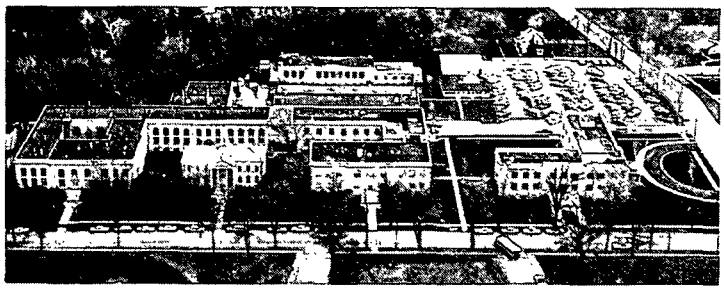

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# ENZYMATIC SYNTHESIS OF LOW POLYDISPERSITY AMYLOSE OF CONTROLLED MOLECULAR WEIGHT USING RABBIT PHOSPHORYLASE A

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# Enzymatic Synthesis of Low Polydispersity Amyloses of Controlled Molecular Weight Using Rabbit Phosphorylase A

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Portions of this work were used by DJG in partial fulfillment of the requirements for the Ph.D. degree at The Institute of Paper Chemistry and has been submitted for consideration for publication in Carbohydrate Research

# ENZYMATIC SYNTHESIS OF LOW POLYDISPERSITY AMYLOSES OF CONTROLLED MOLECULAR WEIGHT USING RABBIT PHOSPHORYLASE A

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## ABSTRACT

A method of enzymatically synthesizing amyloses of controlled number average degree of polymerization ($\overline{DP_n}$) is described. The method uses only commercially available reagents and has successfully produced amyloses of $\overline{DP_n}$ up to 6100 in quantities approaching one gram. The products have very narrow molecular weight distributions and have been shown to be free of α-1,6 branches.

The synthesis is based on the action of the enzyme rabbit phosphorylase a, which catalyzes the building of polymer chains from α-glucose 1-phosphate, the source of glucose monomer units. Maltoheptaose serves as the "primer" substrate, to which the glucose units are attached.

## INTRODUCTION

During the course of a recent investigation of the alkaline degradation reactions of amylose, linear amyloses of known molecular weight were required for calibrating a gel permeation chromatograph and as well-characterized substrates for kinetic studies.

Linear amyloses have been synthesized previously by several researchers[1-5], using enzymatic techniques. The enzyme phosphorylase, which normally depolymerizes polyglucans *in vivo*[6] can, under the proper reaction conditions, be used to build up chains of amylose from an oligosaccharide primer substrate and glucose 1-phosphate. The reaction scheme is shown in Fig. 1. Because the number of growing chains throughout the reaction is fixed by the initial concentration of the primer substrate, the polymerization process is analogous to other "living" polymerization reactions. Flory[7] has shown mathematically that such "living" polymerizations result in products which have molecular weight distributions conforming to the Poisson distribution. The polydispersity index (the ratio of the weight average to the number average molecular weight) for such a polymer is given by

$$\frac{M_w}{M_n} = 1 + \frac{(DP_n - 1)}{DP_n^2} \qquad (1)$$

where $\overline{DP_n}$ is the number average degree of polymerization, and so very low polydispersities are attainable.

Figure 1 here

Equation (1) holds strictly only for an irreversible polymerization process. The action of phosphorylase is reversible, and reaction in the direction of amylose depolymerization becomes more

important as the initial supply of glucose 1-phosphate is depleted. If too high a conversion of glucose 1-phosphate to amylose is attempted, a broad distribution will result[3]. Too low a conversion makes the synthesis expensive, as starting material is wasted. A balance must therefore must be struck between the desire for the narrowest distribution and the desire for a high conversion. Acceptably narrow distributions can be obtained with conversions up to about 50%[3].

The objective of this study was to develop a means of synthesizing amyloses with DPs up to several thousand in quantities approaching a gram or more using commercially available reagents. The previously reported enzymatic amylose synthesis procedures[1-5] could not be used to meet these objectives because they produced milligram-sized quantities at the most, and typical DPs extended only into the hundreds. Furthermore, phosphorylase isolated from potatoes was used exclusively, but the potato enzyme is not widely available commercially and is difficult to store.

The approach taken was to scale up the reactions to increase the yields obtained, to find suitable reaction conditions for producing high DPs, and to investigate alternate sources of phosphorylase. Rabbit muscle phosphorylase is widely available in lyophilized form, and so it was a logical choice for the source of enzyme.

The nature of the primer substrate required to initiate the synthesis varies with the type of phosphorylase used. For the potato enzyme, several references[6,8-10] state that a linear maltooligosaccharide containing at least three or four glucose units can function as the primer. Banks et al.[1] used a mixture of maltohexaose and maltoheptaose in their synthesis using potato phosphorylase, and Bailey and Whelan[2,5] used maltohexaose. The literature is unclear and conflicting on what length of primer molecule is needed for the muscle-derived enzyme. Bourne[9] states that muscle phosphorylases cannot be primed by maltooligosaccharides containing fewer than eight glucose units. More recently, however, Madsen and coworkers[11,12] studied the kinetics of the action of rabbit phosphorylase and the binding of maltoheptaose to the enzyme. These workers report that they were able to get the reaction to proceed in the direction of synthesis using maltoheptaose as the primer, although no product was isolated.

## RESULTS AND DISCUSSION

The first experiments conducted in working toward a practical synthesis used glycogen as the primer substrate. Muscle phosphorylase is primed most efficiently by glycogen, its native substrate[8]. Reaction conditions such as pH, temperature, and reagent concentration could thus be optimized without concern for the effectiveness of the primer. Reactions with glycogen also served as a quick test of the activity of

the enzyme used in other reactions. Measurement of the phosphate liberated during synthesis proved to be an effective means of monitoring the progress of the reactions.

One problem that became apparent in the early experiments was that over the long course of the synthesis reactions (sometimes spanning weeks) the enzyme tended to denature and precipitate out of solution. Degradation of the enzyme by oxidation was kept to a minimum in subsequent reactions by addition of the antioxidant dithiothreitol[13] and by blanketing the reaction vessels with argon. The temperature of the reactions was limited to 30°C, and EDTA was added as a chelating agent to further stabilize the enzyme. Even with these precautions, it was sometimes necessary to add additional enzyme during the course of a synthesis to make up for that lost by degradation.

It was discovered that maltoheptaose could act as the primer substrate, if careful attention was paid to the order of addition of reagents to the reaction vessel. The reaction proceeded most rapidly when the enzyme was first mixed with the maltoheptaose before being added to the glucose 1-phosphate. In contrast, when the enzyme solution was added to an existing mixture of maltoheptaose and glucose 1-phosphate, no reaction took place.

After appropriate reaction conditions were found, four amyloses were successfully synthesized. These ranged in $\overline{DP}_n$ from 41 to 6100.

The synthetic amyloses, as well as maltoheptaose, were analyzed by gel permeation chromatography (GPC) as their tricarbanilate derivatives. The chromatograms are shown in Fig. 2. With the exception of product 4, the chromatograms demonstrate that the molecular weight distributions of the products are very narrow.

Figure 2 here

The molecular weight of the products could theoretically be calculated from the final inorganic phosphate concentration and the initial maltoheptaose concentration. This method failed to give accurate results, however. In particular, the $\overline{DP}_n$ calculated in this way for product 4 was much higher than indicated by the gel permeation chromatogram. Since this product also had the widest distribution of any of the synthetic amyloses, it may be speculated that during the long reaction time involved (9 days) there was some hydrolysis of the product. A reducing end group analysis was therefore used to characterize the size of the synthetic amyloses.

Examination of gel permeation chromatograms before and after treatment with isoamylase was used to check the synthetic amyloses for α-1,6 branches. The analysis verified that the products were completely free of branching.

EXPERIMENTAL

All chemicals and solvents used were reagent grade or better. Whenever washing of amylose or amylose tricarbanilate is mentioned in the following procedures, it was accomplished by thoroughly mixing the wash liquid with the solid, and separating the supernatant by centrifugation and decanting.

Enzymatic synthesis. - Maltoheptaose, α-disodium glucose 1-phosphate, dithiothreitol, and rabbit phosphorylase a were purchased from Sigma Chemical Company (St. Louis, MO).

All reagent solutions used in the synthesis reactions were made up in β-glycerophosphate buffer (pH 6.80, 0.100M) which contained dithiothreitol (0.015M) and EDTA (0.001M).

Four synthesis reactions were conducted, numbered 1 through 4. Reaction 1 was designed to produce the lowest DP material, and reaction 4 was designed to produce the highest.

Phosphorylase solution (17 units/mL, 25 mL) was placed in each of four flasks, and precisely measured volumes of maltoheptaose solution (4.65 mM) were added (25.42 mL for reaction 1, 3.372 mL for reaction 2, 0.5247 mL for reaction 3, and 0.0804 mL for reaction 4). The enzyme/maltoheptaose mixtures were incubated at 30°C for at least 45 minutes prior to initiating the reactions.

Buffer (25 mL) and α-disodium glucose 1-phosphate solution (0.125M, pH 6.80, 100 mL) were added to the four reaction vessels. The enzyme/maltoheptaose solutions were transferred quantitatively to the vessels, and additional buffer was used to bring the total volume up to a constant level (250.0 mL). The vessels were sealed, flushed with argon, and placed in a water bath at 30°C. Throughout the reactions, a gentle mixing was maintained by means of magnetic stirrers.

Periodically, samples were withdrawn from the vessels for inorganic phosphate analysis by the method of Fiske and SubbaRow[14]. Additional enzyme was added as a concentrated solution (425 units/mL) after 46 hours to reaction 2 (1.0 mL), reaction 3 (1.0 mL), and reaction 4 (2.0 mL) and again after 70 hours to reaction 3 (1.0 mL) and reaction 4 (1.0 mL).

The reactions were stopped when phosphate analysis showed that the desired conversion of glucose 1-phosphate to amylose had been reached. This occurred at 28 hours for reaction 1, (33% conversion), 46 hours for reaction 2 (40% conversion), 145 hours for reaction 3 (46% conversion), and 188 hours for reaction 4 (36% conversion). The syntheses were terminated by refluxing the reaction mixtures for 10 minutes under nitrogen, and the denatured enzyme was removed by filtration of the hot solutions through a glass fiber disk.

The filtrate was poured into absolute ethanol (1500 mL), and the precipitate was allowed to settle overnight. The supernatant was removed by centrifugation and the precipitate was washed nine times with absolute aqueous ethanol (50% v/v, 2 x 1500 mL, 7 x 900 mL), once with absolute ethanol (900 mL), and once with anhydrous ethyl ether (500 mL), and filtered onto a glass fiber disk from pentane (250 mL). The final products were dried under vacuum over phosphorus pentoxide at 30°C (yields: 0.66 g of product 1, 0.83 g of product 2, 0.94 g of product 3, and 0.74 g of product 4). Elemental analyses for nitrogen and phosphorus were used to verify that the products were essentially free of protein and phosphates.

Carbanilation and gel permeation chromatography. - Pyridine was dried by refluxing over potassium hydroxide and distillation. Pyridine (4 mL) and phenyl isocyanate (0.25 mL) were added to the amylose samples in glass hypo vials. The vials were flushed with nitrogen and sealed. After 48 hours at 80°C the excess phenyl isocyanate was expended by adding methanol (0.125 mL), and the carbanilate solutions were diluted with dioxane (8 mL) and filtered. The carbanilates were precipitated into a mixture of water (20 mL), methanol (40 mL), and acetic acid (0.4 mL). The precipitates were washed once with a mixture of water (11.5 mL), methanol (21.5 mL) and acetic acid, once with a mixture of water (32 mL) and acetic acid (0.2 mL), and twice with water (65 mL). Final drying was accomplished by freeze drying.

Gel permeation chromatography was performed at 21.1°C on a Jordi-Gel Mixed Bed (High MW) column (Jordi Associates, Mills, MA) using distilled tetrahydrofuran as the mobile phase, and UV detection at 235 nm. The injector loop volume was 200 µL, and the flow rate was 1.50 mL per minute. Sample concentrations ranged from 0.01 to 0.1% (w/v). Elution volumes were measured relative to a low molecular weight, total permeation standard, methyl N-phenyl carbamate.

DP determination by reducing end group analysis. - The method used for end group determination is based on that of Manners et al.[15], but the procedure given here includes several modifications necessary for applying the technique to amyloses of high DP. Sorbitol dehydrogenase and nicotinamide adenine dinucleotide (NAD) were purchased from Boehringer Mannheim Biochemicals (Indianapolis).

Amylose (40 mg) was dissolved in dimethyl sulfoxide (1.0 mL) and sodium borohydride solution (50 mg sodium borohydride in 0.5 mL water and 1.5 mL dimethyl sulfoxide) was added. After shaking for 48 hours at room temperature, excess borohydride was destroyed with hydrochloric acid (2.2M, 0.50 mL), and the samples were poured into absolute ethanol (10-12 mL). The resulting precipitates were washed three times with absolute ethanol (10-12 mL), once with anhydrous ethyl ether (10-12 mL), and dried from pentane (2-5 mL) under vacuum at 40°C over calcium sulfate.

The reduced amyloses were hydrolyzed with trifluoroacetic acid (2M, 15 mL) at 120°C for one hour. The hydrolyzates were dried by vacuum evaporation (40-50°C), and residual acid was removed by three evaporations (40-50°C) to dryness from water (5.0 mL). In order to remove traces of borate which interfered with the glucitol assay, it was necessary to evaporate (30-40°C) the product to dryness four times from methanol (5 mL). This removed the borate as methyl borate. The final mixtures of glucose and glucitol were dried under vacuum at 40°C over calcium sulfate.

The dry hydrolyzates were dissolved in pyrophosphate buffer (pH 9.50, 0.1M, 2-4 mL) and the pH of the resulting solutions was adjusted to 9.50 +/-0.02 with sodium hydroxide solution (2.0M). Duplicate aliquots (0.1 mL) were removed for glucose analysis by the hexokinase method[16]. Further volumetric dilution with pyrophosphate buffer was then done on the product 1 hydrolyzate solution (50X), and the product 2 hydrolyzate solution (5X) to bring the glucitol concentration into the proper range for analysis.

Immediately before conducting the glucitol analysis, a stock assay solution was prepared by combining pyrophosphate buffer (20.00 mL) with NAD solution (20 mM, 4.00 mL) and water (15.00 mL). Stock solution (1.00 mL) and sample (0.50 mL) were added to a spectrophotometer cell, and an initial absorbance reading was taken at 340 nm. Sorbitol dehydrogenase solution (160 U/mL, 25 µL) was then added and the final

absorbance was recorded after 40 minutes elapsed. The glucitol concentration was calculated from the change in absorbance, assuming that one mole of NAD is reduced to NADH per mole of glucitol present, using a molar extinction coefficient for NADH at 340 nm of $6.22 \times 10^6$ $cm^2$ (ref. 15).

When solutions containing glucose but no glucitol were analyzed, a small change in absorbance was noted. Therefore, it was necessary to correct the absorbance readings of unknown samples before calculating glucitol concentrations. A correction curve was prepared for each set of assays by concurrently analyzing several glucose solutions of known concentration (0 to 30 g/L) and plotting absorbance versus concentration.

Duplicate glucitol determinations were done on each hydrolyzate solution, and the DP of the amylose was calculated from the ratio of the glucitol to glucose concentrations. The entire procedure was repeated three times for each of the synthesized amyloses. In general, the determinations agreed within 5%.

The calculated $\overline{DP}_n$ for product 1 was 41.4, for product 2 was 366, for product 3 was 2550, and for product 4 was 6100. Analysis of maltoheptaose by the same technique gave a calculated $\overline{DP}_n$ of 7.03.

Branching analysis. - The debranching procedure described by Kobayashi et al.[17] was modified to allow recovery of the product so that

the carbanilate derivative could be prepared and analyzed by GPC. Isoamylase was purchased from Sigma Chemical Company (St. Louis). Before use, the enzyme (5000 units, 3500 units/mg protein) was washed of ammonium sulfate by diluting it with acetate buffer (pH 3.80, 0.1M, 5 mL) and concentrating it (to 0.5 mL) twice in a collodion bag apparatus. The resulting solution was diluted to a known volume (5.0 mL) with additional buffer and stored under refrigeration.

For each product evaluated, two sample solutions were made. Amylose (30 mg) was dissolved in dimethyl sulfoxide (2.00 mL) and mixed with acetate buffer (2.00 mL) and water (3.00 mL). Isoamylase solution (0.590 mL) was added to one of the solutions (treated) and an equal volume of buffer was added to the other (control).

The samples were incubated at 40°C for 24 hours in a water bath. The polysaccharides were precipitated by pouring the solutions into absolute ethanol (50 mL), washed three times with absolute ethanol (100 mL), once with anhydrous ethyl ether (50 mL), and once with pentane (50 mL), and dried under vacuum at 30°C over phosphorus pentoxide.

All of the samples were carbanilated and analyzed by GPC as described above. A synthetic amylose was judged to be free from $\alpha$-1,6 branches if no differences were detected between the control and treated sample chromatograms. Amylopectin was subjected to the analysis to verify the reliability of the technique.

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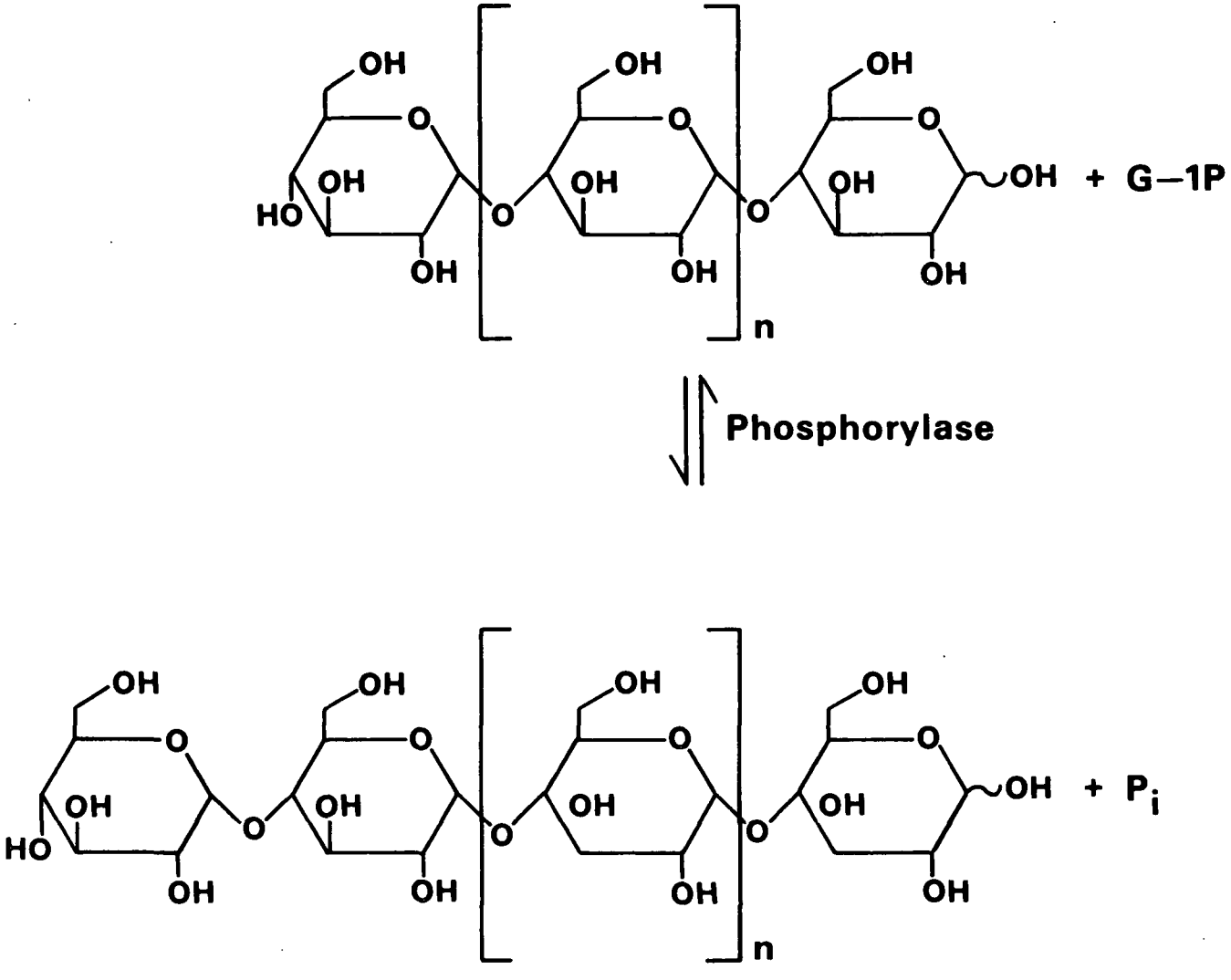


Fig. 1. Phosphorylase-catalyzed lengthening of an amylose chain. (The minimum value of n depends on the source of the enzyme. G-1P is α-glucose 1-phosphate and $P_i$ is inorganic phosphate.)

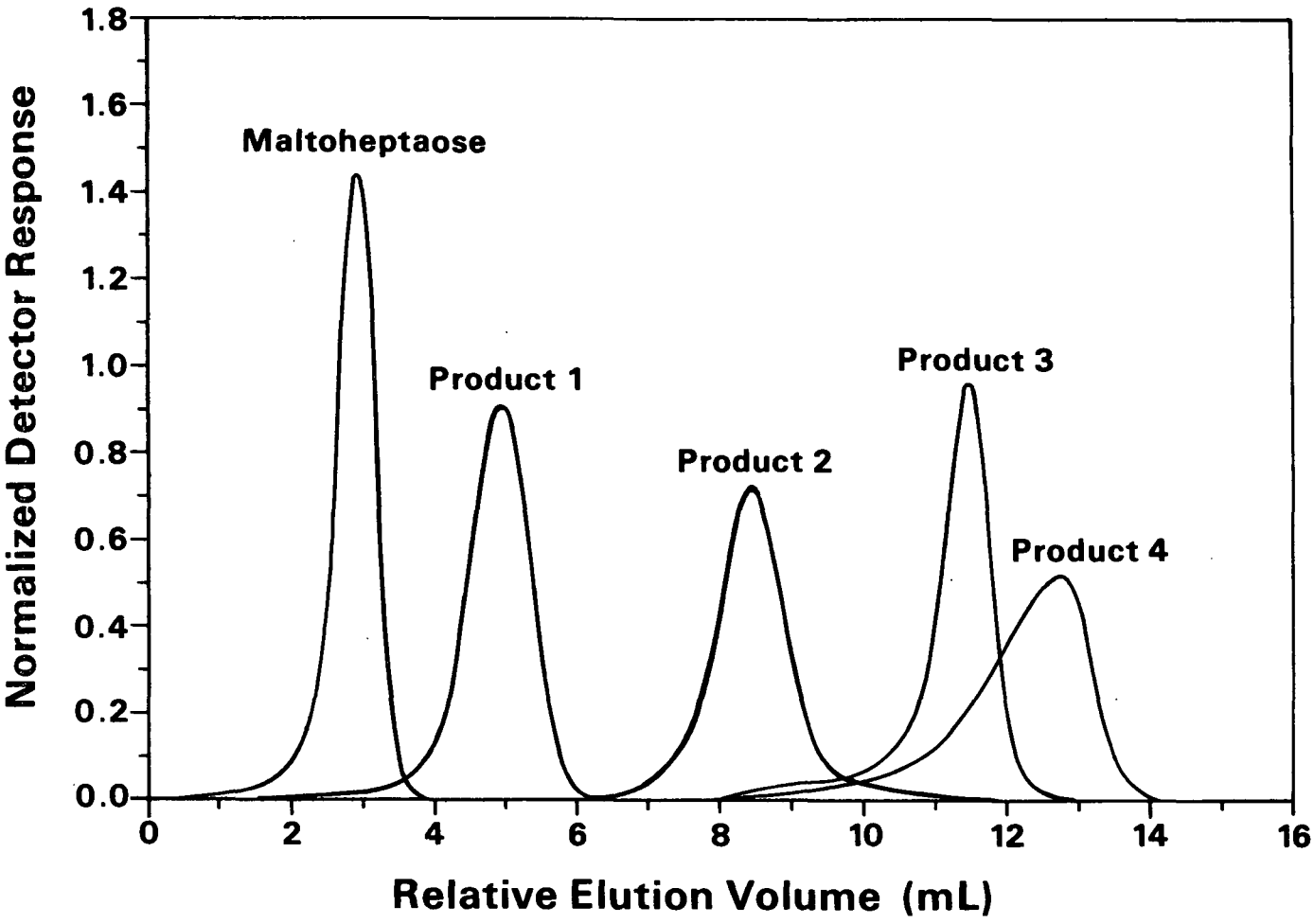


Fig. 2. Gel permeation chromatograms for synthetic amyloses and maltoheptaose. (The peak areas have been normalized to a constant value.)