# POTATO PHOSPHORYLASE

## I. PURIFICATION, PHYSICOCHEMICAL PROPERTIES AND CATALYTIC ACTIVITY

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(Received January 14th, 1960)

SUMMARY

A large scale preparation for the isolation of potato phosphorylase is described. Electrophoresis, sedimentation and diffusion measurements have been employed for the investigation of the homogeneity of the enzyme. The sedimentation coefficient was $7.9_4 \cdot 10^{-13}$ sec, the diffusion coefficient $3.7_6 \cdot 10^{-7}$ cm$^2$ sec$^{-1}$ and the calculated molecular weight, 207,000.

The turnover number at 30° at pH 6.3 was 9400 moles of glucose-1-phosphate per mole of protein/min. MICHAELIS constants, pH optimum, the effect of metals and the stability of the enzyme under various conditions have been determined. The enzyme preparation did not contain any nucleotide and was not activated by 5'-adenylic acid or other nucleotides.

## INTRODUCTION

In order to determine the molecular weight and the phosphate content of potato phosphorylase, it was necessary to prepare a sufficient quantity of enzyme in homogeneous form. Existing methods for the purification of this enzyme[1,2] were not suitable for this purpose. A convenient procedure which yields about 80 mg of enzyme protein from 5 kg of potatoes is described in this paper.

The properties and kinetic behavior of potato phosphorylase have been studied by several investigators with partially purified enzyme preparations. It seemed desirable to repeat some of these observations with the highly purified enzyme preparation described in this paper.

## EXPERIMENTAL

Glucose-1-phosphate (G-1-P) as the dipotassium salt, was obtained from Schwarz Laboratories. Potato amylose and amylopectin were kindly contributed by STEIN, Hall and Co., Inc., New York, N.Y.

Abbreviations used: G-1-P, glucose-1-phosphate; TCA, trichloroacetic acid; 5'-AMP, 5'-adenylic acid; 2'-AMP, 2'-adenylic acid; 5'-CMP, cytidine-5'-phosphate; 5'-GMP, guanosine-5'-phosphate; 5'-IMP, inosine-5'-phosphate; 5'-UMP, uridine-5'-phosphate.

Enzyme assays were carried out at pH 6.3 in a reaction mixture (1 ml) containing 0.1 *M* citrate buffer, 0.01 *M* G-1-P and 0.76 % amylopectin. After 5 min at 30°, the reaction was stopped by the addition of 0.5 ml of 5 % TCA and the pH adjusted to about 4 by the addition of 2 ml of 0.1 *M* sodium acetate solution. The amount of inorganic P formed by the enzymic reaction was determined by the method of LOWRY AND LOPEZ[3]. A suitable blank was prepared for each series of measurements by incubating the reactants without G-1-P and addition of the latter after the protein precipitation.

Under the conditions described, the reaction was first order with respect to G-1-P. Enzyme units were defined as $K \times 1000$, where $K$ is the first order velocity constant ($\log_{10}$, $min^{-1}$). These are the same units which have been used in this laboratory to express the activity of muscle phosphorylase[4]. $K$ was proportional to enzyme activity over a 20-fold dilution of the enzyme. It should be noted that the concentration of G-1-P (0.01 *M*) in the assay of the potato enzyme was lower than in the case of the muscle enzyme (0.016 *M*). Specific activity was expressed as units/mg of protein.

Protein in crude enzyme preparations was determined by the biuret method of GORNALL *et al.*[5]. In more highly purified preparations, protein concentration was calculated from O.D. measurements at 280 mμ in the Beckman spectrophotometer. The log $I_0/I$ reading for a solution containing 1 mg of highly purified enzyme protein/ml in a cell of 1 cm light path was 1.29, the protein concentration having been determined by drying to constant weight.

*Purification procedure*

*Potato juice*: Idaho potatoes, obtained in the open market, were kept in the cold room. The washed potatoes were sliced and homogenized in the Waring blendor for about 1 min in a solution of 0.5 % $Na_2S_2O_4$–0.5 % sodium citrate (100 ml/kg of potatoes). The homogenate was passed through 4 layers of gauze and the residue squeezed out in a laboratory press. In order to settle out the starch, the combined potato juice was allowed to stand for 1 h in the cold room (3°). All further steps were carried out in the cold room. pH adjustments during fractionation with solid ammonium sulfate were made by addition of ammonia.

*Precipitation-adsorption by amylose*: Inert protein was removed by the addition of 20 g of solid ammonium sulfate to 100 ml of the starch-free potato juice at pH 6.5. After standing for several hours or overnight, a clear filtrate was obtained by centrifugation and filtration with filter aid (Celite). To the filtrate was added slowly, with sufficient stirring so as to avoid the formation of lumps, 1 g of finely powdered amylose/100 ml of fluid. After stirring for 1 h, 18 g of solid ammonium sulfate/100 ml was slowly added at pH 6.5 and the stirring continued for another hour. The centrifuged precipitate was extracted with 5 successive portions of 50 ml each of 1.8 *M* ammonium sulfate solution, pH 6.5, per kg of potatoes used. The residue was then eluted with successive portions of 50 ml each of 0.1 *M* citrate buffer pH 6.5 per kg of potatoes used until most of the activity was extracted.

*Second ammonium sulfate fractionation*: To the combined eluate was slowly added 20 g of solid ammonium sulfate/100 ml at pH 6.5. After centrifugation, 18 g of solid ammonium sulfate/100 ml was added to the supernatant fluid. The precipitate was collected by centrifugation and was suspended in a small amount of 0.002 *M* potassium

phosphate buffer, pH 6.8, to give a protein concentration of about 10 %. This suspension was dialyzed against large volumes of the same buffer (saturated with thymol) for about 2 days with 3 changes of the buffer solution. The precipitate which formed during the dialysis was centrifuged off and discarded. The protein concentration of the supernatant fluid was about 6.5 %.

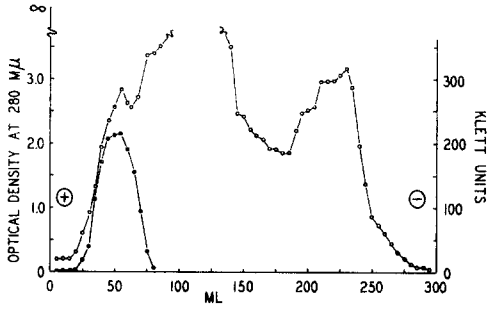


Fig. 1. Electropherogram of the first zone electrophoretic separation. O—O, protein concentration (O.D. at 280 mμ); ●—●, phosphorylase activity expressed in Klett units. 700 mg of the 2nd ammonium sulfate fraction, 38 mA for 22 h.

*First column electrophoresis*: The apparatus used and procedure followed were based on those described by PORATH[6]. About 700 mg of protein solution in 0.002 *M* phosphate buffer, pH 6.8, was applied to a column 600 × 25 mm which contained 70 g of Munktell's cellulose powder for electrophoresis. The protein zone was moved down about 1 cm from the top by the addition of buffer solution. The top of the column was connected to the cathode, the bottom to the anode and a current of 38 mA was applied for 21 to 22 h. The column then was eluted with the same buffer solution at a flow rate of about 50 ml/h. Fractions of 5 ml each were collected for the measurement of protein concentration at 280 mμ and for the determination of enzyme activity. One of the experiments is shown in Fig. 1.

The combined active fractions were treated with solid ammonium sulfate; 28 g/100 ml was added and after 1 h the suspension was centrifuged and 12 g was added/100 ml of the supernatant fluid. The resulting precipitate was collected by centrifugation, suspended in a small amount of 0.002 *M* phosphate buffer pH 6.8 and dialyzed against the same buffer for 1 day with 2 changes of the buffer solution.

*Second column electrophoresis*: This was carried out in the same manner as the first zone electrophoretic separation; one experiment is shown in Fig. 2.

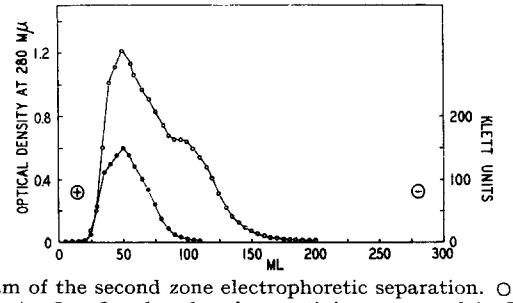


Fig. 2. Electropherogram of the second zone electrophoretic separation. O—O, protein concentration (O.D. at 280 mμ); ●—●, phosphorylase activity expressed in Klett units. 65 mg of the first zone electrophoretic fraction, 38 mA for 22 h.

TABLE I

PURIFICATION OF POTATO PHOSPHORYLASE*

| *Fraction* | *Total protein (mg)* | *Total units* | *Units per mg* | *Recovery (%)* |
|---|---|---|---|---|
| 1. Potato juice | 29,800 | 848,000 | 28.5 | (100) |
| 2. Precipitation-adsorption | 4,160 | 705,000 | 170 | 74.5 |
| 3. 2nd ammonium sulfate fractionation | 2,560 | 487,000 | 191 | 57.5 |
| 4. 1st column electrophoresis | 280 | 193,000 | 687 | 22.3 |
| 5. 2nd column electrophoresis | 80 | 125,000 | 1560 | 14.7 |

* From 5 kg of potatoes.

The combined active fractions were fractionated with ammonium sulfate as described in the preceding section and the final precipitate was dissolved in 0.1 *M* citrate buffer pH 7.

The procedure outlined results in about a 55-fold purification of the enzyme, starting with starch-free potato juice. An example is given in Table I. It should be emphasized that the yield and the specific activity of the final product depend to some extent on the activity of the starting material.

Potato phosphorylase could also be isolated by gradient elution with 1 *M* KCl–0.002 *M* Tris buffer, pH 7.5 from DEAE cellulose columns. The enzyme appeared in the last fractions and the degree of purification achieved was about the same as by zone electrophoresis.

Attempts to crystallize the purified enzyme preparations were not successful; in particular, the ammonium sulfate method described by BAUM AND GILBERT[2] failed to yield a crystalline product.

*Physical measurements*

The purified preparation obtained from the second zone electrophoresis was used in these experiments.

*Electrophoresis*: Measurements were made in the 2-ml open cells of the Perkin-Elmer apparatus, Model 38A. The protein solutions were dialyzed for 24 h with stirring against 3 l of potassium phosphate buffer of ionic strength 0.1 at pH values from 6.6 to 8.0 at 1°. Three of the electrophoretic patterns are shown in Fig. 3. It

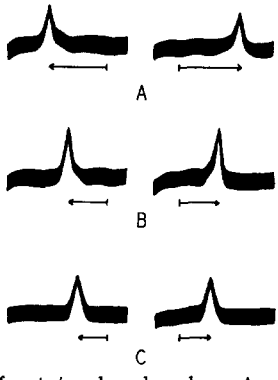


Fig. 3. Electrophoretic patterns of potato phosphorylase. A, pH 6.9; B, pH 7.2; C, pH 7.5; the bases of the arrows indicate the initial boundary.

can be seen that a second component is present in the patterns obtained at pH 6.9 and 7.2. This component is not seen at pH 7.5.

*Sedimentation*: The sedimentation experiments were carried out in a Spinco Model E analytical centrifuge at 20° at 52,640 rev./min. The protein solutions had been dialyzed for 1 day with stirring in the cold room against 3 l of 0.01 *M* potassium phosphate–0.15 *M* KCl, pH 6.7. Protein concentrations of 6.5, 5.0 and 3.5 mg/ml were used. The protein sedimented as a single peak (Fig. 4) and the sedimentation coefficient $S_{20,w}$ (extrapolated to zero protein concentration) was 7.9$_4$.

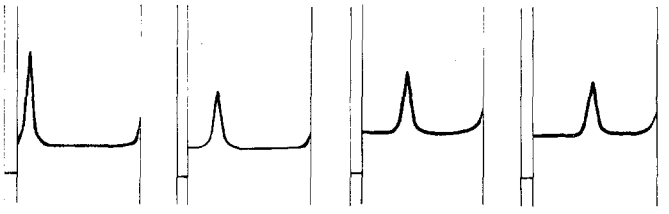

Fig. 4. Sedimentation velocity patterns. 6 mg/ml of protein was centrifuged at 52,640 rev./min at 20°. The pictures were taken at 15, 35, 55 and 70 min.

*Diffusion*: The cell of the Perkin-Elmer apparatus was used for the diffusion measurements which were carried out at 4° at a protein concentration of 6.5 mg/ml. The protein solution had been dialyzed against 0.01 *M* potassium phosphate–0.15 *M* KCl buffer pH 6.7 for 2 days in the cold room. In Fig. 5 is shown a comparison of the normal probability curve with the experimental points. The diffusion coefficient, $D_{20,w}$ calculated by the maximum area method, was $3.76 \cdot 10^{-7}$ cm$^2$ sec$^{-1}$.

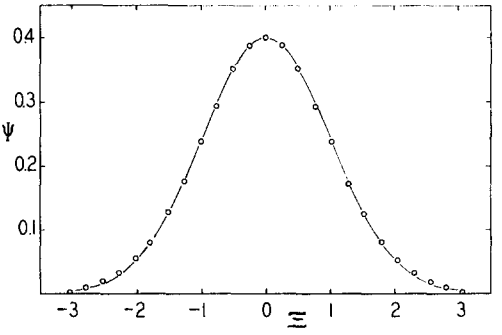


Fig. 5. Graph of the comparison of the experimental curve with the normal probability curve in diffusion measurement.

*Molecular weight*: Assuming a partial specific volume of 0.75, the molecular weight of potato phosphorylase was calculated to be 207,000. The frictional ratio, $f/f_0$, was found to be 1.43.

*Catalytic activity*

*Reaction velocity*: HANES[7] found optimum activity between pH 5.9 and 6.1 in 0.028 *M* maleate buffer. The purified preparation of potato phosphorylase in 0.1 *M* citrate buffer exhibited a relatively sharp pH optimum at 6.5 (Fig. 6). The $Q_{10}$ for polysaccharide synthesis at pH 6.3 was about 1.7 between 15 and 45°.

The effect of concentration of reactants on the initial velocity of the reaction was determined in 0.1 $M$ citrate buffer, pH 6.3 at 30°. The MICHAELIS-MENTEN constant ($K_m$) was evaluated from LINEWEAVER-BURK plots and was found to be $3.5 \cdot 10^{-3}$ $M$ for glucose-1-phosphate, $7.5 \cdot 10^{-3}$ $M$ for inorganic phosphate and 13 mg/100 ml for potato amylopectin. The latter value corresponds to about $0.4 \cdot 10^{-4}$ $M$ glucose end groups. WEIBULL AND TISELIUS[8] reported similar values for glucose-1-phosphate and inorganic phosphate and a significantly higher value for starch.

The turnover number of the enzyme at pH 6.3 and 30° was 6700 moles/mole of protein/min under the conditions of enzyme assay and 9400 moles/mole of protein/min when the enzyme was saturated with respect to glucose-1-phosphate.

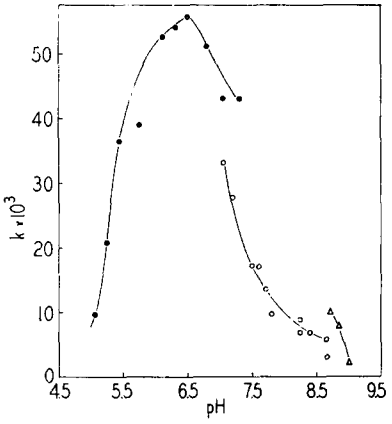


Fig. 6. Effect of pH on enzyme activity. $1 \cdot 10^{-2}$ $M$ glucose-1-P, 0.75% amylopectin and 54 units of enzyme were incubated in 0.1 $M$ buffer at 30°. ●, citrate buffer; ○, Tris buffer; △, glycine buffer.

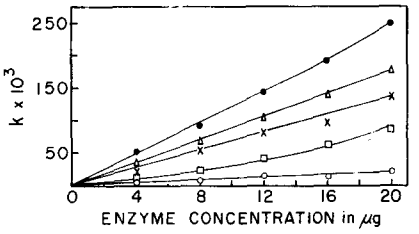


Fig. 7. Effect of heavy metals on enzyme activity. The reaction mixture contained 0.04 $M$ glycerophosphate buffer pH 6.3, $1 \cdot 10^{-2}$ $M$ glucose-1-P and 0.75% amylopectin. ●, no inhibitor; △, $AgNO_3$ ($1 \cdot 10^{-4}$ $M$); ×, $AgNO_3$ ($1 \cdot 10^{-3}$ $M$); □, $HgCl_2$ ($1 \cdot 10^{-4}$ $M$); ○, $CuSO_4$ ($1 \cdot 10^{-3}$ $M$).

*Effect of nucleotides*: One of the significant differences between muscle and potato phosphorylase is that the latter is not stimulated in its activity by the addition of 5'-AMP. This could have been due to the fact that potato phosphorylase contained tightly bound nucleotide, but no evidence could be obtained from spectroscopic observations. The purified preparation had an $E_{280}/E_{260}$ ratio of 1.6 and this value did not change after treatment of the enzyme with Norit. Furthermore, the perchloric acid extract of the enzyme had no absorption peak at 260 mμ. The u.v. absorption spectrum of the protein ($2.5 \cdot 10^{-6}$ $M$) and of 5'-AMP ($1 \cdot 10^{-5}$ $M$) in mixtures did not show any significant change. This indicates that potato phosphorylase, in contrast to muscle phosphorylase[9], does not bind any 5'-AMP.

Other nucleotides (2'-AMP, 5'-AMP, 5'-GMP, 5'-IMP, 5'-UMP) in concentrations of $1 \cdot 10^{-5}$ to $3 \cdot 10^{-5}$ $M$, and yeast extract did not have any effect on the activity of potato phosphorylase.

*Stability of enzyme*: Relevant data on the stability of the enzyme under various conditions are shown in Table II.

*Effect of heavy metals*: FISCHER AND HILPERT[1] reported that potato phosphorylase is inactivated by cupric, mercuric, silver and zinc ions. It was found that the type of buffer used played a role. In 0.1 $M$ citrate buffer none of these ions was inhibitory,

except $Hg^{++}$. Examples of the effect of heavy metals on enzyme activity in glycerophosphate buffer are shown in Fig. 7.

TABLE II

STABILITY OF POTATO PHOSPHORYLASE

Incubated in 0.05 *M* citrate buffer, except where noted.

| *Period of incubation* | *pH* | *Temperature* | *Percent loss of enzyme activity* |
|---|---|---|---|
| 5 min | 6.3 | 50 | 10 |
| 5 min | 6.3 | 55 | 30 |
| 5 min | 6.3 | 60 | 90 |
| 5 min | 6.3 | 65 | 100 |
| 17 h | 5.9–7.7 | 30 | 0 |
| 17 h | 7.7–8.7* | 30 | 0 |
| 10 min | 5.3 | 30 | 10 |
| 60 min | 5.3 | 30 | 20 |
| 10 min | 4.9 | 30 | 75 |
| 60 min | 4.9 | 30 | 100 |
| 10 min | 9.3** | 30 | 20 |
| 60 min | 9.3** | 30 | 35 |

* 0.05 *M* Tris buffer.
** 0.05 *M* glycine buffer.

Metal binding agents ($CN^-$, histidine, 8-hydroxyquinoline, Versene, $1 \cdot 10^{-3}$ *M*) had no effect on enzyme activity. No evidence could be obtained that potato phosphorylase requires a metal for its activity.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. C. F. CORI for his guidance and encouragement during the course of this investigation and Miss C. LOWRY for excellent technical assistance in determining the molecular weight.

This work was supported in part by grants from the Corn Industries Research Foundation and the Nutrition Foundation.

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