 using similar conditions of isolation and assay (Table 3).

C. Growth Study

These studies were designed to correlate bacterial growth and protein production with the production and localization of cyclodextrinase.

1. Bacterial Growth

The two *Bacteroides* strains grew at different rates on CD medium.

Table 2. Results of replicator screening for fermentation of CD by different DNA homology groups of *Bacteroides*

Species	Strain[a]	Fermentation[b]
3452-A[c]	C10-2	-
	C7-8	-
	C14-3	-
B. distasonis	B1-20	+*
	C18-7	+*
	C21-1	+*
B. ovatus	R3-39	+*
	0038-1	+*
	T4-7	+
	C1-45	+*
	B4-11	+*
	3524	+*
B. eggerthii	B8-51	-
B. thetaiotaomicron	7330-1	+*
	B1-46	+*
	5951	+*
	T1-37	+
	6180-A	+*
B. uniformis	C7-17	-
	C31-12	+*
	C20-25	-
B. vulgatus	C43-46B	+*
	C11-25	+*
	OC-13	+
	R1-5	+*
	C10-6	+*
B. fragilis type I	0479	+
B. fragilis type II	A3-18B	+*
	C40-1	+*
	C48-32	+*

[a] Strains obtained from the VPI Anaerobe Lab. culture collection.

[b] Symbols: - negative; + positive reaction as indicated by pH reduction of 1.0 as compared to controls after 7 day 37° C incubation; * indicates pH reduction detected within 24 h.

[c] Unnamed DNA homology groups are designated by the number of the reference strain.

Table 3. Alpha and beta CD hydrolysing activities found in disrupted cells of two *Bacteroides* species from the human colon

Bacteroides Species (VPI strain number)	α-CD (units/mg Protein)	β-CD (units/mg Protein)
B. ovatus 3524	10.74 ± 1.2	12.42 ± 1.6
B. distasonis C18-7	7.45 ± 0.7	6.51 ± 0.7

[a] 1 unit = release of 1 μmole of reducing sugar (as glucose) per 4 h.

B. ovatus 3524 entered logarithmic growth phase after 2 h incubation and after 10 h incubation entered a stationary phase of growth (Fig. 2). *B. distasonis* C18-7 grew slower, entering logarithmic growth phase after 4 h, and stationary growth phase after 14 h incubation (Fig. 3).

2. Cellular Localization of Cyclodextrinase Activity

Cyclodextrinase activity was measured in intracellular and extracellular fractions from the two organisms. The lowest cyclodextrinase activity was found in the extracellular enzyme fraction of *B. distasonis* C18-7, with intracellular levels of cyclodextrinase activity being about 6 times greater than extracellular levels after 18 h growth (Fig. 3). The cyclodextrinase activity showed a similar localization in *B. ovatus* 3524 up to about 16 h. However, *B. ovatus* 3524 showed a sharp increase in extracellular cyclodextrinase activity at about 16 h growth. Total cyclodextrinase activity of *B. ovatus* 3524 was about 3 times that in *B. distasonis* C18-7 with the activity shared between intracellular and extracellular fractions (Fig. 2).

3. Inducibility of Cyclodextrinase Activity

Both organisms were grown on CD medium for induction periods of 0 to 18 h. Assay of intracellular enzyme fraction showed that cyclodextrinase activity was detected within 2 h of growth for *B. distasonis* C18-7 (Fig. 3) and 4 h growth for *B. ovatus* 3524 (Fig. 2). Maximum cyclodextrinase activity was detected within 6 h growth for *B. ovatus* 3524, as compared to 10 h growth for *B. distasonis* C18-7. No cyclodextrinase activity was detected in either strain when cells were grown

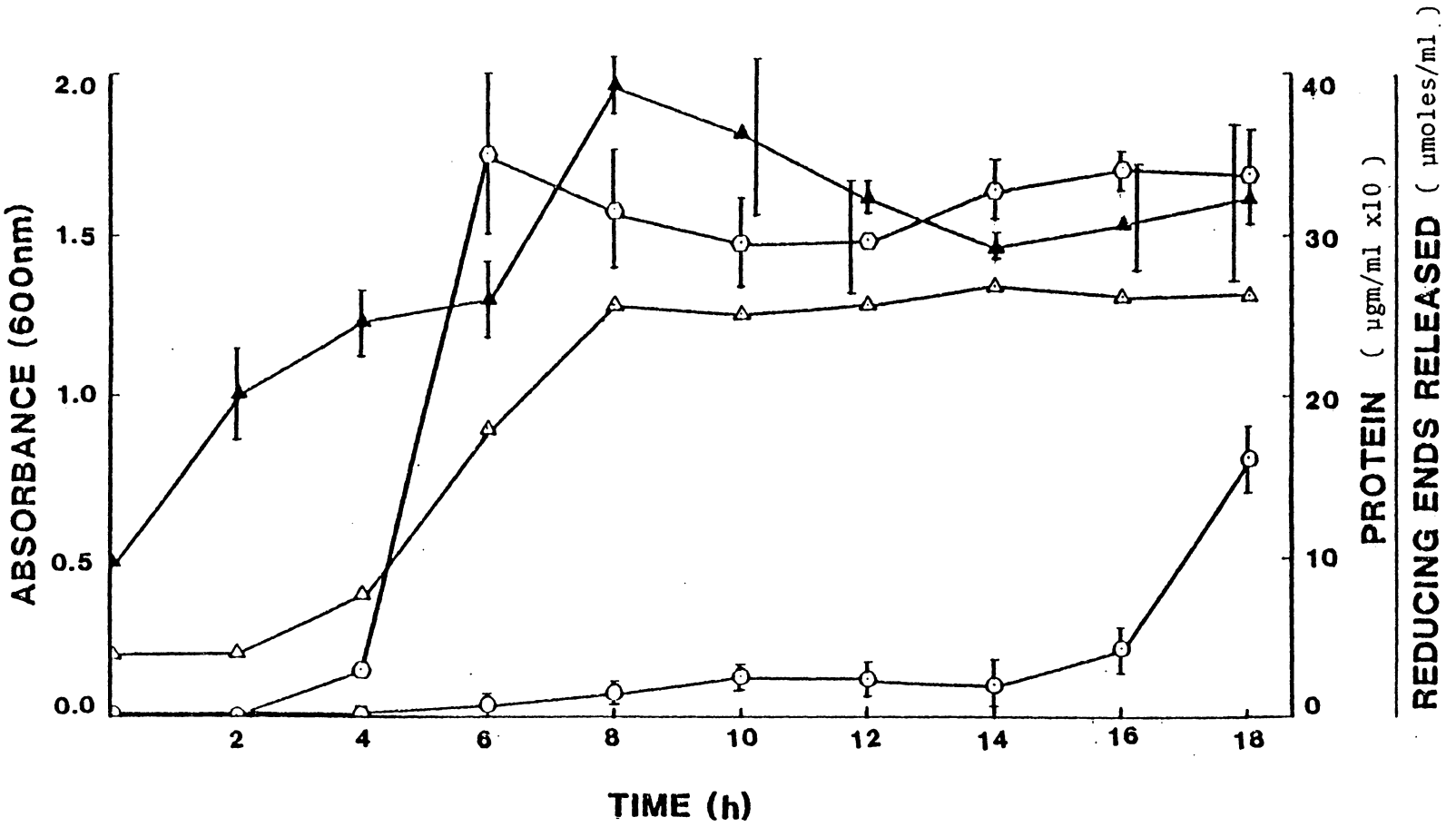


Figure 2. Protein synthesis and cyclodextrinase activity during 18 h (37°C) growth of *B. ovatus* 3524 on CD medium. Symbols: (△) - growth measured by optical density (600nm); (▲) - intracellular protein measured by the method of Lowry et al, (1951); (⬡) - intracellular cyclodextrinase activity, (○) - extracellular cyclodextrinase activity measured by the reducing end assay.

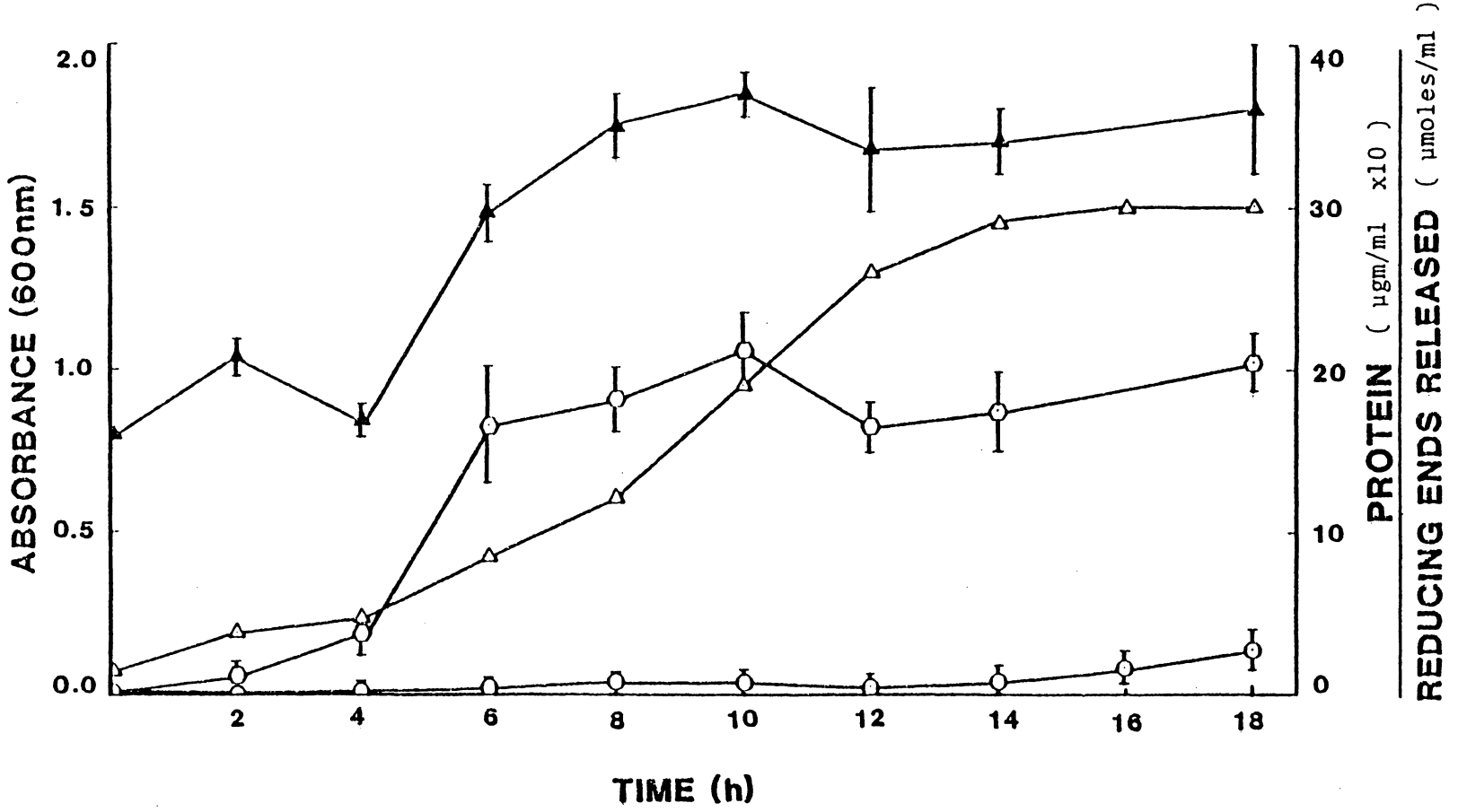


Figure 3. Protein synthesis and cyclodextrinase activity during 18 h (37°C) growth of *B. distasonis* C18-7 on CD medium. Symbols: (△) - growth measured by optical density (600nm); (▲) - intracellular protein measured by the method of Lowry et al, (1951); (⬡) - intracellular cyclodextrinase activity, (○) - extracellular cyclodextrinase activity measured by the reducing end assay.

on medium containing glucose as carbon source. Thus, the cyclodextrinase activity of both strains is induced by growth on CDs.

4. Protein Production

The production of protein increased at a steady and nearly linear rate in both strains, starting after 4 h growth. *B. ovatus* 3524 protein reached a maximum at about 8 h (Fig. 2) as compared to 10 h growth for the same trends in *B. distasonis* C18-7 (Fig. 3).

D. Properties of Crude Cyclodextrinase

1. pH Optimum and Stability

A sharp pH optimum of 7.0 (Fig. 4) is observed for *B. ovatus* 3524 cyclodextrinase after 4 h at 37° C. Also, ≥ 70% relative activity was retained between pH 7.0 and 8.0. Beyond these limits the cyclodextrinase activity dropped sharply approaching 0% at pH 6.0 and 20% at pH 10.0. Cyclodextrinase from this strain showed similar trends for pH stability after 48 h holding periods, with higher stability observed at pH 10.0 (Fig. 4).

B. distasonis C18-7 cyclodextrinase also showed an optimum pH of 7.0 (Fig. 5). However, ≥ 80% activity was maintained over a broader pH range (5.0-7.0). Little or no activity was detected at pH 4.0 or below and cyclodextrinase activity decreased steadily on the alkaline side approaching 0% at pH 10.0. Cyclodextrinase from this strain showed highest stability at pH 6.0 and 7.0 after 48 h. Beyond these limits, cyclodextrinase stability decreased steadily approaching 0% at pH 4.0 and leveling off to 25% at pH 10.0 (Fig. 5).

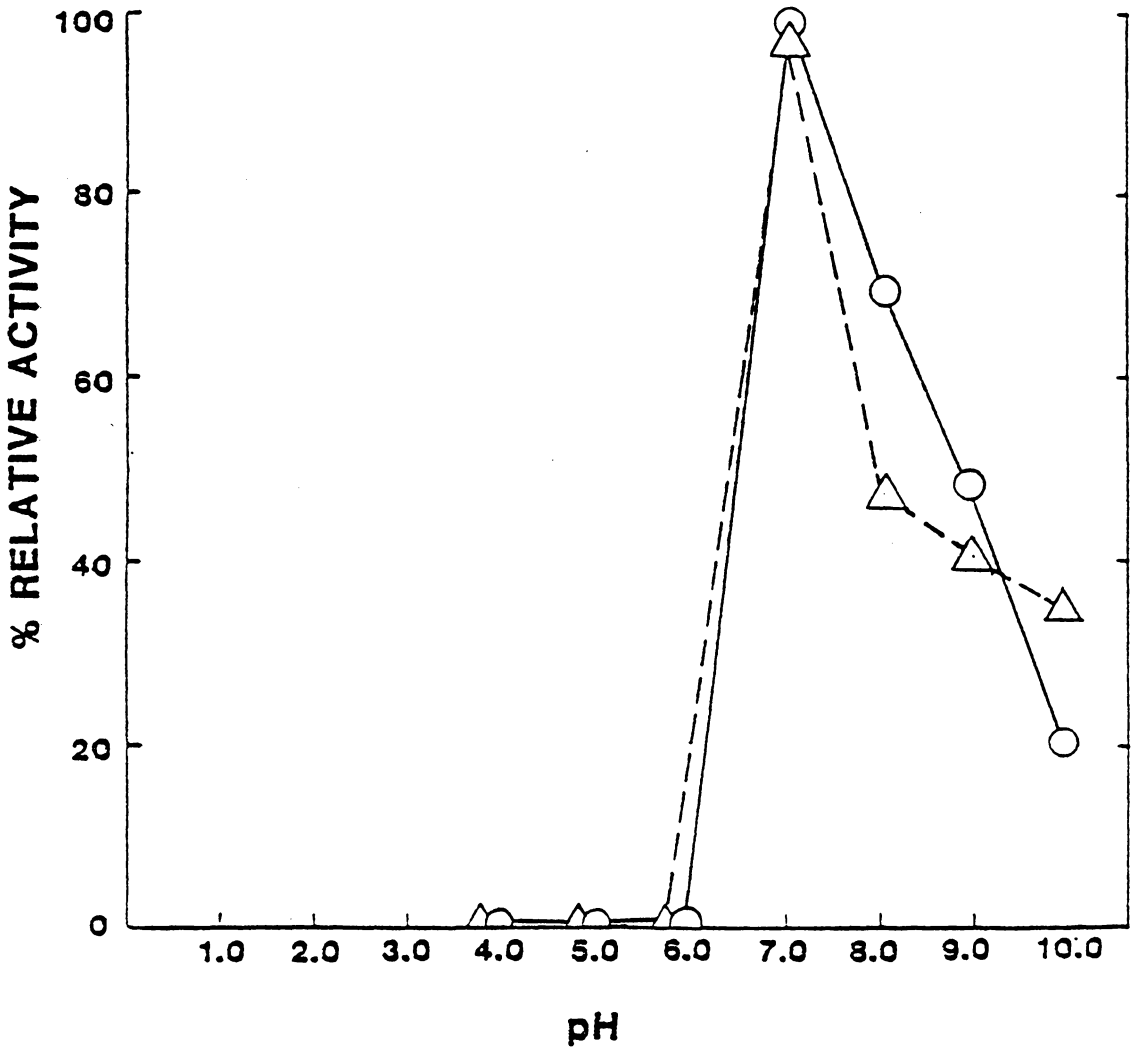


Figure 4. pH optimum (◯) and stability (△) for *B. ovatus* 3524 cyclodextrinase. pH optimum was assayed by measuring the increase in reducing ends (as glucose) after 4 h, 37°C incubation of crude enzyme on CD substrate solution. pH stability was measured by holding the enzyme at the designated pH for 48 h, after which cyclodextrinase activity was measured by the reducing end assay.

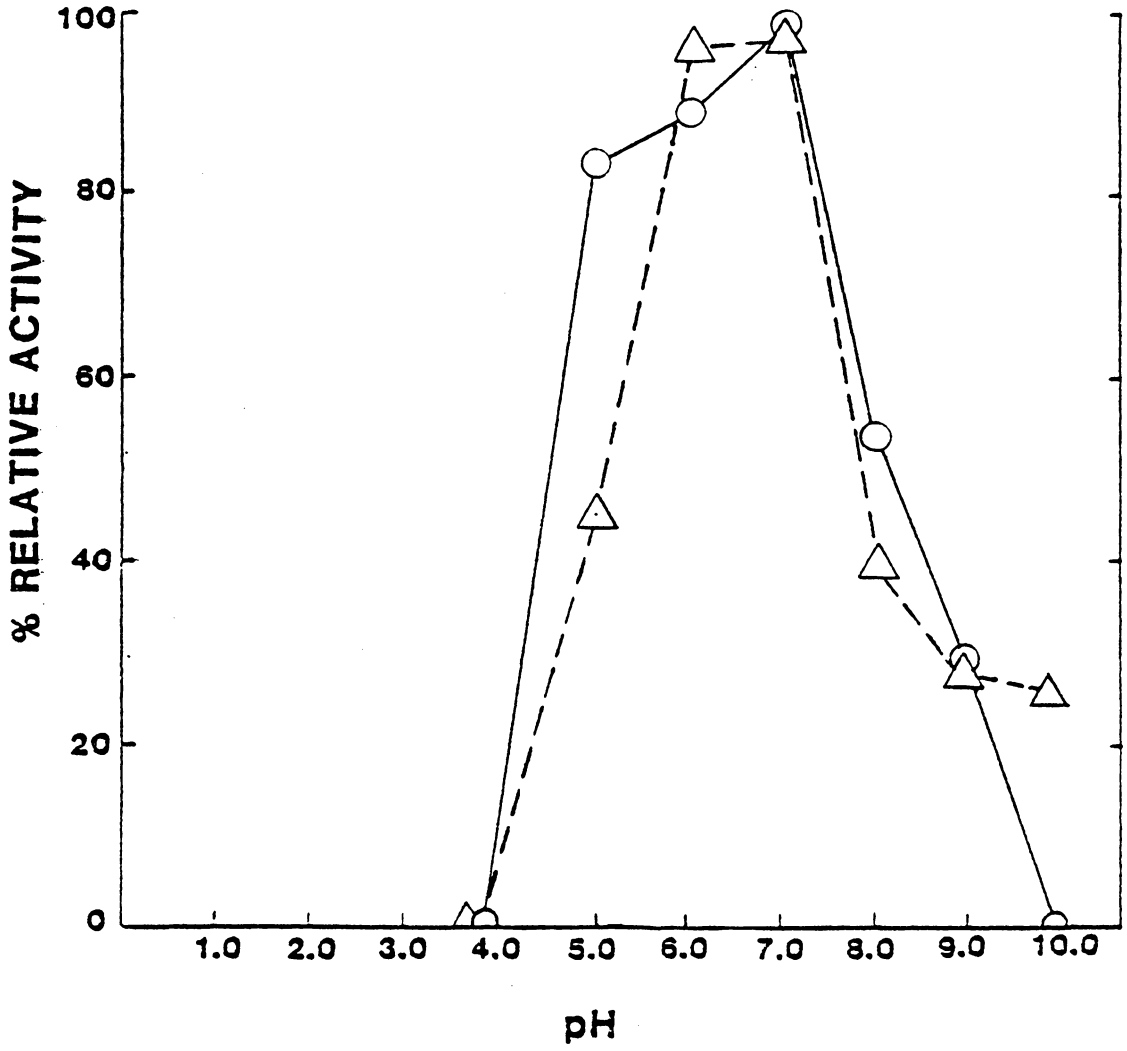


Figure 5. pH optimum (○) and stability (△) for *B. distasonis* C18-7 cyclodextrinase. Conditions as in Fig. 4.

2. Temperature Optimum and Stability

A temperature optimum of 55° C was observed for *B. distasonis* C18-7 cyclodextrinase (Fig. 6), with ≥ 70% activity retained between 37-55° C. Cyclodextrinase from this strain also showed excellent temperature stability with ≥ 75% activity retained between 4° and 55° C after 48 holding periods (Fig. 6).

B. ovatus 3524 cyclodextrinase showed a temperature optimum of 42° C (Fig. 7) and also retained ≥ 70% activity between 37° and 55° C. However, cyclodextrinase from this strain was significantly less stable at higher temperatures with complete inactivation of activity observed after 48 h holding periods at 37° or 55° C. An unusual finding with the *B. ovatus* 3524 enzyme was the higher stability observed at 42° C (Fig. 7). Complete inactivation of cyclodextrinase activity from both strains occured after 4 h when incubated at 80° C (Fig. 6 and 7).

E. Products of CD Hydrolysis

All previous studies of enzyme activity were based on production of reducing ends from CD. The studies in this section were designed to identify the actual products of CD hydrolysis. HPLC proved suitable for rapid chromatographic separation of α-CD and β-CD from each other (Fig. 8) and from the expected products of hydrolysis. However, maltotetrose (DP 4) and α-CD as well as maltopentose (DP 5) and β-CD had similar retention times which made separation difficult. Selectivity was altered by changing the ratio of acetonitrile-water eluent.

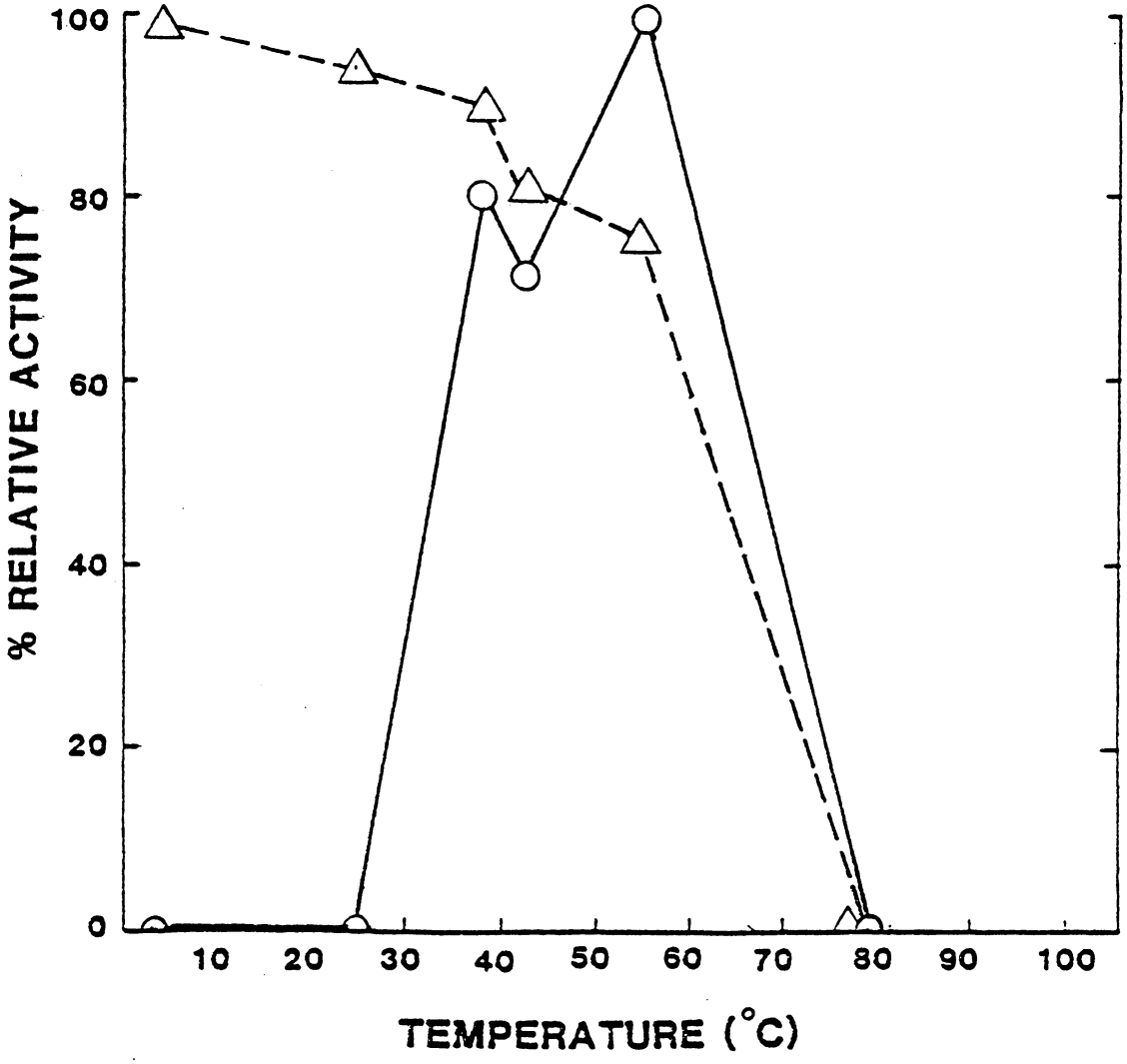


Figure 6. Temperature optimum (○) and stability (△) for *B. distasonis* C18-7 cyclodextrinase. Temperature optimum was assayed by measuring the increase in reducing ends (as glucose) after 4 h incubation (pH 6.8) of crude cyclodextrinase on CD substrate solution at designated temperatures. Temperature stability was measured by holding the enzyme at the designated temperatures for 48 h, after which cyclodextrinase activity was measured by the reducing end assay.

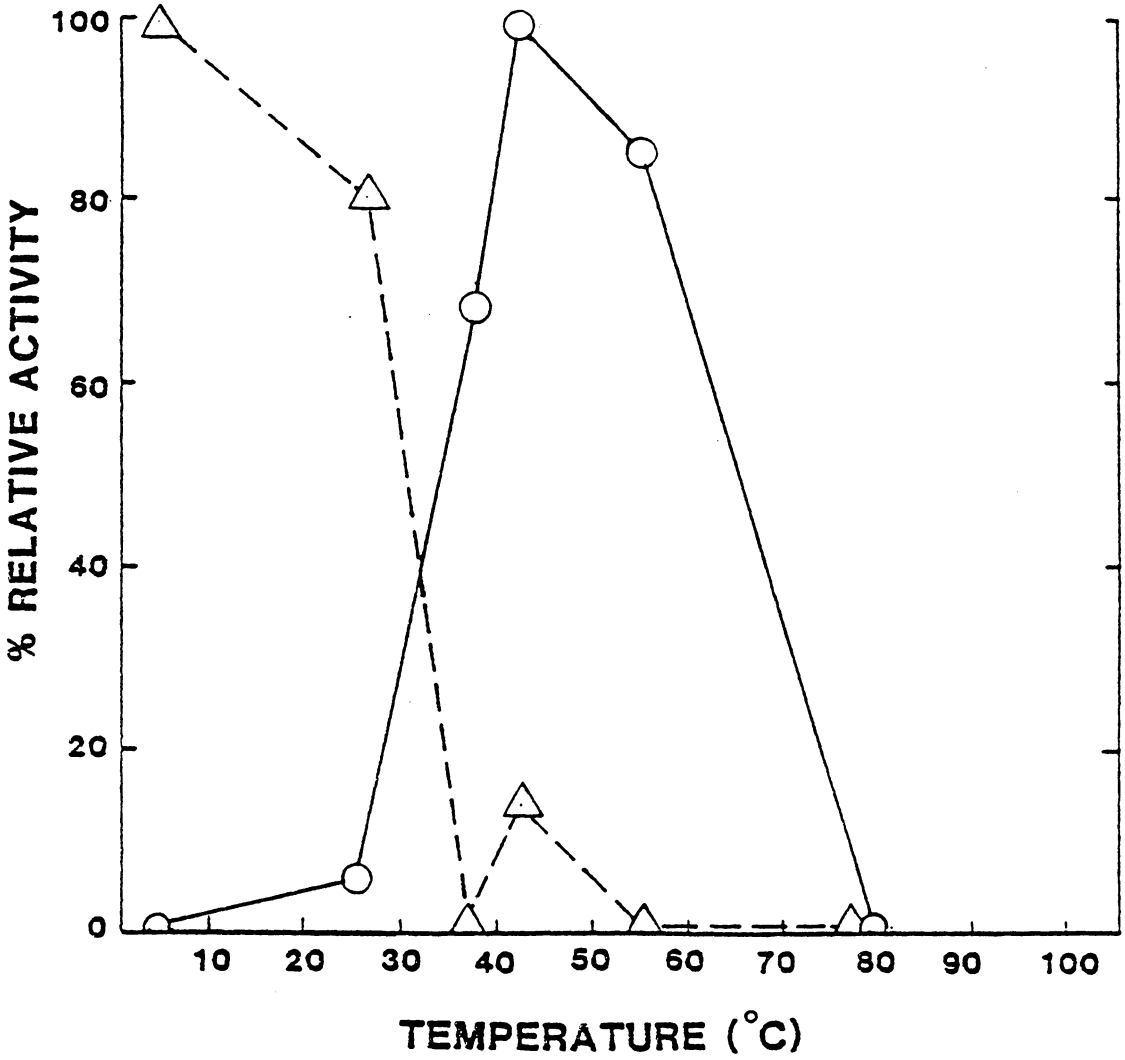


Figure 7. Temperature optimum (○) and stability (△) for *B. ovatus* 3524 cyclodextrinase. Conditions as in Fig. 6.

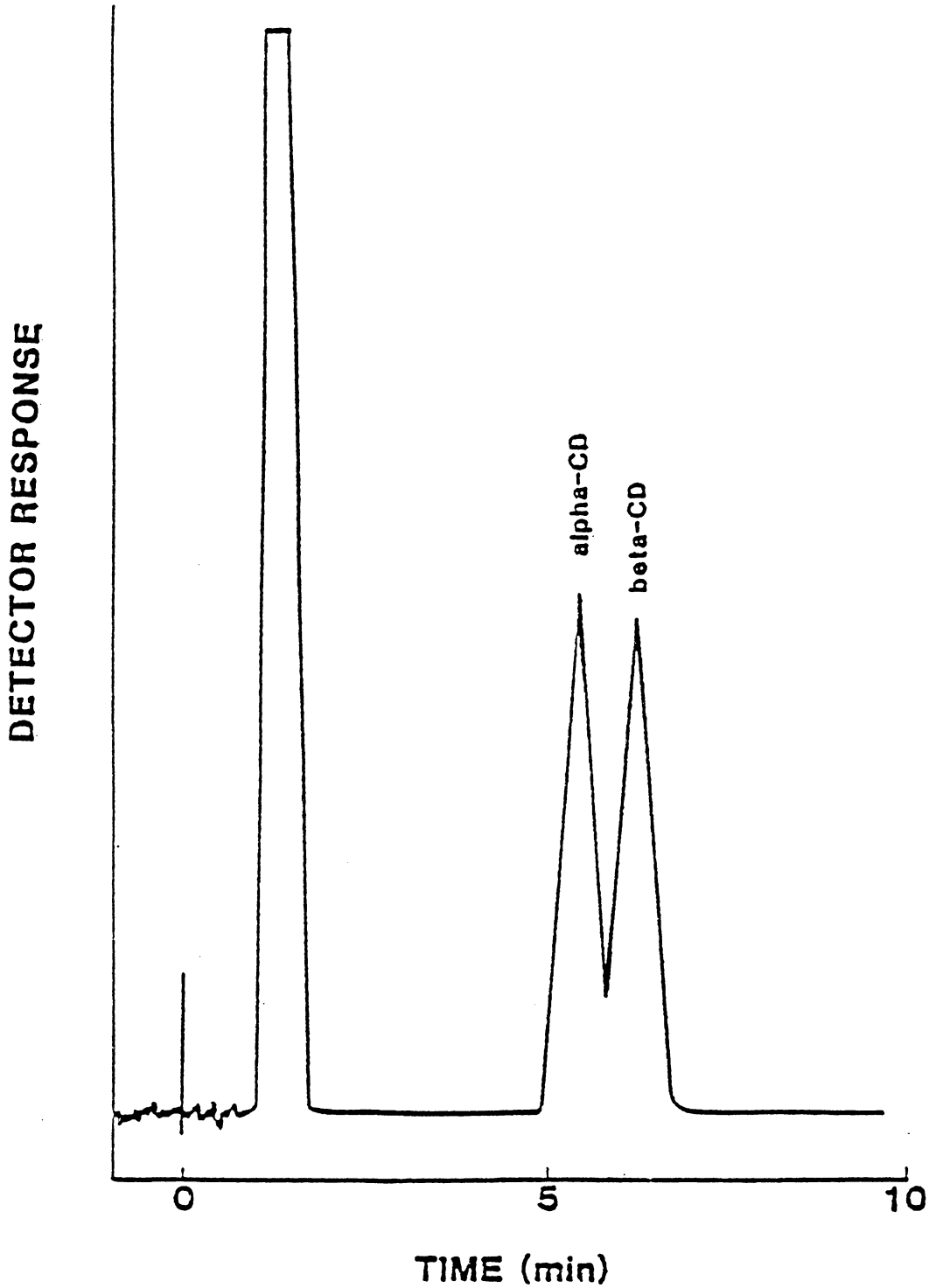


Figure 8. Typical HPLC chromatogram of α- and β-CD. Eluent (70% CH_3CN, 30% H_2O) at a flow rate of 2.0 ml/min, on a μ-Bondapak/carbohydrate column; refractive index detector.

The use of 80:20 v/v acetonitrile-water was successful in separating these compounds.

The products of CD hydrolysis were distinctly different for the enzymes from the two *Bacteroides* strains. The *B. distasonis* C18-7 cyclodextrinase catalyzed breakdown of α-CD to glucose, maltose, and maltotriose, with some undegraded α-CD remaining after 18 h incubation (Fig. 9). β-CD was hydrolyzed similarly by *B. distasonis* C18-7 cyclodextrinase to yield a mixture of glucose, maltose, maltotriose, and maltotetrose after 18 h incubation (Fig. 10). However, the cyclodextrinase from *B. ovatus* 3524 showed a sharply different action pattern hydrolyzing α and β-CD completely to glucose in 18 h incubation (Fig. 11 and 12).

Time course analysis of CD hydrolysis by cyclodextrinase of both *Bacteroides* strains was conducted to examine product formation during early stages of CD hydrolysis. At 1, 3, and 8 h incubation (37° C), *B. ovatus* 3524 cyclodextrinase again showed only production of glucose, with no significant presence of intermediate short chain oligosaccharides (Fig. 13 a, b, c). In contrast, *B. distasonis* C18-7 cyclodextrinase catalyzed formation of glucose-oligosaccharide mixtures at all stages, with the relative proportions of the individual products remaining approximately the same throughout (Fig. 14 a, b, c). Thus, it appears that breakdown of CD by *B. ovatus* 3524 crude cyclodextrinase involves different enzymes than that of *B. distasonis* C18-7 cyclodextrinase.

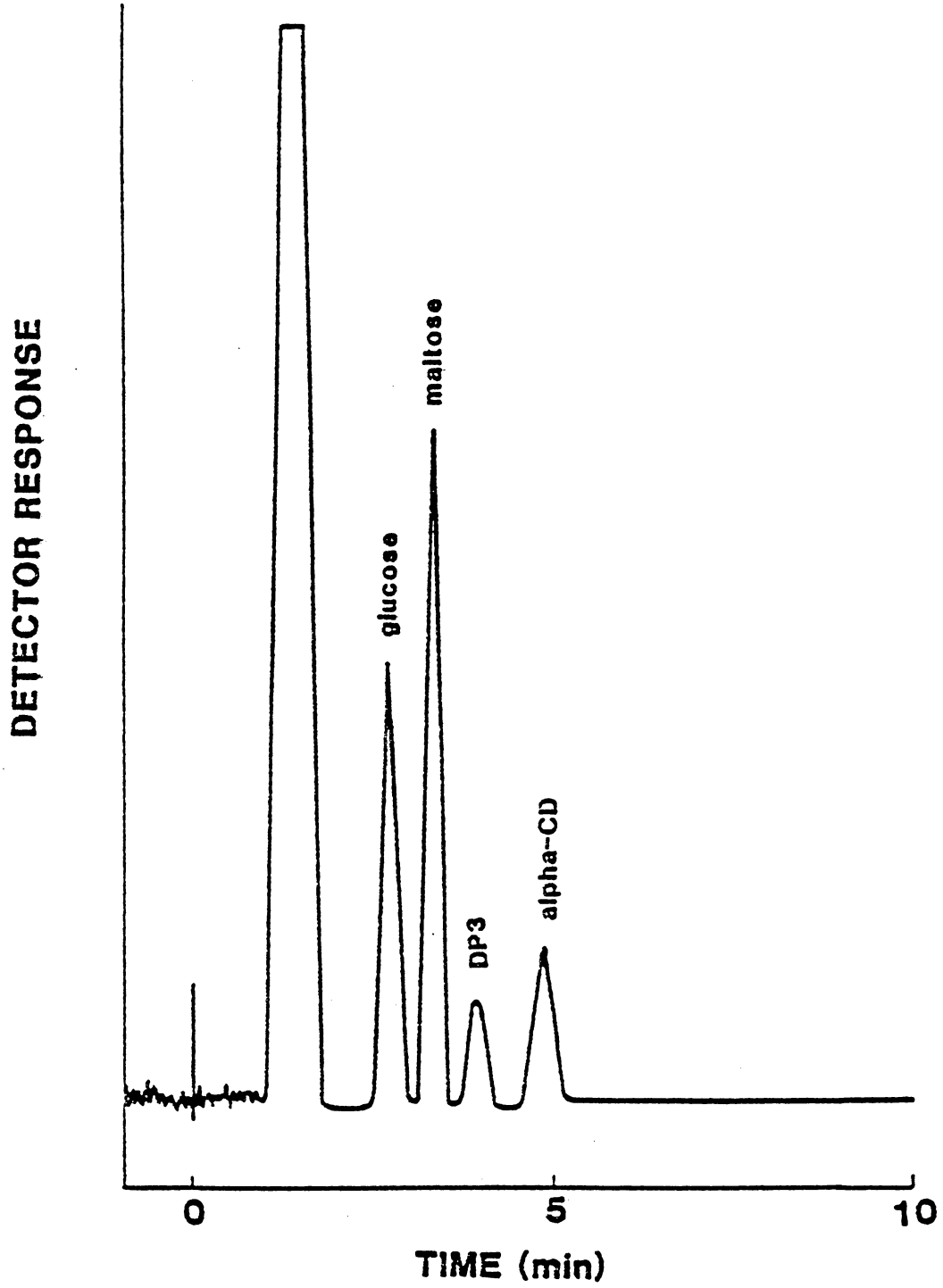


Figure 9. HPLC chromatogram of the products of α-CD hydrolysis by *B. distasonis* C18-7 cyclodextrinase. Conditions as in Fig. 8, (18 h incubation).

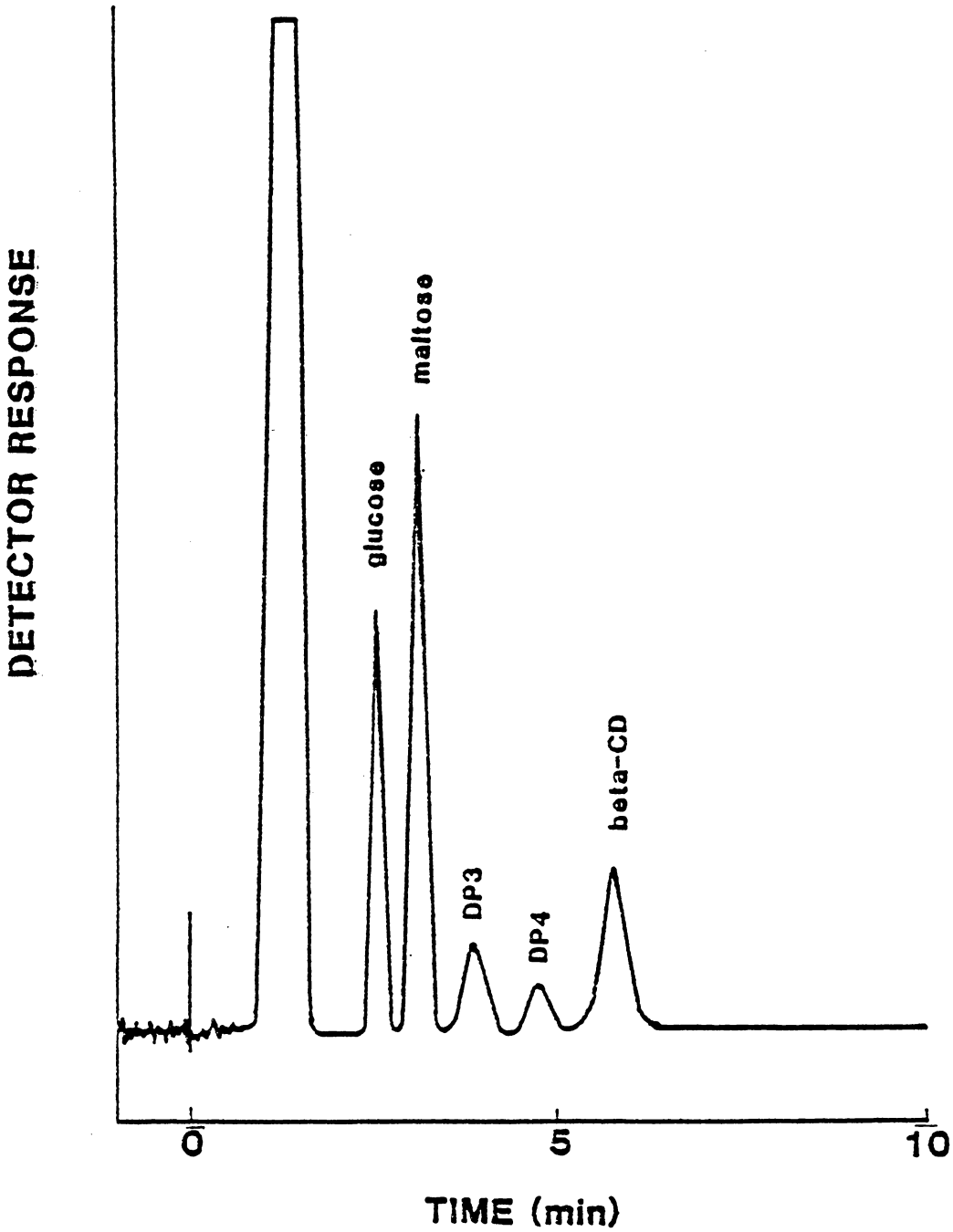


Figure 10. HPLC chromatogram of the products of β-CD hydrolysis by *B. distasonis* C18-7 cyclodextrinase. Conditions as in Fig. 8, (18 h incubation).

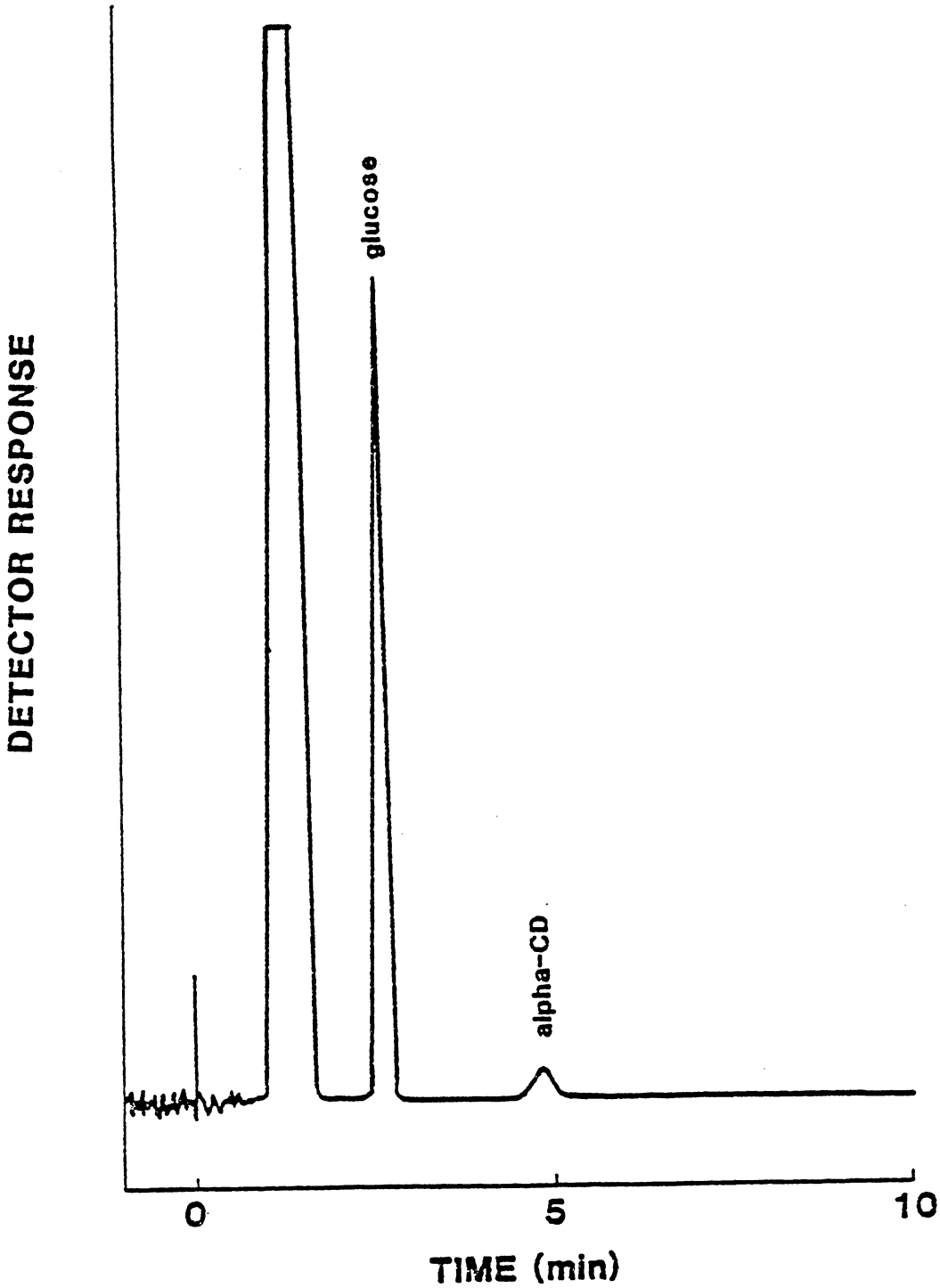


Figure 11. HPLC chromatogram of the products of α-CD hydrolysis by *B. ovatus* 3524 cyclodextrinase. Conditions as in Fig. 8, (18 h incubation).

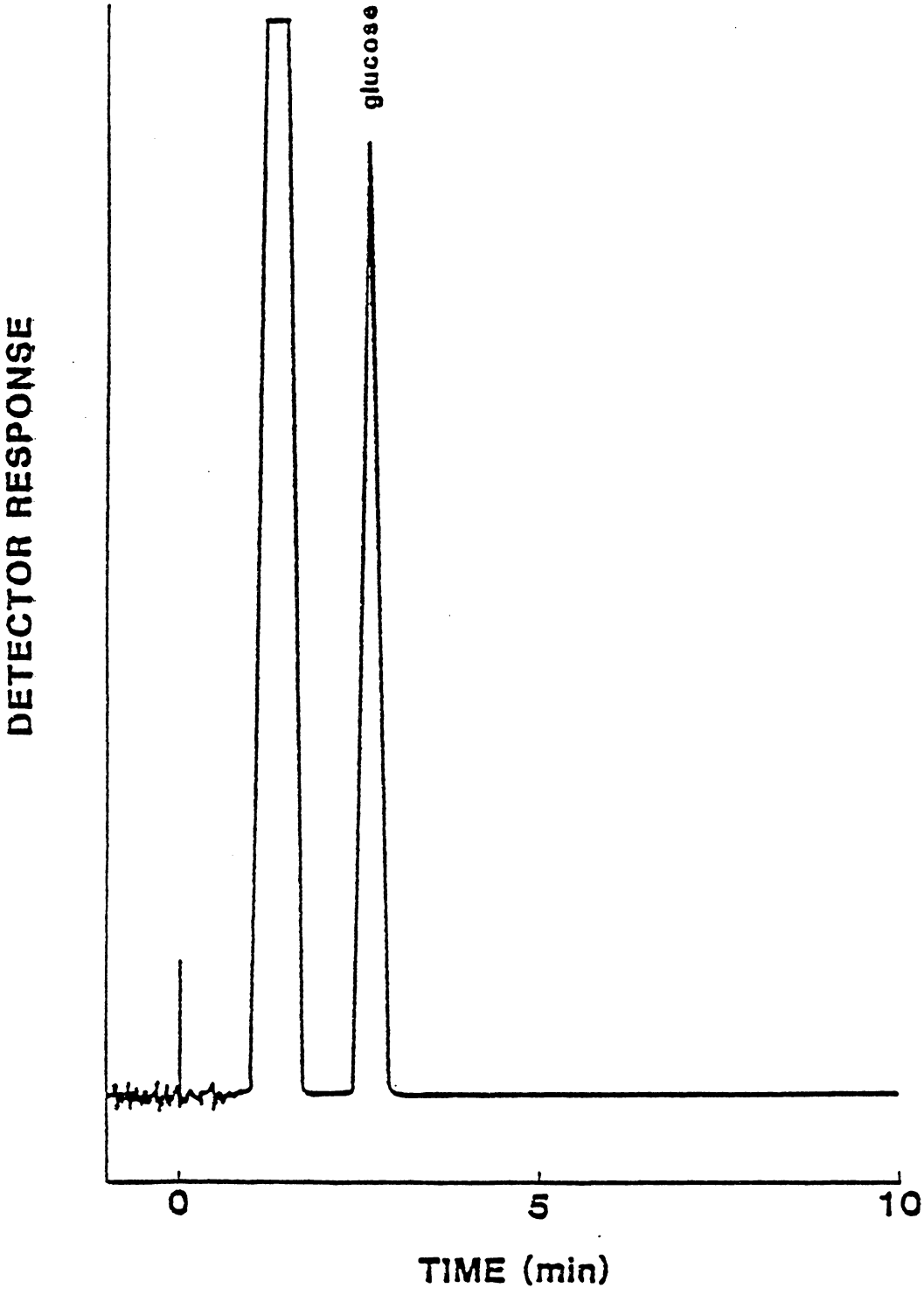


Figure 12. HPLC chromatogram of the products of β-CD hydrolysis by *B. ovatus* 3524 cyclodextrinase. Conditions as in Fig. 8, (18 h incubation).

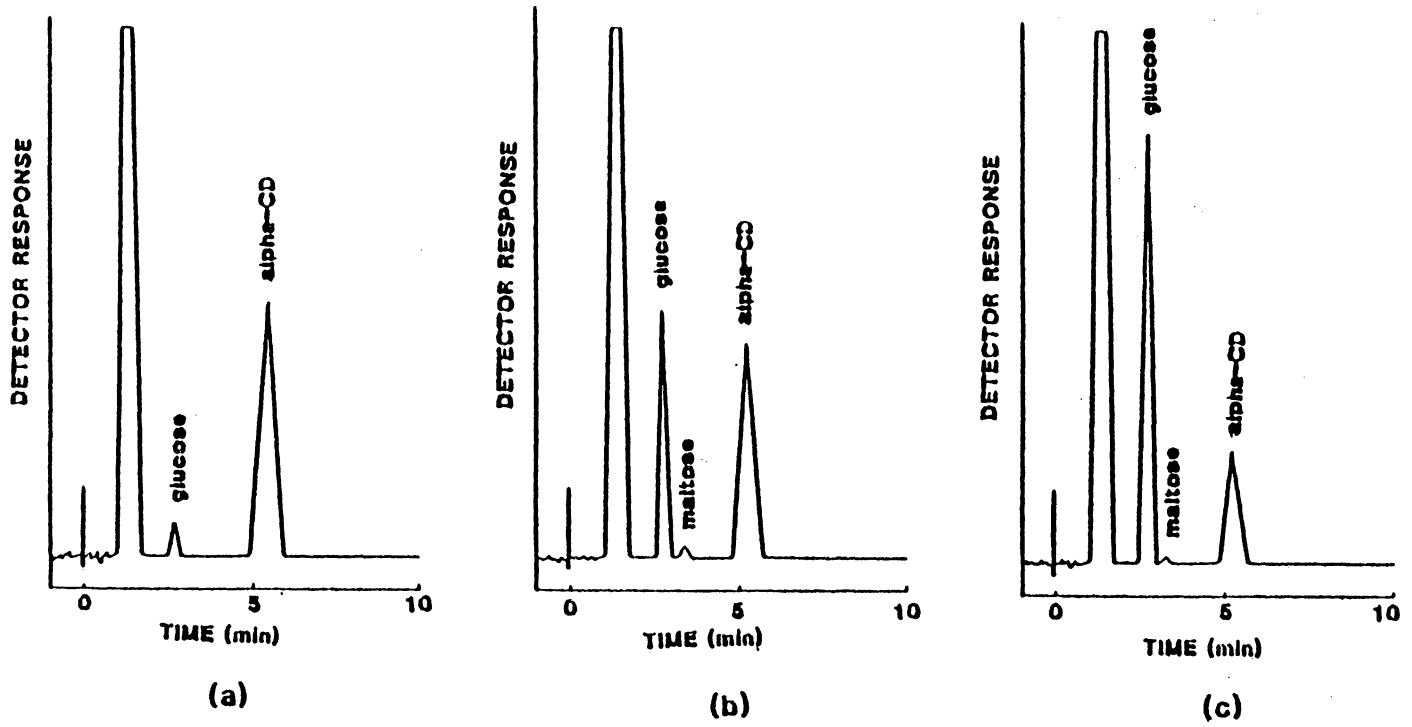


Figure 13. Time course analysis via HPLC of α-CD hydrolysis by *B. ovatus* 3524 cyclodextrinase at (a) 1 h, (b) 3 h, (c) 8 h. Conditions as in Fig. 8.

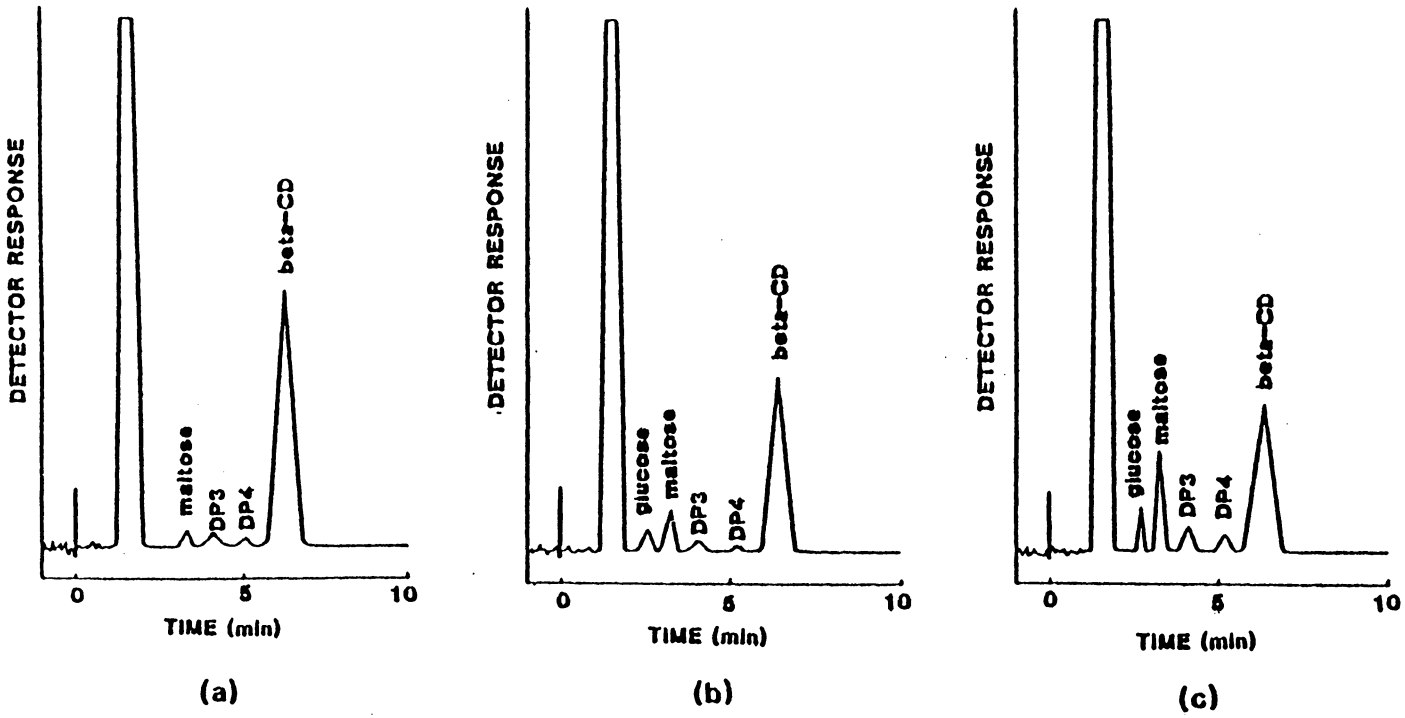


Figure 14. Time course analysis via HPLC of β-CD hydrolysis by *B. distasonis* C18-7 cyclodextrinase at (a) 1 h, (b) 3 h, (c) 8 h. Conditions as in Fig. 8.

F. Enzyme Purification

1. Hydrophobic Interaction Chromatography (HIC)

Results of purification for *B. ovatus* 3524 and *B. distasonis* C18-7 crude cyclodextrinase are shown in Table 4. HIC of *B. distasonis* C18-7 enzyme yielded a 15-fold increase in specific activity of cyclodextrinase/mg protein and *B. ovatus* 3524 enzyme showed a 17-fold increase. Typical HIC elution profiles for cyclodextrinase of both strains are shown on Fig. 15. Gradient salt elution removed inactive protein and cyclodextrinase activity was then eluted from the column when β-CD (5 mg/ml) was utilized as eluent.

2. Electrophoresis

Under identical conditions of SDS-PAGE, patterns were different for HIC fractionated cyclodextrinase from the two strains. Facsimilies of slab gel electrophoretic patterns, and calculated molecular weights of protein components in enzyme preparations from both strains are presented in Fig. 16. Presence of multiple bands indicates that neither cyclodextrinase was pure. Also, there is no evidence indicating which protein band(s) represents cyclodextrinase activity, possible iso-enzymes, contaminating enzymes, or inactive proteins.

3. Product Formation by HIC Fractionated Cyclodextrinases

A significant change in product formation was noted for the *B. ovatus* 3524 cyclodextrinase after certain purification steps. After treatment with 0.1 M $MnSO_4$ (designed to remove nucleic acids), *B. ovatus* 3524 cyclodextrinase hydrolyzed β-CD to produce glucose, mal-

Table 4. Results of purification procedures

Strain	Purification Stage	Activity (units/ml)[a]	Protein (µg/ml)	Specific Activity (units/µg protein)	Fold-Purification
C18-7	Crude liquor	1524.00	398.30	3.82	
	$MnSO_4$	1847.00	358.26	5.15	1.30
	$(NH_4)_2SO_4$	3220.00	330.40	9.74	2.55
	Phenyl-sepharose CL-4B	3524.00	61.80	57.02	14.92
3524	Crude liquor	4213.00	1063.20	3.96	
	$MnSO_4$	8054.00	968.50	8.31	2.09
	$(NH_4)_2SO_4$	7519.00	654.80	11.48	2.89
	Phenyl-sepharose CL-4B	4005.60	58.80	68.11	17.19

[a] Units = release of 1 µmole of reducing sugar (as glucose) per 4 h.

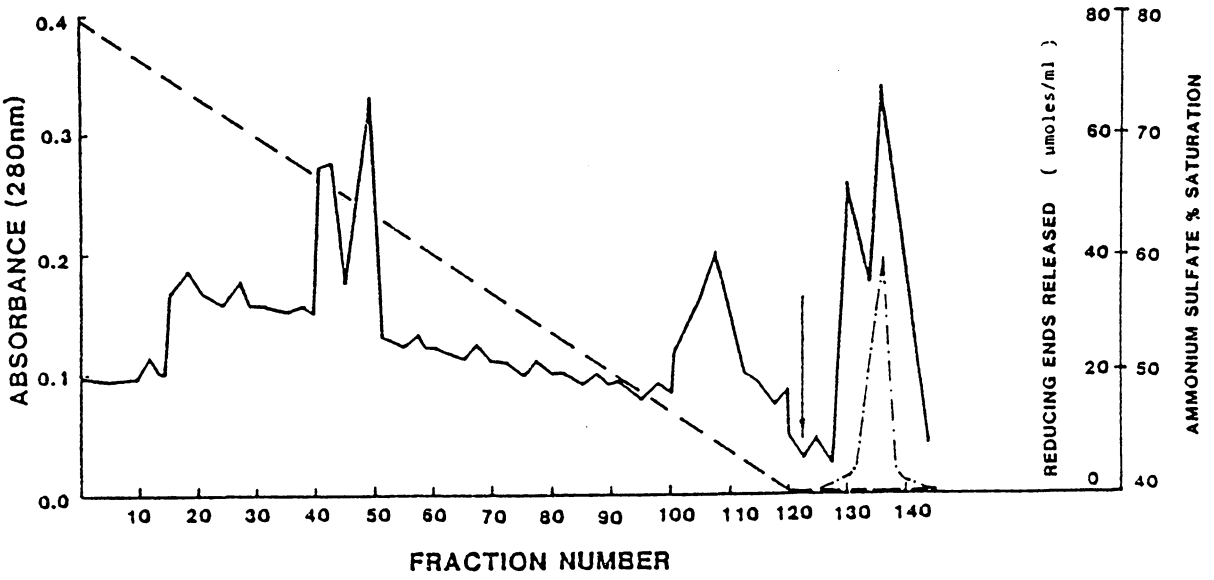


Figure 15. Typical elution profile of cyclodextrinase by hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B. Sample, 50 mg; bed volume, 60 ml; flow rate, 50 ml/h; eluent, gradient of decreasing ammonium sulfate concentration (phosphate buffer, pH 6.8) shown by dashed line. Solid line indicates protein measured by absorbance at 280nm. Arrow indicates addition of CD solution (5 mg/ml, 200 ml). Dash-dot line indicates cyclodextinase activity measured by the reducing end assay.

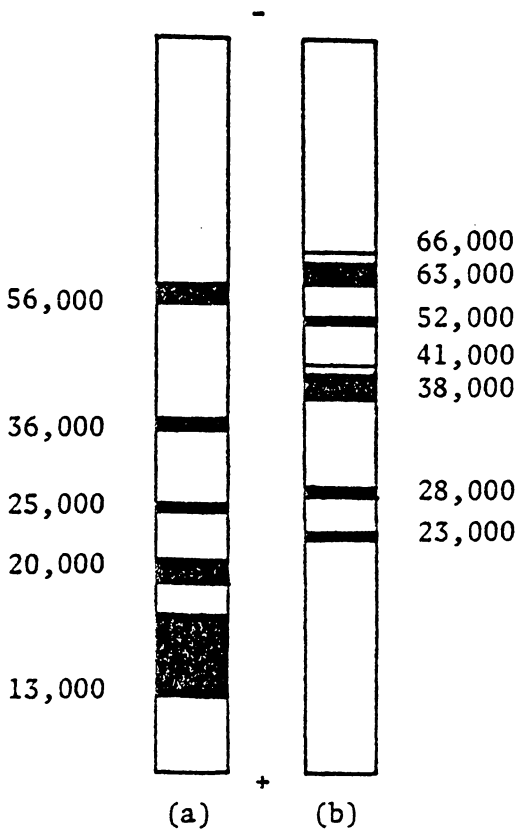


Figure 16. SDS-PAGE electrophoretic patterns and calculated molecular weights of HIC fractionated cyclodextrinase from (a) *B. ovatus* 3524 and (b) *B. distasonis* C18-7.

tose, maltotriose, and maltotetrose (Fig. 17 b), as compared to production of only glucose by the crude enzyme (Fig. 17 a). An additional change in product formation was detected after HIC of *B. ovatus* 3524 cyclodextrinase. Incubation with β-CD resulted in production of maltose, maltotriose, and maltotetrose, but no significant glucose formation (Fig. 17 c). Thus, the enzyme(s) responsible for sole production of glucose by crude *B. ovatus* 3524 cyclodextrinase were apparently removed during purification. No significant change in product formation occured from *B. distasonis* C18-7 cyclodextrinase after similar treatment.

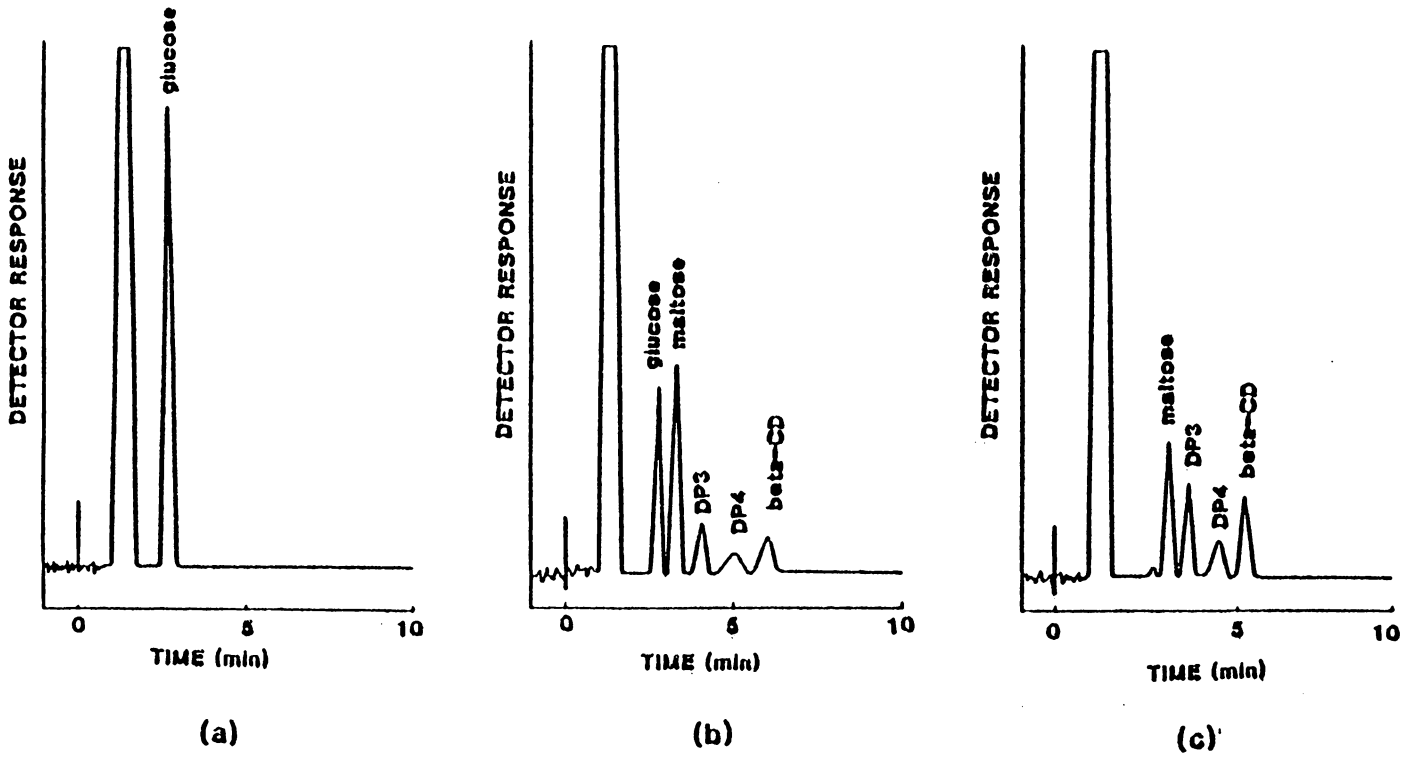


Figure 17. HPLC chromatograms of the products of β-CD hydrolysis by *B. ovatus* 3524 cyclodextrinase after certain purification stages: (a), crude cyclodextrinase; (b), after treatment with 0.1 M $MnSO_4$; (c), after HIC. Conditions as in Fig. 8, (18 h incubation).

DISCUSSION

CD use in food products raises concern about the metabolic fate and toxicity in humans. Earlier evidence shows that CDs are metabolized in the rat and suggests the colon as a possible location of CD metabolism (Anderson et al, 1962, Szabo et al, 1981). Since human colon anaerobes (especially *Bacteroides* species) have shown ability to degrade a variety of complex carbohydrates (Salyers et al, 1977), CDs might also be degraded. Our research approached this hypothesis by testing the ability of isolated human colon anaerobes to degrade CDs. A survey of 30 *Bacteroides* showed that 24 were able to degrade CDs as evidence by their ability to grow on CDs as sole carbon source. Furthermore, the majority of the 24 CD hydrolyzing strains show evidence for degradation and growth within 24 h of inoculation. This time course is consistent with the hypothesis that CDs are degraded in the colon, since the colon transit time is estimated at approximately 40 h (Burkitt and Trowell, 1975). The results of this screening suggest that CDs are not metabolically inert in humans, but may be susceptible to microbial degradation in the colon.

Additional studies involving 2 colon anaerobic organisms (selected from those which grew on CD in the screening study) were designed to characterize the enzymes presumably responsible for CD degradation. Detailed examination of *Bacteroides ovatus* 3524 and *Bacteroides distasonis* C18-7 showed that CDs were degraded by enzymes induced in these organisms by growth on CDs. The majority of enzyme activity was

cell bound for *B. distasonis* C18-7. The enzyme activity in *B. ovatus* 3524 was cell bound in the earlier stages of growth, but partly extracellular in cultures grown between 16 and 18 h on CD. Whether this increase in extracellular activity resulted from lysis of the cells or represented enzymes actively excreted from the organism remains to be determined.

Further investigation of the properties of the crude isolated enzymes indicated that little of the original activity was lost when C18-7 was incubated at 4-55° C for 48 h. There was significant loss of original enzyme activity (>50%) from *B. ovatus* 3524 when the enzyme was incubated at 37° C and higher for longer than 24 h. However, despite this loss, complete hydrolysis of CDs by *B. ovatus* 3524 enzyme occurs within 18 h at 37° C in the assay system employed. The enzymes from both strains also show maximum activity and stability at neutral pH. Thus, it would appear that both enzymes would be stable and active in the colon environment.

Growth studies show that cyclodextrinase activity could be detected in both organisms as early as 4 h after low level (1-5%) inoculation on CD-containing medium. The enzymes isolated from either organism catalyzed essentially complete hydrolysis of CDs in 16-28 h. These results suggest that 40 h transit time through the colon would be adequate time to induce enzyme production and allow partial or complete CD hydrolysis by the induced enzymes.

Degradative capabilities of the crude enzymes were further ana-

lyzed using HPLC to examine the products of CD hydrolysis. Results indicate that the products resulting from enzymatic hydrolysis of α and β-CD were significantly different for *B. distasonis* C18-7 and *B. ovatus* 3524. The products of CD hydrolysis (18 h) by *B. distasonis* C18-7 enzyme were glucose, maltose, maltotriose from α-CD. β-CD was hydrolyzed similarly forming glucose, maltose, maltotriose, and additionally maltotetrose. When hydrolysis was continued for 32-48 h, the predominant products were glucose and maltose. In contrast, the *B. ovatus* 3524 enzyme hydrolyzed α and β-CD entirely to glucose by 18 h. Time course studies of product formation by enzymes from both strains showed that the products were qualitatively the same throughout hydrolysis, for times of up to 18 h. This indicates that crude cyclodextrinase from both strains contain different enzymes.

The data for enzyme preparations from both *B. distasonis* C18-7 and *B. ovatus* 3524 indicate that these enzymes do not require linear glucosidic chains, reducing ends or non-reducing ends for CD hydrolyzing activity. Also, the enzymatic hydrolysis of the DP 6 α-CD by both *Bacteroides* strains showed the formation of products having an odd number of glucose units (DP 3 and glucose). This suggests that both crude enzymes are unlike the CD hydrolyzing enzymes of *Bacillus polymyxa* (Robyt and French, 1963) and *Aspergillus oryzae* (Hanrahan et al, 1952), which produce only maltose from substrates containing an even number of glucose units like α-CD. Hydrolysis of α and β-CD by both crude enzymes also did not produce maltooligosaccharides larger

than DP 4. Therefore, CD hydrolyzing enzymes of *B. distasonis* C18-7 and *B. ovatus* 3524 suggests a high degree of "multiple attack", in which the enzyme attacks a single substrate molecule several times before attacking another substrate molecule (Robyt and French, 1963). Since all of the studies on product formation were performed with crude enzyme preparations, it is possible that the products formed resulted from the continued attack of more than one enzyme. Isolation of purified individual enzymes will be required to further elucidate the actual mechanism of hydrolysis.

Enzyme purification procedures in this study utilized slat concentration and various chromatographic methods with the initial goal of isolating the enzyme responsible for cleaving the cyclic ring of CD. Purification proved difficult. However, hydrophobic interaction chromatography (HIC) provided the best enzyme separation for both *Bacteroides* strains. The specific activity of cyclodextrinase was increased 15-fold and 17-fold for *B. distasonis* C18-7 and *B. ovatus* 3524, respectively. However, SDS polyacrylamide gel electrophoresis of the fractionated enzymes failed to show homogeneity, indicating that enzymes of both *Bacteroides* strains were not of pure form. Further purification is needed before other aspects of these enzymes can be studied, such as enzyme active site, configuration, molecular weight, enzyme inhibition, and mechanism of cleavage.

Certain purification steps showed a significant alteration of CD hydrolysis by *B. ovatus* 3524 enzyme. HPLC analysis of the products of

CD hydrolysis by *B. ovatus* 3524 enzyme after one stage of purification ($MnSO_4$ treatment, Fig. 2) showed the formation of glucose, maltose, maltotriose, and maltotetrose, in contrast to formation of only glucose by the crude enzyme. This production of intermediate short chain oligosaccharides by *B. ovatus* 3524 enzyme is similar to products formed by *B. distasonis* C18-7 crude enzyme. Another change in product formation also occured after HIC, with *B. ovatus* 3524 enzyme hydrolyzing β-CD to form maltose, maltotriose, and maltotetrose without significant formation of glucose.

These changes in product formation during purification suggest that a multiple enzyme system is involved in the complete hydrolysis of α and β-CD to glucose by *B. ovatus* 3524 crude enzyme. Also, though no change in the pattern of CD hydrolysis was observed when partially purified enzyme from *B. distasonis* C18-7 was tested under similar conditions, the possibility of a multiple enzyme system existing for this organism should still be given consideration. Finally, no direct evidence for presence of a specific ring cleaving enzyme has been obtained.

Overall, the results of this research indicate that CD can be degraded by certain human colon anaerobes. These results are also in accordance with the findings of Szabo et al (1981) who found that CD could be degraded by the colon bacterial flora of rats and rabbits and suggested that the colon may be the major location of CD hydrolysis.

The findings of this research are also consistent with other studies that showed *Bacteroides* were able to induce enzyme activity to degrade various complex food carbohydrates (Salyers et al, 1977, Dekker, 1979, Balascio, 1976, Vercilloti, 1977). The cyclodextrinase activity of the *Bacteroides* strains tested was found to be induced by growth of the organisms on CD. These results support the hypothesis that members of the *Bacteroides* genus are able to adapt to available substrates in order to compete for survival in the human colon (Salyers et al, 1977).

Single bacterial cultures were used in this research to examine breakdown of CD *in vitro* by human colon anaerobes. These results may help predict *in vivo* action of intact *Bacteroides* cells encountering CD in the colon. The factors *in vivo* which determine if CD could be hydrolyzed would involve examination of the effect of a competitive environment and the ability of these organisms to synthesize enzymes and hydrolize CD in the presence of other substrates. The use of mixed bacterial cultures would also better approximate the actual colon environment. However, limited information about the colon environment makes simulation difficult.

CD use in food and pharmaceutical products shows great possibilities. However, more work is needed to better understand the fate of ingested CD as well as CD inclusion complexes. The results of this research show that CD can be hydrolyzed by certain human colon anaerobes well within the time required for transport through the colon.

Further research is needed to understand the utilization of CD degradation products. Also, the fate of ingested CD-inclusion compounds needs further investigation. Currently it is assumed that the guest molecule will be released into the mouth, or into the neutral or slightly alkaline portion of the intestinal fluid, depending on the nature of the included compound (Fromming, 1981). However, if a portion of the CD-complex were to pass intact into the colon, colon anaerobes could possibly degrade the CD thus releasing the guest molecule in the colon.

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ENZYMATIC DEGRADATION OF ALPHA AND BETA CYCLODEXTRINS BY *BACTEROIDES* FROM THE HUMAN COLON

by

Robert N. Antenucci

(ABSTRACT)

Thirty *Bacteroides* strains from the human colon were tested for ability to degrade cyclodextrins (CD). Twenty four strains were able to degrade CD. Cyclodextrinase in two of these strains *B. ovatus* 3524 and *B. distasonis* C18-7 has been studied.

Organisms were grown on a minimal medium containing CD (0.5%), and cyclodextrinase activity was assayed by measuring the increase in reducing sugar (as glucose) when CD was incubated at 37° C for 4 h with crude enzyme preparations. Cyclodextrinase activity was predominantly cell bound and induced in both organisms by growth on CD. Analysis via high performance liquid chromatography showed that products of CD hydrolysis by the crude enzyme preparations from the 2 strains were sharply different. *B. ovatus* 3524 cyclodextrinase yielded glucose only, while the *B. distasonis* C18-7 enzyme catalyzed production of a series of maltooligomers. Cyclodextrinase of both strains was stable at 4° C for at least 48 h. *B. distasonis* C18-7 cyclodextrinase showed greater than 75% retention of activity at temperatures up to 55° C after 48 h, whereas the *B. ovatus* 3524 enzyme was labile above 25° C. Optimum activity and stability of cyclodextrinase from both strains occured at pH 7.0.

Salt precipitation and chromatographic methods were utilized in

an attempt to purify the enzyme(s) in crude cyclodextrinase. No enzymes were purified to homogeneity, but a 15- to 17-fold increase in specific cyclodextrinase activity was obtained via hydrophobic interaction chromatography. Also, the products obtained by the action of cyclodextrinase from *B. ovatus* 3524 were markedly altered during purification, suggesting that the crude cyclodextrinase contains a mixture of enzymes.