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POTATO PHOSPHORYLASE ISOENZYMES

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; phosphorylase; isoenzymes; gel electrophoresis; glycogen.

Abstract—Potato phosphorylase isoenzymes were separated by gel electrophoresis, and DEAE-Sephadex chromatography. Electrofocusing experiments showed a heterogeneity in isolelectric point. Molecular weights and Stokes-radii were estimated using Sephadex G200. The adsorption on glycogen of two low molecular weight forms, probably dimers, was investigated by means of gel electrophoresis. The dissociation constants were 5×10^{-5} and 2×10^{-3}% glycogen.

INTRODUCTION

THE OCCURRENCE of phosphorylase isoenzymes has been demonstrated in several plants and correlated with the physiological age of leaves,[1] plant differentiation[2] or developmental stages of seeds.[3–5] In a previous paper[6] phosphorylase isoenzyme patterns of various plants were described in relation to starch synthesis and breakdown, and the association of one isoenzyme with amyloplasts from potato tubers was demonstrated.

Although the physicochemical and catalytic properties of purified potato phosphorylase have been studied intensively[7–10] little is known about the structure and properties of the different isoenzymes. The present paper deals with some physicochemical properties of potato tuber phosphorylase isoenzymes.

Separation of Phosphorylase Isoenzymes by DEAE-Sephadex

The phosphorylase activity was eluted in three peaks from a DEAE column at pH 7·2, by a linear NaCl gradient (Fig. 1). Disc electrophoresis of aliquots of column fractions revealed that the first peak, eluted at 0·15 M NaCl, consisted of the isoenzymes 1 and 3 (Fig. 1). The main peak, eluted by 0·25 M NaCl, contained isoenzyme 6. Isoenzymes 7, 8 and 9 were eluted together with some isoenzyme 6 just before the main peak. The third peak, eluted by 0·32 M NaCl, consisted of several isoenzymes dominated by 6 and 4.

Determination of the Isoelectric Point of Phosphorylase Isoenzymes

Preliminary electrofocusing experiments with ampholite at pH 3–10 showed that all phosphorylase isoenzymes of crude extract concentrate around pH 5. Other experiments

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using ampholite at pH 4–6 showed a phosphorylase peak at pH 4·8 (Fig. 2) and after electrophoresis of the ampholite fractions, this could be separated into two bands, 6 and 6a, the latter migrating a little faster. The isoenzymes 1 and 3 have an isoelectric point of 5 (fraction 15). Isoenzyme 4 seems to be unstable under these conditions and could only be

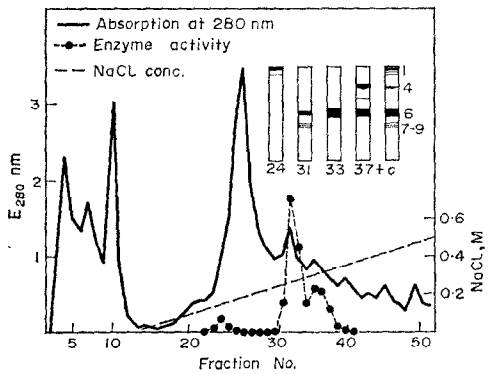

FIG. 1. CHROMATOGRAPHY OF CRUDE POTATO EXTRACT ON DEAE SEPHADEX A25, ELUTED WITH A LINEAR NACl GRADIENT.

Absorption at 280 nm. Disc electrophoresis patterns of aliquots from fraction 24, 29, 32, 36 and crude extract (c) are inserted.

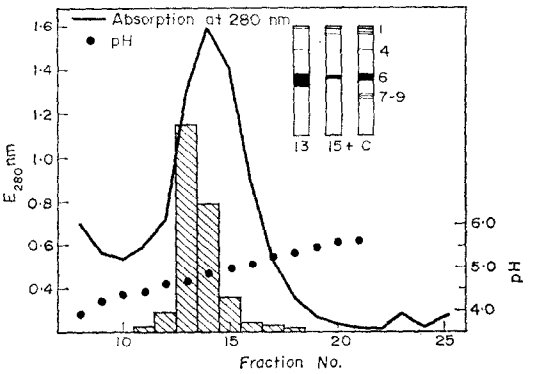

FIG. 2. ELUTION PATTERN OF AN ELECTROFOCUSING COLUMN.

Phosphorylase activity is indicated by the histogram. Disc electrophoresis patterns of aliquots from fraction 13, 15 and crude extract (c) are inserted.

detected when twice the quantity of extract was applied to the column. In this case it showed an isoelectric point of 4·8. Bands 7, 8 and 9 could not be found in these experiments. The nature of band 6a is not understood. When ampholite fractions were chromatographed on Sephadex G200 and the eluted fractions analyzed with disc-electrophoresis, band 6a proved to be eluted together with or directly after band 6.

Gel Filtration

The molecular weights of the isoenzymes were determined with crude potato extract on a Sephadex G200 column.[11] An elution pattern of crude potato extract is shown in Fig. 3. The phosphorylase activity was found in a single peak. Electrophoresis of aliquots from column fractions revealed that the main activity corresponds with isoenzyme 6. The position of the peaks of activity of each of the isoenzymes 1, 2, 3 and 4 was determined by electrophoresis and the aliquots in which these peaks occurred were used to calculate the elution volumes. From these data approximate MWs were calculated: 180 000 for isoenzymes 1 and 3, 320 000 for isoenzyme 6, 520 000 for isoenzyme 4, and over 600 000 for isoenzyme 2. Activity of isoenzymes 7, 8 and 9 could hardly be detected after gel filtration. Their molecular weight could not be estimated.

Stokes Radius

The Stokes radii were calculated by plotting the gel filtration data according to the correlation of Laurent and Killander[12] used in the form given by Siegel and Monty.[13] With

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bovine serum albumin, aldolase, catalase and apoferritin as references, the following Stokes radii for the phosphorylase isoenzymes were calculated: 48·5 Å for isoenzyme 1 and 3; 63·5 Å for isoenzyme 6; 85 Å for isoenzyme 4.

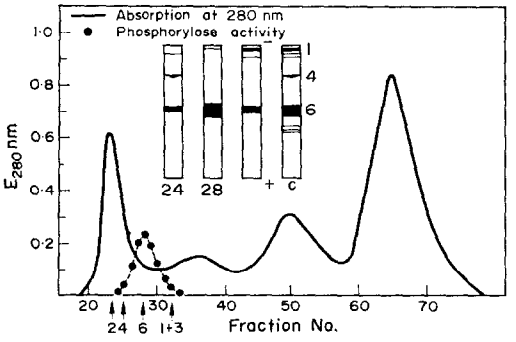

FIG. 3. GEL FILTRATION OF CRUDE EXTRACT WITH SEPHADEX G200.

The elution volume of the different isoenzymes are indicated by numbered arrows. Disc electrophoresis patterns of aliquots from fraction 24, 28, 32 and crude extract (c) are inserted.

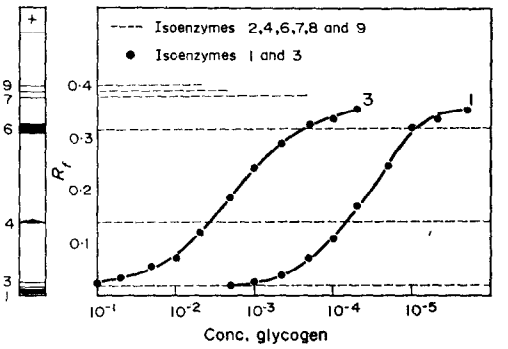

FIG. 4. DISC ELECTROPHORESIS OF ISOENZYMES IN GELS WITH DIFFERENT AMOUNTS OF GLYCOGEN.

The pattern with 0·1% glycogen is drawn at the left.

Adsorption on Glycogen

Adsorption on glycogen was studied by determining the R_fs of the isoenzymes after disc electrophoresis in gels containing different concentrations of glycogen. Using crude extract the R_fs of isoenzymes 1 and 3 alter with changing glycogen concentrations, while the R_fs of the other isoenzymes are not affected. Further experiments were performed with partly purified isoenzymes 1 and 3, by using fractions eluted at 0·15 M NaCl from a DEAE-Sephadex column. The results are given in Fig. 4.

To be able to calculate the dissociation-constant for both isoenzymes, the results are plotted in a linear relationship, comparable with a Lineweaver–Burk plot. The adsorption processes therefore, can be considered as an equilibrium between enzyme (E), glycogen (S) and enzyme-substrate complex (ES), $E + S \rightleftharpoons ES$ (equation 1), with the dissociation constant $K = S(e - c)c^{-1}$ (equation 2), where e is the total amount of enzyme, c the concentration of the enzyme substrate complex and s the concentration of glycogen in the gels. To describe the effect of glycogen on electrophoretic mobility the quotient R_f/R_fm is used, where R_fm is the maximal R_f obtained without glycogen in the gel. At low enzyme concentrations this quotient is equal to the ratio of free enzyme concentration to total enzyme concentration $R_f/R_fm = e^{-1}(e - c)$ (equation 3). Rearranging and combining equations 2 and 3 yields $(R_fm - R_f)^{-1} = K(R_fm \times S)^{-1} + R_fm^{-1}$ (equation 4). This equation represents a linear relationship between $(R_fm - R_f)^{-1}$ and S^{-1}. The intercept on the ordinate corresponds with R_fm^{-1}, the intercept on the abscissa with $-K^{-1}$.

The sigmoidal curve for isoenzyme 3 (Fig. 4) is a symmetrical one, which corresponds with a straight line in the reciprocal plot. The curve for isoenzyme 1 in Fig. 4 is not symmetrical; the curve in the low glycogen concentration range is sharper than that shown by high glycogen concentrations. Consequently in the reciprocal plot the linearity does not hold at low glycogen concentrations, where enzyme concentrations are relatively high. This

indicates that equation 3 is not valid in this concentration range, because competition between the enzyme molecules for the scarce binding places on the glycogen results in an increased R_f. From the reciprocal plots K-values were obtained, corresponding with a glycogen concentration giving an $R_f = \frac{1}{2} R_f m$: for isoenzyme 1 $K = 5 \times 10^{-5}$ g/100 ml and for isoenzyme 3 $K = 2 \times 10^{-3}$ g/100 ml.

DISCUSSION

The experiments described in this paper indicate that the phosphorylase isoenzymes found by disc electrophoresis[6] are almost certainly not artefacts produced as a result of the electrophoresis technique itself. The isoenzymes appear to be stable after chromatography with DEAE-Sephadex and most can also be found after chromatography with Sephadex G200. Although the possibility remains that some of the isoenzymes are artefacts, it is likely that most are present *in vivo*.

Our results demonstrate that there is good agreement of some properties of potato and muscle phosphorylases. The Stokes radii for the isoenzymes 1 and 3 (48·5 Å) and for isoenzyme 6 (63·5 Å) are in agreement with the values 49·3 Å for muscle phosphorylase *b* (dimer) and 63·0 Å for phosphorylase *a* (tetramer).[14] Other molecular properties of potato and muscle phosphorylases such as amino acid composition, circular dichroism and bound pyridoxal phosphate are also similar.[15] It is reasonable therefore to suppose that there is little difference between the molecular weights of potato and muscle phosphorylases. The gel filtration data represented in Fig. 3 give only rough information about MWs in so far as other physicochemical parameters of the standard proteins are comparable with the investigated proteins.[15] The isoenzymes 1 and 3 might represent dimers, with a MW of *ca.* 180 000, isoenzyme 6 could be a tetramer, MW *ca.* 360 000 and isoenzyme 4 an octomer, isoenzyme 2 might be a highly aggregated form.

The adsorption of potato phosphorylase on glycogen has not been previously described. Fischer and Hilpert[16] state that potato phosphorylase has practically no affinity for muscle or oyster glycogen; it seems likely that the isoenzymes that adsorb on glycogen were lost during the purification procedure which they employed. De Fekete reports a phosphorylase isoenzyme from *Vicia* that adsorbs on potato starch grains.[17] It is possible that some structural similarity exists between the surface of a starch grain, with many glucose end groups, and the highly branched glycogen. The affinity for glycogen of isoenzyme 1 and 3 (dimers) is therefore comparable with the physiologically interesting phenomenon of adsorption on amyloplasts, supposing that the binding in both cases is at the end groups.

These adsorption phenomena cannot be observed with isoenzyme 6 (tetramer) and isoenzyme 4 (octomer). This may be caused by steric properties, which make the active site inaccessible for glycogen.[18] The fact that the two isoenzymes 1 and 3 have the same molecular properties, excepting their affinity for glycogen, suggest that this difference is probably caused by allostery. The interconversion of tetramer into dimer, and the interconversion of a phosphorylase with great affinity for glycogen into an isoenzyme with less, or no affinity, may represent a fine mechanism for the regulation of carbohydrate metabolism in potatoes.

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EXPERIMENTAL

Preparation of tissue extracts. Potato tubers (*Solanum tuberosum* L. var. Bintje) stored in the dark at 8°, were cut into small pieces and homogenized in a blender for 1 min, with an equal volume of the buffer used for the different columns to which 4 mM Na_2SO_3, 3 mM $Na_2S_2O_5$ and 2 mM EDTA had been added. The homogenate was pressed through perlon gauze and centrifuged at 10 000 *g* for 15 min. The supernatant was used as crude extract.

Disc electrophoresis. This was performed as previously desrcibed,[6] with 7% acrylamide and usually 0·1% glycogen (Merck). The phosphorylase activity was demonstrated by incubation of the gels at 20° for 5 h in 0·1 M Na–citrate, pH 5·1, containing 25 mM glucose-1-phosphate. The gels were stained with 0·01 M iodine in 0·014 M KI.

Assay of phosphorylase activity. This was determined by adding 0·5 ml of column effluent to 0·5 ml medium consisting of 0·1 M Na–Citrate, pH 6·1, 12·5 mM glucose-1-P and 0·1% glycogen. After incubation for 30 min at room temp., 0·2 ml I-KI was added and A was measured at 600 nm.

DEAE–Sephadex column chromatography. 40 ml crude extract was applied to a DEAE-Sephadex A25 column (2·3 × 35 cm), equilibrated with 2 mM Tris-HCl buffer, pH 7·2, containing 0·02 M NaCl. After washing with the same buffer the column was eluted with a linear NaCl gradient, 0·02–0·5 M. Fractions of about 10 ml were collected at a flow rate of 35 ml/hr.

Electrofocusing. This was performed in a LKB 8101 column. The final Ampholine concentration was 4%, the crude potato extract, *ca.* 3 ml, was mixed with the less dense solution. The lower electrode solution consisted of 0·4 ml ethylenediamine, 14 ml H_2O and 12·0 g sucrose and the upper solution was 0·5 N H_2SO_4 in 14 ml H_2O. After applying 300 V for 64 hr at 5°, fractions of 2·5 ml were collected; the pH, A at 280 nm and phosphorylase activity were measured.

Sephadex G200 chromatography. Samples (3 ml) were applied to a 2·5 × 40 cm column[11] and eluted with 0·05 M Tris-HCl, pH 7·5, with 0·1 M KCl. Fractions (3·6 ml) were collected at a flow rate of 15 ml/hr. The column was calibrated with blue dextran to estimate the void volume, and with the standard proteins bovine serum albumin, apoferritin, R-phycoerythrin, catalase and aldolase. The phycoerythrin used for calibration was prepared from *Acrochaetium virgatulum.* All column operations were carried out at 4°.