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# INTERACTION OF POTATO PHOSPHORYLASE WITH CYCLOAMYLOSES

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SUMMARY

1. Pure potato phosphorylase ($\alpha$-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) has been prepared by chromatography on DEAE-Sephadex A-50 and Sephadex G-200.

2. The synthesis of starch by potato phosphorylase from glucose 1-phosphate and primer component is inhibited by cyclohexa-, cyclohepta- and cyclooctaamylose, $K_i$ being 1.6, 2.9 and $9.8 \cdot 10^{-5}$ M, respectively.

3. Enzymolysis of potato phosphorylase by trypsin (EC 3.4.4.4) is retarded by cyclohexaamylose and by the priming glycogen of the starch reaction. The kinetic investigation shows that several molecules of the cyclic carbohydrates interact with phosphorylase and that these complexes are attacked by trypsin like free phosphorylase but at a slower rate. Complete protection of phosphorylase cannot be achieved.

## INTRODUCTION

Potato phosphorylase ($\alpha$-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) catalyzes synthesis and breakdown of starch according to the equation ($\alpha$-1,4-glucosyl)$_{n-1}$ + Glc-1-$P \leftrightharpoons$ ($\alpha$-1,4-glucosyl)$_n$ + $P_i$. Starch or a polysaccharide of lower molecular weight with non-reducing glucose end groups must be present for the attachment of the additional glucose molecule. Amylose or amylopectin-type starches normally are such primers but maltotriose or -tetraose can also fulfill this function. Branched oligosaccharides inhibit the enzymatic polymerization. It was concluded that a minimum of 3, or better, 4 glucose units is required as terminal chain for the enzymatic polymerization reaction[1-4].

GREEN AND STUMPF[5] found that potato phosphorylase is inhibited by cyclohexa- and cycloheptaamylose and their results indicated that the inhibition is competitive with starch. The lack of any terminal group in cyclohexa- and cycloheptaamylose[6] is the reason for their not being primers. However, the inhibition by cyclo-

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amylose is not explained merely by reference to their structure. Cycloamyloses exhibit a great variety of inclusion and association phenomena[7,8] which are a consequence of their rather rigid conformation and are not encountered with open-chain oligosaccharides. For example, it has been shown that cycloamyloses undergo inclusion association in aqueous phase with certain organic molecules[9]. However, their inhibitory effect is then diminished and with appropriate cosolutes it is virtually cancelled[10]. Such aspects instigated a more detailed study of the interaction of cycloamyloses with potato phosphorylase.

When investigating the deinhibition by inclusion association with cosolutes, it was observed that non-complexed cycloamyloses have a protective effect for potato phosphorylase in enzymatic proteolysis[10]. This offered another parameter for studying the association.

Possible extreme types of association are attachment of cycloamylose molecules to specific sites of phosphorylase, or non-specific association of a multitude of molecules with the enzyme. Inhibition of the starch synthesis by phosphorylase reaction apparently is caused by specific association of one molecule cycloamylose to the active sites while the protective effect against proteolysis is caused by multiple association.

## EXPERIMENTAL

### *Isolation of potato phosphorylase*

Phosphorylase was prepared from potato tuber. In the first steps, the procedure followed the purification described by LEE[11]. However, salt gradient elution on DEAE-Sephadex A-50 instead of electrophoresis was applied after the second fractionation with $(NH_4)_2SO_4$. The enzyme was further purified by gel filtration on Sephadex G-200. Data on the purification of the enzyme are summarized in Table I and details of chromatographic fractionations are described in Figs. 1 and 2.

Fractions containing enriched phosphorylase (Fig. 1, upper part) from two chromatograms were combined and the protein was precipitated with $(NH_4)_2SO_4$, 40 g/100 ml. After centrifugation the precipitate was dissolved in 20 ml Tris buffer (pH 6.9) and residual $(NH_4)_2SO_4$ was removed by dialysis against buffer which was changed 3 times during 36 h. The preparation was rechromatographed (Fig. 1,

TABLE I

PURIFICATION OF POTATO PHOSPHORYLASE FROM 3800 g POTATOES

| *Fractionation* | *Total units recovered* | *Units/mg protein* | *Recovery (%)* |
|---|---|---|---|
| Potato juice | 694 000 | 34 | (100) |
| 1st $(NH_4)_2SO_4$ precipitation–adsorption | 640 000 | 202 | 92 |
| 2nd $(NH_4)_2SO_4$ precipitation | 446 000 | 322 | 64 |
| 1st chromatogram on DEAE-Sephadex A-50* | 287 000 | 885 | 42 |
| 2nd chromatogram on DEAE-Sephadex A-50* | 233 000 | 1560 | 34 |
| 1st gel filtration on Sephadex G 200** | 142 000 | 1950 | 22 |
| 2nd gel filtration on Sephadex G-200** | 138 000 | 2050 | 20 |

* See Fig. 1.
** See Fig. 2.

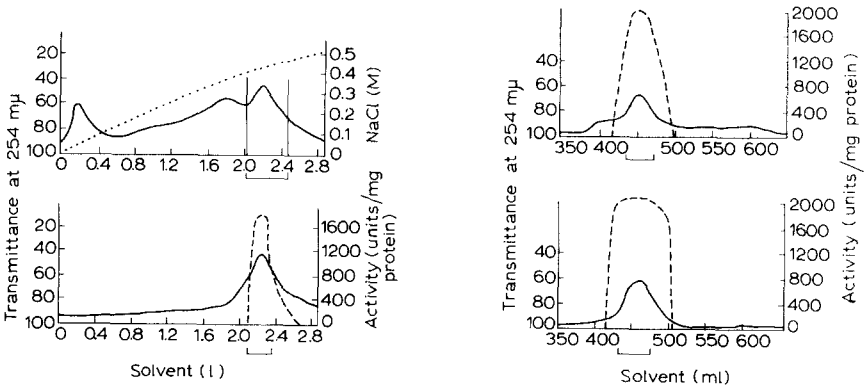


Fig. 1. Chromatographic enrichment of potato phosphorylase on DEAE-Sephadex A-50. The packing was equilibrated with buffer (pH 6.9) of 0.02 M Tris, 0.02 M NaCl and 0.15 mM EDTA, the solution being one-fourth saturated with thymol. Active material from the $(NH_4)_2SO_4$ fractionation was dialyzed against the buffer and about 700 mg was applied to a column, 3.8 cm × 86 cm (980 ml bed volume). The salt gradient was produced by pumping buffer containing 1 M NaCl, 45 ml/h, into a mixing reservoir with 4.2 l original buffer and from there to the column which was kept at 8°. The eluant was collected under cooling. Chromatography was repeated with the most active portion (lower part of figure). The full lines give the recorded transmittance at 254 mμ (LKB Uvicord). The dotted line in the upper part indicates the salt concentration. This line is omitted in the lower part where the broken line gives units/mg protein.

Fig. 2. Final purification of potato phosphorylase by chromatography on Sephadex G-200. The packing was equilibrated for 4 days with Tris buffer and then filled into a column, 2.7 cm × 175 cm (1000 ml bed volume) at 8°. The flow rate was reduced to 12 ml/h with a Teflon needle-valve stopcock and remained then constant for many runs. The most active fraction was rechromatographed (lower part of figure). The full lines show the recording at 254 mμ; the broken lines show specific activity, units/mg protein.

lower part). The active fraction was then chromatographed twice in two portions on Sephadex G-200 (Fig. 2) and recovered.

Aliquots from Chromatograms 2–4 were electrophoretically tested and showed uniformity of the final product (Fig. 3).

*Reagents*

The di-potassium salt of glucose 1-phosphate (Nutritional Biochemicals Corp.) was recrystallized twice from water–ethanol. Amylopectin (Calbiochem), trypsin (EC 3.4.4.4) (Worthington Enzymes, No. TRL6253) and casein (vitamin-free, Fisher Scientific Co.) were used as obtained commercially. The inhibitors, cyclohexa-, cycloheptа- and cyclooctaamylose, were preparations of this laboratory.

*Quantification methods*

The activity of potato phosphorylase was determined at 30° in 0.1 M citrate buffer (pH 6.3) with 10 mM glucose 1-phosphate and 0.8% amylopectin[11]. Samples of 1 ml were pipetted from the reaction mixture into 4 ml ice-cold 10% trichloroacetic acid after a time period during which about 50% phosphate would be released. $P_i$ was usually determined in a volume of 50 ml according to FISKE AND SUBBAROW[12], using a Klett photometer with filter No. 66. Blank values were determined with citrate buffer and amylopectin, the standard sample contained 5 ml of 1 mM $Na_2HPO_4$

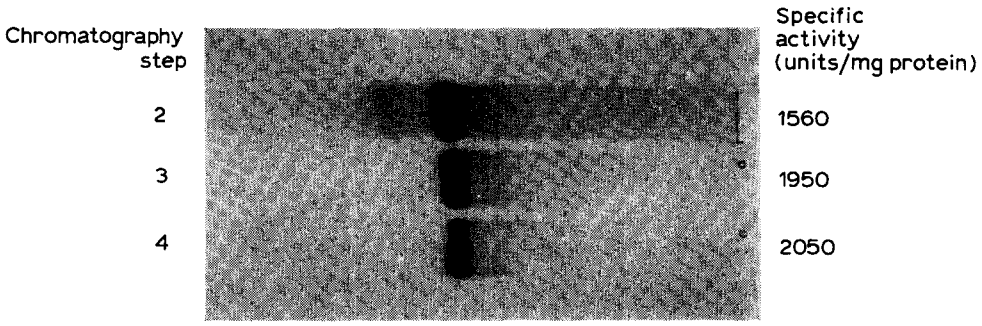


Fig. 3. Electrophoresis of potato phosphorylase. Vertical acrylamide gel, Tris buffer (pH 6.9), 15 V/cm, 3 h. Materials from Chromatograms 2, 3 and 4 are represented. The protein migrates to the anode.

in addition, and any $P_i$ from glucose 1-phosphate was considered by measuring 1 ml of the substrate solution without enzyme.

The enzyme activity was calculated according to CORI, CORI AND GREEN[13] and HANES[1]. The equilibrium of $P_i$ and glucose 1-phosphate under the described conditions was found to be at 86.5 mole % $P_i$ liberated.

Protein concentrations were measured according to LOWRY *et al.*[14] with Folin's phenol reagent in a Klett photometer with filter No. 54. The reference was bovine serum albumin. The absorbance ratio serum albumin: potato phosphorylase was found to be 1:0.92 by drying and weighing the materials. This was considered when calculating specific activities.

Tryptic digestions were carried out at 30° and pH 6.9. The mixtures contained 1.0 $\mu$M (24 $\mu$g/ml) trypsin, 2.0 $\mu$M (415 $\mu$g/ml) potato phosphorylase in 0.04 M citrate buffer (pH 6.9) and 5.0 mM $CaCl_2$. At time intervals, 50-$\mu$l samples were pipetted with a syringe into 2.0 ml of the substrate solution for determining the residual activity of the phosphorylase. Tryptic action is stopped by the presence of glucose 1-phosphate[15].

Procedures were essentially the same when measuring the influence of cycloamylose and of amylopectin on trypsin activity. A 1% casein solution was incubated with 10 $\mu$M (240 $\mu$g/ml) trypsin at 30° and pH 6.9. At appropriate time intervals 0.2 ml were pipetted into 3.0 ml of 10% trichloroacetic acid. The precipitate was centrifuged and the material soluble in trichloroacetic acid was measured at 280 m$\mu$. Procedures in presence of amylopectin, cyclohexa- and cycloheptaamylose were under the same conditions.

## RESULTS AND DISCUSSION

The purity of potato phosphorylase is of crucial importance in this study. The course of purification developed here (Table I and Figs. 1–3) yielded a preparation with an activity of 2050 units/mg protein which compares favorably with that obtained by LEE[11]. Perhaps more significant for the purity is the shape of the curve for specific activity in the final chromatogram and the homogeneity in electrophoresis at pH 6.9. A minor component which probably is identical with that retained in the earlier preparations is eliminated by the column chromatographic procedures.

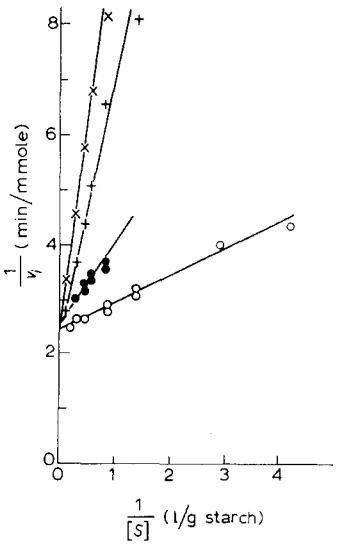


Fig. 4. Lineweaver–Burk diagram from different concentrations of starch: ○—○, no inhibitor; ×—×, 0.238 mM cyclohexaamylose; +—+, 0.238 mM cycloheptaamylose; ●—●, 0.238 mM cyclooctaamylose. Assays were carried out with 28.6 mM glucose 1-phosphate in 0.1 M citrate buffer (pH 6.3).

The Lineweaver–Burk diagram of Fig. 4 shows that besides cyclohexa- and cycloheptaamylose, the cyclooctaamylose compound also competitively inhibits potato phosphorylase acting on starch. The inhibition constants are $K_i$ = 1.6, 2.9 and $9.8 \cdot 10^{-5}$ M, respectively.

The affinities of phosphorylase follow the order cyclohexa- > cyclohepta- > cyclooctaamylose[16]. This decrease is in contrast to the interaction of cycloamylose with sweet potato $\beta$-amylase where cyclohexa- and cycloheptaamylose are equally tightly bound[17].

When interacting with the enzyme, cycloamylose molecules simulate terminals of the starch chain which are more flexible in conformation than cycloamylose. Therefore, one may interpret the highest affinity to the smallest ring, cyclohexaamylose, as due to the relatively better steric compatibility of enzyme and carbohydrate of this size while the conformational equilibria of cyclohexa- and cycloheptaamylose involve forms which are not as compatible. On the other hand, the difference of affinities can also be interpreted on the basis of a partial inclusion of the active site by forces that are strongest with the smallest ring. The latter mechanism appears more likely in view of the deinhibition by molecules which *per se* are inert to phosphorylase but undergo inclusion association with cycloamylose[9,10]. Association with the priming starch can be visualized as a partial wrapping of 4 terminal glucose units around the active site. They are then like a segment of the cycloamylose ring but are open for addition of another glucose molecule. The $K_m$ of potato phosphorylase for non-reducing end groups of amylopectin has been estimated by LEE[11] as $4.0 \cdot 10^{-5}$ M carbohydrate end groups which is of the same magnitude as $K_i$ of the cycloamylose.

The hydroxyl groups are essential for the enzyme–inhibitor complex since

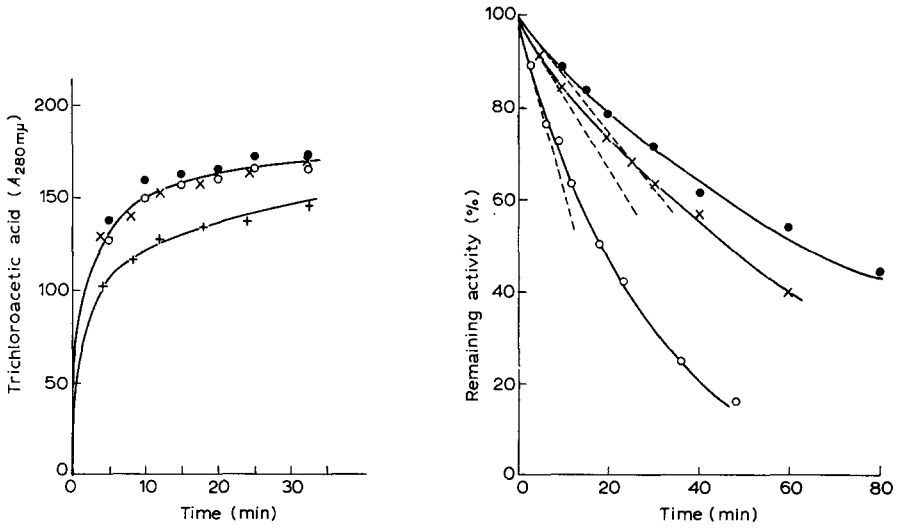


Fig. 5. Action of trypsin on casein. 10 mM trypsin in citrate buffer (pH 6.9) with 1% casein: ○—○, without complexing agent; ×—×, 5.8 mM cyclohexaamylose; +—+, 5.8 mM cycloheptaamylose; ●—●, 8 mg amylopectin/ml (approx. 2.4 mM end groups).

Fig. 6. Action of trypsin on potato phosphorylase. 1.0 $\mu$M trypsin in citrate buffer (pH 6.9) with 2.0 $\mu$M potato phosphorylase. $v_i$ was determined by the tangential method as indicated by the broken lines: ○—○, no complexing agent ($v_i = 8.1 \cdot 10^{-8}$ M·min$^{-1}$); ×—×, 0.24 mM cyclohexaamylose ($v_i = 3.2 \cdot 10^{-8}$ M·min$^{-1}$); ●—●, 0.62 mM non-reducing end groups of amylopectin ($v_i = 2.3 \cdot 10^{-8}$ M·min$^{-1}$).

cycloheptaamylose tetradecamethyl ether does not inhibit the phosphorylase reaction. Two-thirds of the hydroxyl groups of cycloheptaamylose are methylated in this compound and with all likeliness the free hydroxyl groups are in Position 2 of the glucose units[18].

The protection of potato phosphorylase by cycloamylose from enzymolysis was studied with trypsin instead of ficin which had been used in earlier experiments[10]. Commercial preparations of the latter enzyme turned out to be multicomponent mixtures in chromatography on DEAE-Sephadex columns. Trypsin was first tested for direct inhibition by cycloamylose or amylopectin with casein as substrate. Fig. 5 shows that cyclohexaamylose and amylopectin are inert but some inhibition was found with cycloheptaamylose. Therefore only the former ones were investigated with the system trypsin–potato phosphorylase (Fig. 6).

Initial velocities, $v_i$, were determined at various concentrations of cyclohexaamylose and amylopectin by the tangential method as indicated in Fig. 6. The results are shown in Fig. 7. $v_i$ decreases first equally with both carbohydrates but constant values are reached with increasing concentrations. The final $v_i$ is smaller with cyclohexaamylose than with amylopectin and complete protection cannot be achieved.

The equilibrium of phosphorylase ($E$) and cyclohexaamylose ($I$) is, according to the former inhibition experiments,

$$[E] + [I] \rightleftharpoons [EI] \qquad (1)$$

Two principles can be foreseen when the proteolytic enzyme acts upon this

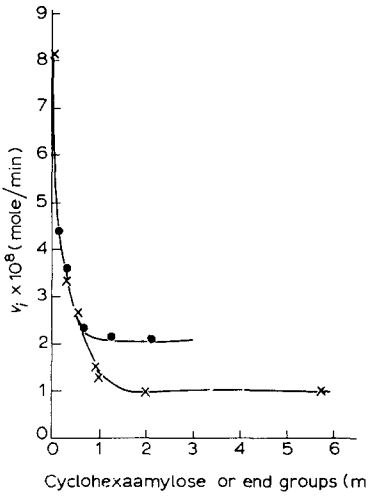


Fig. 7. Initial velocity of proteolysis of 2.0 $\mu$M potato phosphorylase by 1.0 $\mu$M trypsin at various concentrations of carbohydrates: ×—×, cyclohexaamylose; ●—●, amylopectin.

equilibrium: trypsin may attack only $E$ (Assumption $A$) or it may attack $E$ and $EI$ concurrently (Assumption $B$). In the latter case, the reaction with $EI$ is slower than with $E$ since the proteolysis is slower in the presence of carbohydrate. $v_i$ from Fig. 7, therefore, represents either (A) $v_{iE}$ or (B) $v_{iE} + v_{iEI}$.

The following calculations strongly favor the simultaneous proteolytic attack on $E$ and $EI$ and bear out the same surmise which may be deduced qualitatively from the fact that potato phosphorylase is not completely protected at high concentrations of cyclohexaamylose.

*A. Only free phosphorylase is attacked*

$$v_i = k_E[E] \tag{2}$$

The portion of free phosphorylase, $E$, of the total, $E_T$, is given by

$$[E] = \frac{K_i[E_T]}{[I] + K_i} \tag{3}$$

Introducing Eqn. 3 into Eqn. 2 results in

$$v_i = k_E \frac{K_i[E_T]}{[I] + K_i} \tag{4}$$

In the equilibrium of cyclohexaamylose, $[I] = [I_T] - [EI]$, one can accept $[I] \approx [I_T]$ since $[I_T] \gg [EI]$. Eqn. 4 is then modified to

$$v_i = k_E \frac{K_i[E_T]}{[I_T] + K_i} \tag{5}$$

Assumption $A$ relates $k_E$ only to proteolysis of free phosphorylase. When cyclohexaamylose is not present, $k_E$ is obtained from Eqn. 2 and introduced into Eqn. 5

$$k_E = \frac{v_{io}}{[E_T]} \tag{6}$$

$$v_i = \frac{v_{io} \times K_i}{[I_T] + K_i} \tag{7}$$

Initial velocities found experimentally (Figs. 6 and 7) and calculated from Eqn. 7 are listed in Table II. The former values are larger than the latter by a factor of about 10. This rules out that trypsin acts only upon free phosphorylase.

TABLE II

PROTEOLYSIS OF POTATO PHOSPHORYLASE BY TRYPSIN IN PRESENCE OF CYCLOHEXAAMYLOSE

| *Cyclohexa-amylose (mM)* | $10^8 \times v_i$ *(M/min)* | | $10^2 \times k_{EI}$/*min (Eqn. 7)* |
|---|---|---|---|
| | *Experimental* | *Eqn. 7* | |
| 0 | 8.1 | — | (4.0, $10^2 \times k_E$) |
| 0.35 | 3.3 | 35 | 1.5 |
| 0.55 | 2.7 | 23 | 1.3 |
| 0.95 | 1.5 | 13 | 0.7 |
| 2.0 | 1.0 | 7 | 0.5 |

*B. Free and bound phosphorylase are attacked*

$$v_i = k_E[E] + k_{EI}[EI] \tag{8}$$

$$v_i = v_{iE} + k_{EI}[EI] \tag{9}$$

From Eqn. 9, the velocity constant for proteolysis of $EI$ is

$$k_{EI} = \frac{v_i - v_{iE}}{[EI]} \tag{10}$$

Entering $[EI] = [E_T] - [E]$ from the equilibrium of phosphorylase and cyclohexaamylose inhibitor yields

$$k_{EI} = \frac{v_i - v_{iE}}{[E_T] - [E]} \tag{11}$$

$E$ is expressed in Eqn. 3. Introduction and rearrangement leads to

$$k_{EI} = \frac{(v_i - v_{iE})([I_T] + K_i)}{[E_T][I_T]} \tag{12}$$

In this equation, $K_i$ is the inhibition constant of the complex phosphorylase–cyclohexaamylose and has been determined; $v_i$ is the initial velocity of proteolysis and has been measured; $v_{iE}$ is the initial velocity of proteolysis of free phosphorylase and has been calculated according to Eqn. 7. Values of $k_{EI}$ calculated according to Eqn. 12 are listed in Table II for respective concentrations of cyclohexaamylose, $I_T$.

The value of $k_E$ is about 3 times that of $k_{EI}$ at conditions closest to those used for determining $K_i$. However, $k_{EI}$ is not constant within the range of concentrations measured. This negates Eqn. 1 which, on the other hand, has been shown valid for the inhibition of potato phosphorylase by cycloamylose. Association of additional molecules of cyclohexaamylose does not become apparent from the inhibition but must be concluded from the proteolysis of phosphorylase. The decreasing rate of proteolysis with increasing concentration of cyclohexaamylose is explained

when $k_{EI}$ refers to a sum of complexes, $EI$, $EI_2$..., all of which are attacked by trypsin at different rates which probably decrease in this order.

Some experiments were made to compare the proteolysis products from phosphorylase and phosphorylase in the presence of cyclohexaamylose or amylopectin. The three systems were incubated with trypsin until 50% of the phosphorylase activity was consumed. Neither electrophoresis of proteins nor finger print patterns by thin-layer chromatography[19] of fractions soluble in trichloroacetic acid showed marked differences for the course of proteolysis in presence or absence of carbohydrates.

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