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CYCLOHEPTAAMYLOSE AS AN AFFINITY LIGAND OF CEREAL ALPHA AMYLASE. CHARACTERISTICS AND A POSSIBLE MECHANISM OF THE INTERACTION

RANDALL J. WESELAKE AND ROBERT D. HILL
Department of Plant Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2 (Canada)
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ABSTRACT

The characteristics of alpha amylase purification on a column of cycloheptaamylose-substituted, epoxy-activated Sepharose 6B were investigated. The enzyme was recovered in high yield from crude triticale and wheat extracts. Enzyme activity assessed after elution from the column was 132% of that measured prior to chromatography. There was no evidence of beta amylase isozymes in the purified alpha amylase. Neither barley beta amylase nor sweet-potato beta amylase was retained by the column. Cycloheptaamylose did not inhibit triticale or wheat alpha amylase activity, but did inhibit barley beta amylase activity, yielding a K_i of 4.5mM. Equilibrium-dialysis experiments showed that alpha amylase did interact with cycloheptaamylose. The dissociation constant for the enzyme–ligand was 19μM. It was concluded that cycloheptaamylose bound at a non-catalytic site on the alpha amylase molecule.

INTRODUCTION

The effect of cycloamyloses on the enzyme activity of amylases is varied and, at times, has been a controversial topic[1,2]. Thoma *et al.*[3] reported that hog-pancreatic alpha amylase was not detectably inhibited by cyclohexaamylose, yet others[4] have found inhibition by cycloheptaamylose and have even demonstrated the binding of 3 mol of the ligand per mol of enzyme near saturation. The alpha amylase of *Bacillus subtilis* has been shown to be inhibited by cycloheptaamylose[5], whereas both cyclohexa- and cyclohepta-amylose inhibited sweet-potato beta amylase[1,6], although there has been disagreement on the inhibition constant[2].

Cycloamylose-substituted, epoxy-activated Sepharose 6B has been used to purify sweet-potato beta amylase[7] and cereal alpha amylase[8,9] by affinity chromatography. Because of the strong, competitive inhibition of the beta amylase by the ligand[1,6], it is possible that beta amylase may interact with the affinity column at the active site of the enzyme. Although the evidence with pancreatic alpha amylase[4] would suggest a similar interaction for that enzyme, there have been no studies on a cereal enzyme to confirm this hypothesis.

The present study was undertaken to investigate some of the characteristics of alpha amylase purification by affinity chromatography and to determine, in the case of the cereal enzyme, the nature of the interaction between cereal alpha and beta amylase and cycloheptaamylose.

MATERIALS AND METHODS

Enzyme purification and detection. — Triticale 6A190 (X *Triticosecale* Wittmack) and wheat (*Triticum aestivum* cv Neepawa) were used. Germinated and freeze-dried triticale was prepared as described by Silvanovich and Hill[8]. The wheat kernels were germinated for 3–4 days at 21°, freeze-dried, and stored at 4°.

The purification of alpha amylase was conducted according to a modification of the method of Silvanovich and Hill[8]. The crude extract prepared by suspending 30 g of flour in 100 mL of 0.2M sodium acetate buffer (pH 5.5, mM Calcium chloride) was stirred for 30 min in the presence of 10 mg/mL of polyvinylpolypyrrolidone (PVP). The PVP was then removed by filtration of the treated extract through glass wool. The extract was then dialyzed overnight against 8 L of 0.02M sodium acetate (pH 5.5, mM calcium chloride). After dialysis, the extract was centrifuged at 10,000*g* for 20 min to remove any protein that had precipitated as a result of the decrease in ionic strength. This supernatant solution was in turn applied to a column (1.6 × 7.6 cm) of cycloheptaamylose–epoxy-Sepharose 6B equilibrated with 0.02M sodium acetate buffer (pH 5.5, mM calcium chloride). The column was washed with ~6 bed-volumes of equilibrating buffer, 30 mL of 0.3M sodium chloride, and a final 30 mL of equilibrating buffer. The enzyme was eluted with 8 mg/mL of cycloheptaamylose in equilibrating buffer. The enzyme peak was pooled immediately after elution from the affinity column. The cycloheptaamylose was separated from the enzyme by gel filtration on a column of Bio-Gel P-4 (200–400 mesh) pre-equilibrated with 0.05M sodium acetate (pH 5.5, mM calcium chloride), using the same buffer to elute the enzyme. Enzyme used in kinetic studies was concentrated to ~800 μg/mL in an Amicon concentrator-cell equipped with a YM10 membrane. The concentrated enzyme was stored frozen in the presence of 0.1% bovine serum albumin until being thawed for use. The affinity column was cleaned with 6M urea intermittently and equilibrated with several bed-volumes of equilibrating buffer before reuse. The degree of ligand substitution in the affinity gel was determined by measuring radioactivity in two 0.1-mL portions of sedimented [^{3}H]cycloheptaamylose–epoxy-Sepharose 6B. The gel was mixed with Aquassure (New England Nuclear) and radioactivity measured in a Searle Analytic Mark III Liquid Scintillation Spectrometer.

Activity determinations were conducted by Briggs' method[10] and protein estimated by the method of Lowry *et al.*[11]. The activity of the purified enzyme was also determined by measuring the appearance of reducing activity from the hydrolysis of gelatinized starch at 30° in the presence of 0.05M sodium acetate (pH 5.5, mM calcium chloride). Reducing activity was determined by Nelson's[12] adaptation of the Somogyi method for the determination of glucose, using reagents recommended

by Robyt and Whelan[13]. The reducing-activity assay was also used in inhibition studies with cycloheptaamylose (Sigma Chemical Company). The purity of the enzyme preparations was examined by gel electrophoresis[17] with sodium dodecylsulfate and by comparative zymograms on β-limit dextrin and starch after isoelectric focusing of the enzyme[15]. Total carbohydrate was determined by the phenol–sulfuric acid method[16].

Columns (1 mL) of cycloheptaamylose–epoxy-Sepharose 6B were used to study the effect of D-glucose and D-glucose oligomers on the release of enzyme from the affinity gel. Columns were washed with 3 mL of the solutions indicated in Table II. The small columns were also used to determine the behavior of purified preparations of beta amylase with respect to the immobilized cycloheptamylose. Sweet-potato beta amylase was obtained from the Sigma Chemical Company and purified barley beta amylase was a gift from Dr. A. W. MacGregor.

Binding studies. — The details for preparing [^{3}H]cycloheptaamylose are described elsewhere[17]. In order to study the reversibility of the interaction, purified wheat alpha amylase (3.5 mg) was applied to the preparative affinity-column and partially eluted with 15 mL of [^{3}H]cycloheptaamylose (which had a specific activity of 1.32×10^5 d.p.m./mg). The eluted enzyme was then pooled and applied to a column (2.5 × 28 cm) of Bio-Gel P-4 that had been equilibrated with 0.05M sodium acetate (pH 5.5, mM calcium chloride). Fractions of 4 mL were collected, and radioactivity determined, throughout the elution. Carbohydrate was analyzed in the region where cycloheptaamylose would be expected to appear.

Equilibrium dialysis was conducted at 4° in the presence of equilibration buffer by using dialysis cells having two compartments of 1 mL separated by a membrane of 10,000 molecular weight cut-off. Freshly prepared and concentrated enzymes were used in these studies. Two batches of [^{3}H]cycloheptaamylose were used, having specific activities of 8.2×10^5 and 1.2×10^6 d.p.m./μmol. The dialysis cells were never completely filled, so that optimum movement of the ligand through the membrane was allowed. A control cell containing [^{3}H]cycloheptaamylose, originally introduced into one side of the dialysis unit, was used to determine when equilibrium was established. The cells were placed on a horizontal shaker. Both compartments of the control cell achieved equal radioactivity within 66 to 110 h. After equilibrium had been reached, duplicate samples from each compartment were assayed for radioactivity. Protein concentration was re-estimated because minor changes of volume were found to occur in the compartments after dialysis.

The least-squares fit method was used to obtain the "best fit" for Scatchard[18] and Dixon[19] plots.

RESULTS

Purification of alpha amylase by affinity chromatography. — Cereal alpha amylase may be purified in high yield from crude extracts by using a column of cycloheptaamylose-substituted, epoxy-Sepharose 6B (Table I). The procedures are

TABLE I

PURIFICATION OF ALPHA AMYLASE FROM EXTRACTS OF GERMINATED TRITICALE KERNELS

Fraction	*Volume (mL)*	*Protein (mg/mL)*	*Activity (IDC) (units/mL)*	*Specific activity (units/mg)*	*Total activity (units)*	*Purification (fold)*	*Recovery (%)*
Crude extract	74	24	24,800	1,033	1,835,200	1	100
PVP treatment	81.5	23	19,200	835	1,564,800	0.8	85
Dialysis step	138	5.6	11,400	2,036	1,573,200	1.97	85
Affinity peak	54	0.135	38,500	285,000	2,079,000	276	113
Unretained	284	n.d.[a]	1,160		329,440		18

[a]Not determined.

similar to those described previously[8,15], except that glycogen precipitation has been eliminated and the extract has been treated with PVP followed by dialysis at low ionic strength. The specific activity of the purified enzyme was 285,000 units/mg protein according to Briggs' assay[10], and 212 μmol D-glucose/min/mg protein when appearance of reducing groups was monitored. There was an apparent activation of the enzyme on purification, as there was ~32% increase in the total activity eluted from the column as compared with that applied to it. Similar results were obtained on purification of wheat alpha amylase, although the yields prior to affinity were lower because of precipitation in the extract at low ionic strength. Maltose and D-glucose were not effective in eluting the enzyme, and maltotriose only eluted ~14% of the enzyme (Table II). Glycogen and β-limit dextrin were both effective in eluting the enzyme.

TABLE II

EFFECT OF D-GLUCOSE AND D-GLUCOSE OLIGOMERS ON THE RELEASE OF CEREAL ALPHA AMYLASE FROM THE AFFINITY GEL

Treatment	*Release (%)*
D-Glucose (44mM)	4
Maltose (22mM)	4
Maltotriose (16mM)	14
β-Limit dextrin (8 mg/mL)	100
Glycogen (8 mg/mL)	100

Zymograms with starch and β-limit dextrin obtained after isoelectric focusing of the purified enzyme gave characteristic alpha amylase isozyme profiles[15] (Fig. 1), with no evidence of contamination by beta amylase. Additional bands should have been apparent on the starch zymogram if beta amylase were present. Separate experiments showed that neither sweet-potato beta amylase nor barley beta amylase was retained by the affinity column.

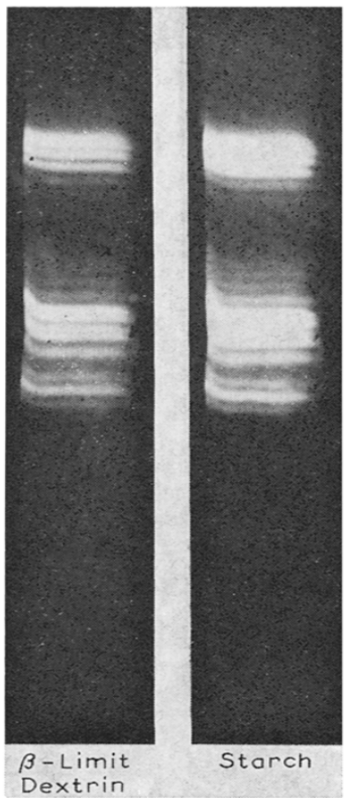

Fig. 1. Isoelectric-focusing zymograms of alpha amylase purified by affinity chromatography.

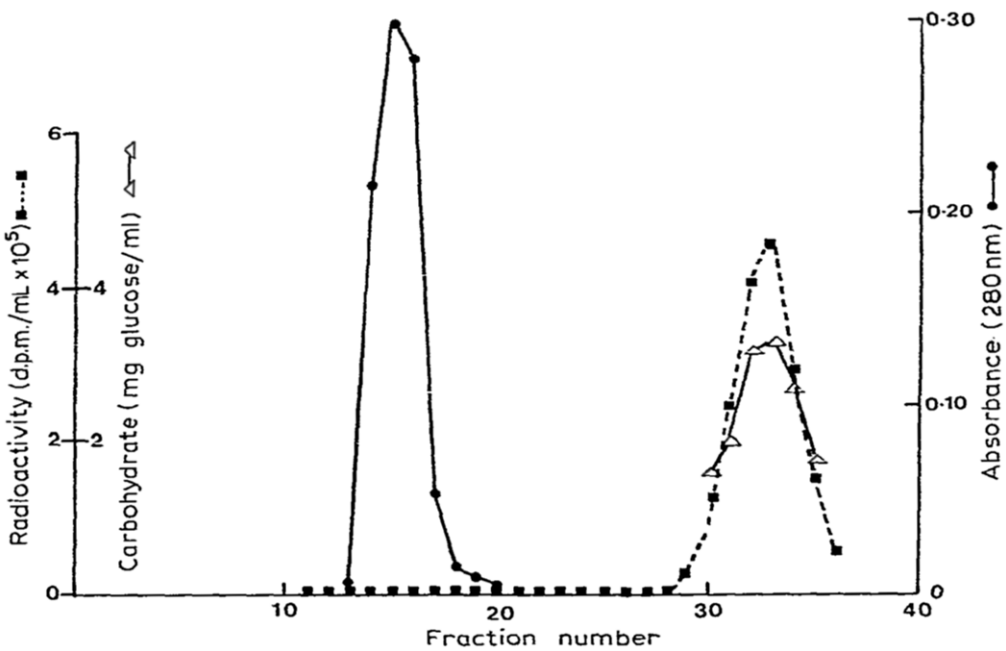

Fig. 2. Separation of [^{3}H]cycloheptaamylose from alpha amylase on Bio-Gel P-4.

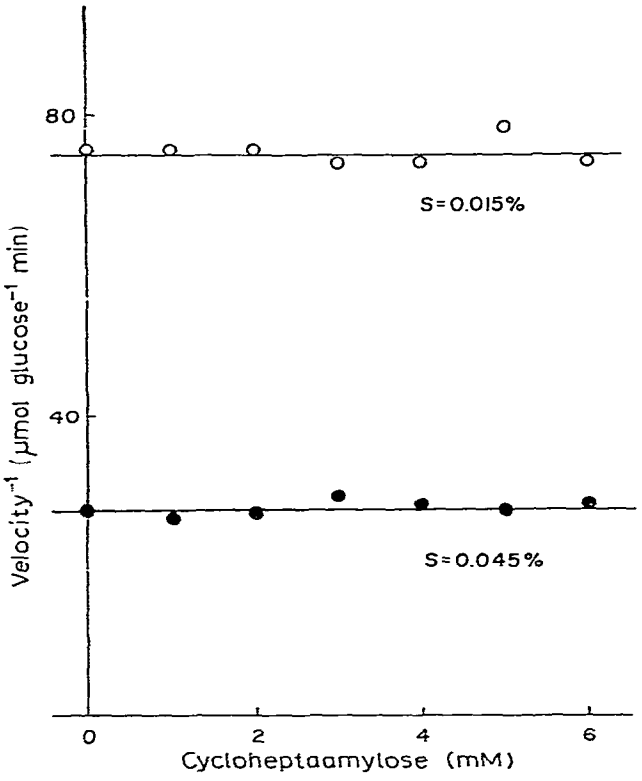

Fig. 3. Dixon plots for the hydrolysis of gelatinized starch by triticale alpha amylase in the presence of increasing concentrations of cycloheptaamylose. S = starch concentration; reaction time = 10 min.

The eluted enzyme had a tendency to precipitate on being kept for a few h in the presence of cycloheptaamylose. The ligand appeared to cause irreversible precipitation of the enzyme when both were present in high concentration. The enzyme remaining in the supernatant solution, however, retained the same or higher specific activity. The precipitate was a mixture of amorphous material and fine, needle-like crystals. Precipitation could be prevented by dilution or by removing cycloheptaamylose by Bio-Gel P-4 chromatography. Studies with [^{3}H]cycloheptaamylose indicated that there was no cycloheptaamylose bound to the enzyme after gel filtration (Fig. 2).

By treating [^{3}H]cycloheptaamylose with epoxy-activated Sepharose 6B, the ligand concentration of the affinity column was determined to be 3.2 μmol of cycloheptaamylose per mL of swollen gel. This result indicated that ~21 % of the available spacer arms were substituted, assuming the manufacturer's value of 15–20 μmol of spacer arm per mL.

Interaction of cycloheptaamylose with cereal amylases. — Cycloheptaamylose had no effect on the hydrolysis of gelatinized starch, as shown by the Dixon[19] plots in Fig. 3. The reaction velocities were constant at concentrations of cyclohepta-

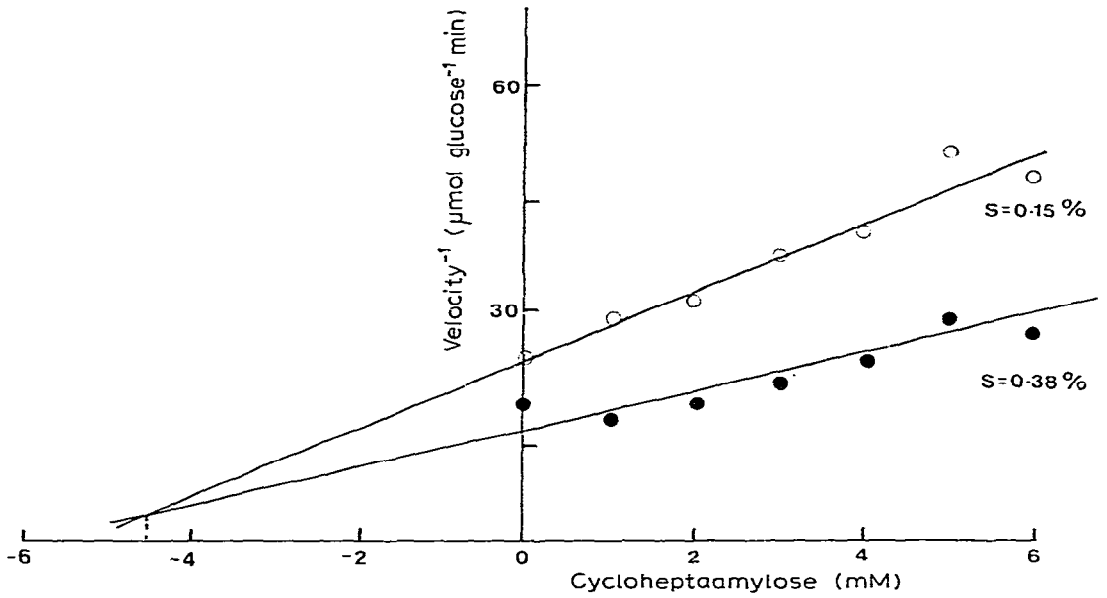

Fig. 4. Dixon plots for the hydrolysis of gelatinized starch by barley beta amylase in the presence of increasing concentrations of cycloheptaamylose. S = starch concentration; enzyme activity = 0.138 μmol glucose/min/mL; reaction time = 5 min.

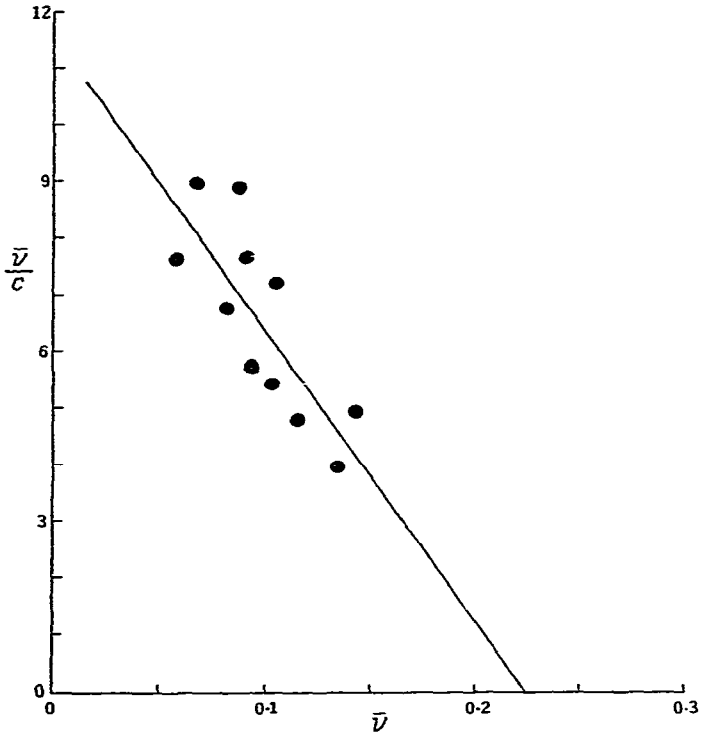

Fig. 5. Scatchard plot for binding of [^{3}H]cycloheptaamylose to alpha amylase at 4°. Legend: $\bar{\nu}$ = μmol cycloheptaamylose bound per μmol of alpha amylase. c = Free concentration of cycloheptaamylose (μmol/mL).

amylose up to 6mM and at two different starch concentrations. Similar results were obtained for a purified wheat alpha amylase. Barley beta amylase, on the other hand, was inhibited by cycloheptaamylose with an apparent K_i of 4.5mM as estimated from the Dixon plots in Fig. 4. Based on the intersection of the lines, the inhibition would be classed as competitive, although the Dixon method may not properly discern between competitive and non-competitive inhibition[20].

As there was no evidence of inhibition of alpha amylase by cycloheptaamylose, and yet a column of cycloheptaamylose-substituted, epoxy-Sepharose 6B retained the enzyme, equilibrium-binding studies using [^{3}H]cycloheptaamylose were performed. The results of equilibrium-dialysis experiments are depicted in the Scatchard plot in Fig. 5. At concentrations of cycloheptaamylose up to 70μM, the plot was linear. The reciprocal of the slope gave a dissociation constant of 19μM. Extrapolation of this line to the x-axis gave a binding capacity of 0.23 mol of cycloheptaamylose bound per mol of enzyme, using a mol. wt. of 41,000 for alpha amylase[15]. Studies at higher concentrations (up to 0.2mM) with wheat alpha amylase gave a Scatchard plot parallel to the x-axis, indicating a continual increase in the apparent binding of cycloheptaamylose with increasing concentration.

DISCUSSION

The results from this study and previous work[8] clearly indicate that purification of alpha amylase on cycloheptaamylose-substituted Sepharose 6B involves an interaction of the enzyme with the ligand. High concentrations of salt did not elute the enzyme from the column, indicating that the interaction was not a result of charge effects.

The interaction in the case of crude cereal extracts was specific for alpha amylase as beta amylase from wheat, triticale, or barley did not bind. Elution required a D-glucose oligomer larger than maltotriose and, as β-limit dextrin and glycogen were as effective as cycloheptaamylose, a cyclic oligomer is not required. The theoretical capacity of the column packing is 131 mg of enzyme per mL of swollen gel assuming a 1 : 1 interaction and a molecular weight of 41,000 for alpha amylase. The apparent activation of the enzyme during purification raises the question of the possibility of an endogenous inhibitor of cereal alpha amylase, similar to the animal alpha amylase inhibitor, located in the wheat kernel[21,22].

Lack of inhibition of alpha amylase-catalyzed hydrolysis of soluble starch by cycloheptaamylose indicates the interaction must occur at a point removed from the active site. The fact that beta amylase is inhibited by the ligand and yet will not bind to the column suggests that there is some hindrance to binding of the epoxy-Sepharose 6B-linked ligand at the active site of either enzyme. Although cycloheptaamylose did not inhibit the hydrolysis of soluble starch by alpha amylase, we have evidence that it will inhibit the binding of alpha amylase to starch granules and the release catalyzed by the enzyme of soluble starch from granules[23].

Further evidence that there is ligand binding to alpha amylase is provided by

the equilibrium-dialysis experiment. The dissociation constant of 19 μM indicates that there is a high affinity of the enzyme for the ligand. A fractional binding-capacity of 0.23 mol ligand per mol is difficult to explain without invoking aggregation of the enzyme or multiple enzyme-binding to the ligand. One other aspect of the study that still require clarification is the precipitation of the enzyme when both ligand and enzyme are present at high concentration. This occurred upon elution of the enzyme with cycloheptaamylose, and a related problem may have occurred in equilibrium-dialysis experiments at high concentration. In the case of enzyme elution, activity was irreversibly lost with no change in specific activity, suggesting that the enzyme had precipitated. In equilibrium dialysis, the enzyme appeared to continue to accumulate ligand as a function of ligand concentration, with no loss of enzyme activity. It is possible that the enzyme can accommodate a number of ligand molecules, as has been reported for pancreatic alpha amylase[4] and that these, in turn, may aggregate to cause precipitation in a manner similar to the precipitation by glycogen[24].

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