Purification and Properties of Potato Phosphorylase Isozymes

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Two multiple forms of α-glucan phosphorylase which migrate about half way in polyacrylamide-gel electrophoresis (named "slow" and "fast" isozyme), were isolated by combined chromatography and preparative electrophoresis after freezing the tissue from freshly harvested and from sprouting potato tubers respectively. Depending on the primer used for the synthesis reaction their pH optimum varied between 5.2 and 6.0 and the optimum temperature was 30 and 35 °C. The isoelectric point for the slow isozyme was at pH 5.0 ± 0.1 and for the fast isozyme, pH 5.5 ± 0.1, the molecular weights were 209 000 ± 10 000 and 165 000 ± 5000 and for their subunits 104 000 ± 4000 and 40 000 ± 2000 respectively. Both isozymes were inhibited by Hg^{2+}, Ag^{+} and *p*-chloromercurobenzoate (p-CMB). Fe^{2+} ions inhibited them partially. Mg^{2+}, Mn^{2+} and sulfhydryl compounds activated both.

K_m values for the slow and fast isozymes with glucose-1-phosphate in presence of soluble starch was 6.7 and 8.0 mM, of amylose 14.3 and 20.0 mM and of glycogen 22.2 and 40.0 mM respectively. The affinity of the primer for the slow and the fast isozymes were as follows: soluble starch 0.5 and 1.0 mM, amylose 2.6 and 3.8 mM, glycogen 6.2 and 7.7 mM respectively. K_m values of phosphorolysis with soluble starch was 0.8 and 0.5 mM, with amylose was 3.1 and 1.1 mM, and with glycogen was 6.5 and 1.3 mM respectively. As substrate and primer the soluble starch was superior and the glycogen was inferior. Amylose was in between.

Kinetic parameters suggested the existence of α-glucan phosphorylase isozymes with different specificities: the slow one being more active in the direction of starch synthesis and the fast isozyme degrading faster the polyglucans. These observations suggest that the polyglucan synthesis and degradation in potato tubers may be regulated by the change in the proportion of slow and fast isozymes.

Phosphorylases (α-1,4-glucan: orthophosphate glucosyltransferase, E.C. 2.4.1.1.) have been extensively studied in mammalian tissues, particularly in those of rabbit [1]. In contrast to animal phosphorylases, relatively less attention has been given to the plant phosphorylases. Multiple forms (the term "isozyme" is used synonymously) of α-glucan phosphorylase have been demonstrated in blue-green algae [2, 3], maize [4], corn [5], *Phaseolus vulgaris* [6], banana [7], *Solanum tuberosum* [6–11], spinach and *Vicia faba* [6, 12]. The potato is one of the richest sources of plant phosphorylase and it is the best studied plant phosphorylase [13–17]. Stegemann *et al.* [8, 9, 18–20] have shown by their primer technique and activity staining that the isozyme pattern vary according to the time of harvest, conditions of storage and part of the tuber samples taken from. Among the isozymes a slow and a fast one which migrate about half way in the polyacrylamide (PAA)-gel are of special interest. The slow moving isozyme which is abundent in young or freshly harvested tubers, but vanishes in sprouting tubers with concomitant appearence of the fast moving isozyme if the sample is taken from frozen tubers [8, 9]. We have designated them as "slow" and "fast" phosphorylase isozyme respectively. Main object of the present investigation is to purify the afore mentioned two isozymes in order to get more information about the function of them in the regulation of carbohydrate metabolism from white potato tubers. Preliminary reports of this work have already been presented [21–24].

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Abbreviations: p-CMB, *p*-chloromercuribenzoate; DEAE-cellulose, diethylaminoethyl-celluose; EDTA, ethylenediamine tetraacetate; G-1-P, glucose-1-phosphate; G-6-P dehydrogenase, glucose-6-phosphate dehydrogenase; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PAA-gel, polyacrylamide-gel; Pi, inorganic phosphate; SDS-gel, sodium dodecyl sulphate-gel; Tris-HCl, tris (hydroxymethyl) aminomethane-hydrochloride.

Materials and Methods

1. *Plant materials:* Harvested white potato tubers (*Solanum* cv. Adelheid) and sprouting tubers (cv. Rosa) were used as the source of slow and fast isozymes respectively. Potatoes were frozen at appropriate time and thawed for 12 hours before use. Unless otherwise stated all further operations were done at 4 °C.

2. *Extraction of the crude sap:* The peeled and sliced potatoes (400 g) were pressed in a hydraulic press (SKG-Tinkturenpresse von Seifert KG, D-7550 Rastatt, POB 606) to obtain the sap. The browning due to oxidation was avoided by adding 2% v/v sulphite solution containing 3.75% sodium bisulphite and 7.44% EDTA [25]. After allowing to stand for an hour the solution was clarified by centrifugation at $12000 \times g$ for 20 min. The supernatant was checked for the presence of the respective single isozyme by analytical PAA-gel electrophoresis using the primer technique of Siepmann and Stegemann [8] in slabs of 3 mm thickness (for details see paragraph "electrophoresis"). The supernatant was used for further purification.

3. *Heat treatment:* The temperature of the supernatant was brought to $50\,^{\circ}C \pm 1$ and maintained for 10 min with constant stirring. It was rapidly cooled in an ice bath and the resulting precipitate was removed by centrifugation for 20 min at $12000 \times g$.

4. *Ammonium sulphate fractionation:* Supernatant obtained after heat treatment was brought to 40% saturation by the addition of solid ammonium sulphate with constant stirring. Suspension was allowed to stand for one hour at $4\,^{\circ}C$ and clarified by centrifugation for 20 min at $12000 \times g$. The precipitate was discarded, the supernatant was collected and brought to 50% saturation. The mixture was allowed to stand for one hour, centrifuged and the sediment was used for further purification.

5. *Dialysis:* The precipitate was dissolved in the minimum amount of proper buffer (citrate buffer, pH 6.2, 0.05 M for the slow enzyme and Tris-HCl buffer, pH 7.5, 0.05 M for the fast enzyme) and dialysed for 6 hours. These buffers were found to stabilize the respective isozymes [26]. The retentate was centrifuged as before and the supernatant was used for column chromatography studies.

6. *DEAE-cellulose chromatography:* 5 ml of the supernatant was applied to DEAE-cellulose (Merck 3201) column (2.5×40 cm) equilibrated with Tris-HCl buffer, pH 7.5, 0.05 M. Elution was effected with a linear gradient of 1 M sodium chloride in Tris-HCl buffer, pH 7.5, 0.05 M, by using 250 ml of each solution at a rate of 60 ml per h. Fractions of 10 ml were collected and analysed for phosphorylase activity. It was observed that the fast enzyme elutes between 140 and 170 ml and the slow enzyme elutes between 220 and 250 ml. The elutes containing the enzyme were pooled and concentrated in collodium bags to about 7 ml as described by Francksen and Garadi [27].

7. *Preparative electrophoresis:* 7 ml of the concentrated elute was further purified by PAA-gel electrophoresis according to the method of Stegemann [28] in a PANTA-PHOR apparatus (Labor-Müller, D-3510 Hann. Münden) using PAA-gel blocks of $100 \times 120 \times 16$ mm and applying 120 V and 100 mA at $10\,^{\circ}C$ for 16 hours. After electrophoresis the gel was washed twice with chilled citrate buffer, pH 5.1, 0.1 M, for about 15 min each to remove the alkaline electrophoretic buffer. A longitudinal gel strip of about 1 cm breadth was cut out and incubated in 50 ml citrate buffer, pH 5.8, 0.1 M containing 0.2 M glucose-1-phosphate as substrate (G-1-P, Boehringer, Mannheim) and 0.1% glycogen as primer (Merck 4202, Darmstadt) at $30\,^{\circ}C$ with gentle shaking for half an hour. The enzyme was located by staining the synthesized polyglucans with dilute iodine [8]. The slow and the fast isozymes from the unstained part of the gel were eluted with an equal amount of [w/v] citrate buffer, pH 6.5, 0.1 M and Tris-HCl buffer, pH 7.5, 0.05 M respectively after pressing the gel through a sieve (0.5×0.5 mm mesh) in a plastic syringe. The elution time was 10 min. The supernatant obtained after centrifugation at $12000 \times g$ for 15 min was used as "free enzyme" for further studies. The purity and stability of the isozymes during the course of all purification steps was checked by analytical PAA-gel electrophoresis in 3 mm thick slabs [29].

8. *Analytical electrophoresis:* The electrophoresis was carried out in a PANTA-PHOR-apparatus [29] employing 5% Cyanogum® in 0.04 M borate buffer, pH 8.2, at 300 V and 100 mA for 3 hours at $0\,^{\circ}C$. A portion of the gel was stained for proteins with coomassie blue [25]. Phosphorylase activity in the remaining portion of the gel was detected by incubating the gel with glycogen (0.1%) and G-1-P (0.2%) in citrate buffer, pH 5.8, 0.1 M at $30\,^{\circ}C$ and staining with dilute iodine solution as mentioned before. Whenever the gel was needed only for activity staining electrophoresis was carried out according to Siepmann and Stegemann [8]. For protein electrophoresis 5% Cyanogum® was used in 0.4 M Tris-borate buffer, pH 8.9 at 600 V and 60 mA for 90 min at $0\,^{\circ}C$ [25].

5 to 10 μl of fresh potato sap, variety ROSA, prepared in May just before sprouting containing both the isozymes were electrophoretically separated in a PAA-gel blocks of 3 mm containing glycogen according to the primer technique [8]. Certain parameters like pH profile and temperature optimum (incubation time 120 min) were determined by incubating the enzyme loaded gel strips "isozymes in gel" in the corresponding mixtures. The gel strips were al-

ways preincubated in the buffer solution without substrate for 15 min before studies.

9. *Molecular weight determination:* Molecular weights of the isozymes were estimated by chromatography on a Sephadex G-200 column according to Andrews [30]. SDS-gel electrophoresis of Shapiro *et al.* [31] modified by Koenig *et al.* [32] was employed for the molecular weight determination of their subunits.

10. *Isoelectric point* was determined in PAA-gel using Ampholine®, pH 3 – 10 (LKB) according to Macko and Stegemann [33].

11. *Enzyme assay:* The activity of the free enzyme in the synthetic direction was determined by incubating the reaction mixture (total volume 1 ml) containing 25 μg of the enzyme protein in citrate buffer, pH 6.5, 0.1 M, 10 μM of G-1-P and soluble starch according to Zulkowsky (Merck 1257) containing 3 μmol of anhydrous glucose for one hour at 30 °C. Liberated inorganic phosphate (Pi) was determined according to Fiske and Subbarow [34] modified by Bartlet [35]. Readings were corrected for blank values by mixing 25 μg enzyme protein in citrate buffer with 0.5 ml of 5% trichloroacetic acid and adding the substrate solution thereafter. The activity is expressed in μmol of Pi liberated in one hour per mg of enzyme protein under the above assay conditions if not otherwise started. Protein content of the enzyme samples were determined according to Lowry *et al.* [36] modified by Stegemann [37]. The temperature- and pH-optima of the free isozymes were also measured by the same method by changing the respective parameter.

12. *Effect of cations and other compounds:* The "synthetic" activity of the isozymes in presence of different primers was studied by preincubation of the respective isozyme with the specific cation or the compound for 15 min followed by the activity determination as mentioned before. Values were corrected against suitable blanks.

13. *Kinetic procedures:* Synthetic reaction of phosphorylase isozymes were studied by measuring the amount of Pi liberated under standard conditions by using varying amounts of G-1-P (1 to 120 μmol per ml) in presence of a primer namely soluble starch, amylose (Merck 4561) or glycogen containing a constant number of anhydrous glucose molecules (3.0 μmol per ml). Similarly the affinity of the phosphorylase isozymes to the primer was estimated by keeping the amount of G-1-P constant (10 μmol per ml) in presence of varying amounts of primer containing 0.3 to 9.0 μmol of anhydrous glucose per ml. Kinetic experiments in the direction of polysaccharide degradation were carried out according to Franken *et al.* [16] in presence of varying amounts of soluble starch or amylose or glycogen, at 30 °C and pH 7.0 by a coupled assay in which the reaction was coupled to a large excess of phosphoglucomutase and G-6-P dehydrogenase so that the rate-limiting step in the formation of NADPH was the phosphorylase reaction. In soluble starch and amylose the amount of anhydrous glucose per ml varied between 0.1 and 10 μmol and in glycogen it varied between 0.1 and 80 μmol per ml.

For determination of kinetic constants at least ten different substrate concentrations were used in each case. 25 μg of phosphorylase isozyme per ml was taken for all kinetic measurements. The initial rates were expressed in mmol of product formed per min per mg of enzyme per l. The synthetic and phosphorolytic activity of the isozymes were measured with the same batch of enzyme simultaneously as the enzyme activity slightly varies from batch to batch and also the activity of the enzyme decreases rapidly in a very highly purified state.

K_m values and V_{max} were determined by plotting $1/V$ against $1/S$ according to Lineweaver-Burk [38]. Similarly the primer affinity of the phosphorylase isozymes were determined by plotting reciprocal of the respective primer concentration against reciprocal of liberated Pi [39]. All values for polyglucans were expressed in concentration of anhydrous glucose.

Conversion of phosphorylase isozymes from slow to fast form and vice versa was achieved by dialysing the partially purified isozyme by heat treatment and ammonium sulphate fractionation of the crude sap obtained from 100 g of the tubers containing the respective single isozyme. The precipitate obtained between 40 and 50% ammonium sulphate concentration was dissolved in 4 ml of 0.05 M Tris-HCl buffer, pH 7.5 and dialyzed against the buffer at 4 °C for an hour to remove the ammonium sulphate. The retentat was redialyzed at 4 °C for 80 hours in buffer solutions of 0.1 M and of different pH values varying from 5.0 to 8.0 with a difference of 0.5 pH units each time. Citrate buffer was taken to convert the fast isozyme into the slow form and the phosphate buffer was used to convert the slow isozyme into the fast form. Each time 0.1 ml of the retentant was pippeted at an interval of 5 hours and subjected to analytical PAA-gel electrophoresis for activity staining as mentioned before.

Results

Purification data of the preparations derived from phosphorylase isozymes from potatoes are sum-

Table I. Purification of potato phosphorylase isozymes.

Step No.	Fraction	Total protein [mg]		Specific activity [Units][a]		Total activity [Units][a]		Purification (Fold)	
		Slow	Fast	Slow	Fast	Slow	Fast	Slow	Fast
1	crude extract	2850	2265	1.1	0.8	3135	1812	1.0	1.0
2	heat treated	1812	1420	1.6	1.1	2899	1562	1.5	1.4
3	ammonium sulphate fractionation (40–50%)	62	44	42.0	28.5	2604	1254	38.2	35.6
4	DEAE-cellulose column eluate	25	16	89.2	58.3	2230	933	81.1	72.9
5	preparative gel electrophoresis	11	6	160.0	96.5	1760	579	145.5	120.6

[a] Specific activity is expressed in μmol of product formed per h per mg of enzyme at specified assay conditions. See methods.

marized in Table I. Starting with 400 g of the tubers (variety Adelheid), 11 mg of purified slow isozyme which could hydrolyze 160 μmol of G-1-P per hour per mg of protein under standard conditions was obtained. 6 mg of the fast isozyme (variety Rosa) was isolated in a similar fashion. This isozyme could hydrolyse 96.5 μmol of G-1-P per mg of protein per hour. Phosphorolytic activity of the slow and fast isozymes measured by formation of reduced $NADP^+$ by a linked assay system based on the rate of G-1-P formation was 90 and 260 μmol per hour per mg of enzyme per l, respectively. However, slight variations in the activity were observed in different preparations.

The optimum pH and temperature of both the "isozymes in gel" were found to be between 5.0 and 5.5 and 30 °C respectively after incubation of 120 min. pH optimum for the slow enzyme *in vitro* at 30 °C in presence of soluble starch was 6.0 whereas in presence of amylose and glycogen it dropped to 5.5. The fast enzyme had a pH optimum of 5.2 in presence of all primers (Fig. 1). In presence of soluble starch the temperature optimum of the free slow isozyme was 35 °C whereas in presence of amylose and glycogen it was 30 °C. The fast enzyme had an temperature optimum of 35 °C in presence of all primers (Fig. 2). The isoelectric points of slow and fast enzymes were at pH 5.0 and 5.5 ± 0.1 respectively.

By gel filtration on Sephadex G-200 the molecular weights were determined to 209000 ± 10000, for the slow enzyme and to 165000 ± 5000 for the fast enzyme. The molecular weights of their subunits were found to be 104000 ± 4000 and 40000 ± 2000 respectively by SDS-gel electrophoresis (Fig. 3). Inhibition and activation data for both isozymes are presented in Table II.

In presence of 0.1 M citrate buffer at pH 6.5 the partially purified fast isozyme was converted into the slow form in 20 hours and remained stable even at 80 hours. The conversion of the fast isozyme into slow form was incomplete in solutions lower than pH 6.5 and at a pH higher than 6.5 the enzyme was inactivated within 10 to 15 hours. At pH 5.5 in

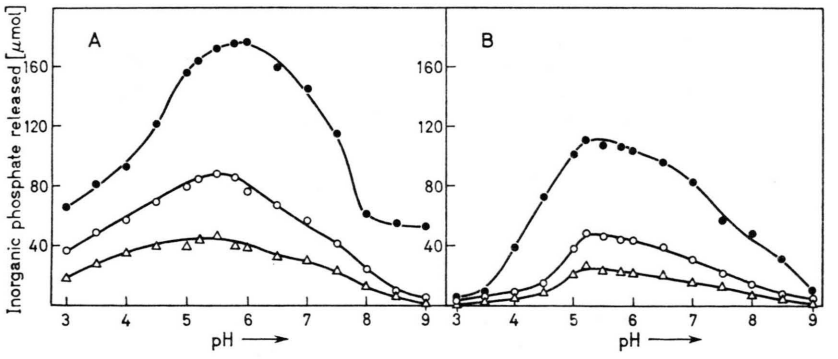

Fig. 1. Effect of pH on the activity of slow (A) and fast (B) potato phosphorylase isozymes in presence of different primers. (For details see methods.) Soluble starch (●-●), amylose (○-○), glycogen (△-△).

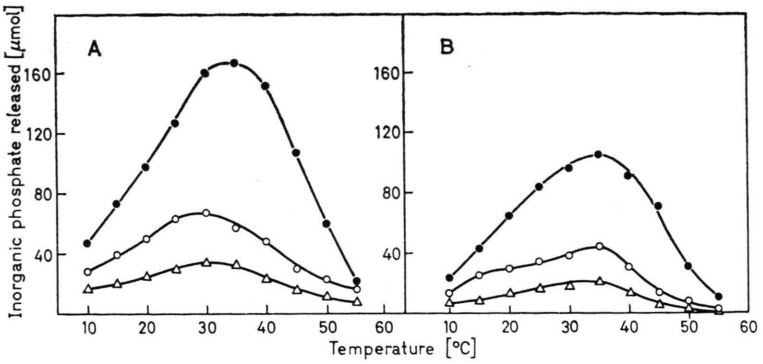

Fig. 2. Effect of temperature on the activity of slow (A) and fast (B) potato phosphorylase isozymes in presence of different primers. (For details see methods.) Soluble starch (●-●), amylose (○-○), glycogen (△-△).

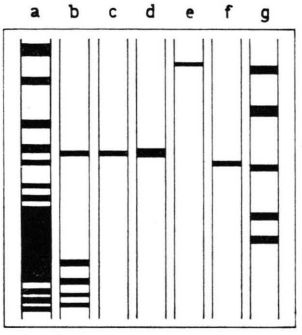

Fig. 3. Purification (a—d) and molecular weight (e—g) of potato phosphorylase isozymes. PAA-gel electrophoresis of the proteins from tubers at pH 8.2: a. crude extract; b. DEAE cellulose column eluate; c. purified slow enzyme by preparative electrophoresis; d. same as c, activity stain. SDS-gel electrophoresis: e. purified slow enzyme; f. purified fast enzyme; g. marker proteins from top to bottom: rabbit muscle phosphorylase b (100 000), bovine serum albumin (67000), alcohol dehydrogenase from yeast (37000), coat protein of tobacco-yellow-mosaic virus (20400) and of tobacco-mosaic virus (17800). Stained with coomassie blue except d.

Table II. Effect of various compounds [+] on the synthetic activity [++] of potato phosphorylase isozymes in presence of different primers.

Name of the compound	Soluble starch	Amylose	Glycogen	Soluble starch	Amylose	Glycogen
Mg^{2+}	110	113	116	110	111	116
Ca^{2+}	101	101	103	100	100	102
Mn^{2+}	114	117	118	116	118	116
Fe^{2+}	57	59	51	60	50	61
Co^{2+}	102	100	100	100	102	100
Ni^{2+}	97	102	96	99	100	102
Cu^{2+}	100	101	101	100	99	100
Zn^{2+}	101	100	101	100	100	102
Ag^{+}	0	0	0	0	0	0
Hg^{2+}	0	0	0	0	0	0
p-CMB	10	10	9	9	10	9
iodoacetamide	101	101	99	100	100	100
mercaptoethanol	110	108	110	110	110	110
cysteine	106	107	106	106	107	109
glutathione	117	109	112	109	110	109
gibberellic acid	100	101	100	101	102	100
indolyl-3-acetic acid	102	101	102	102	98	100
agarose	105	104	104	105	104	104
control	100	100	100	100	100	100

Column 2, 3, and 4: slow enzyme.
Column 5, 6, and 7: fast enzyme.
[+] Concentration of the additives: One µmol per ml.
[++] Conditions as in standard assay. The activity in absence of additions was taken as 100.

0.1 M phosphate buffer the slow isozyme was completely transformed into the fast form in 55 hours. However, the fast isozyme lost its activity completely after 65 hours in the phosphate buffer. At pH 5.0 the conversion of the slow to fast isozyme was incomplete and lost its activity after 70 hours. In

phosphate buffer at pH 6.0 and above the slow isozyme lost its activity rapidly and it was almost inactive within 10 hours.

K_m values and V_{max} obtained in presence of various concentrations of different primers and substrates are shown in Tables III – V. The slow isozyme synthesized polyglucans at a faster rate than the fast isozyme whereas the faster isozyme degraded all the substrates faster than the slow isozyme. Soluble starch is the best substrate and primer whereas glycogen is a poor substrate and primer for both isozymes.

Table III. Kinetic constants of synthesis for potato phosphorylase isozymes.

Primer [a]	Slow		Fast	
	K_m [b] [mM]	V_{max} [c] [mmol]	K_m [b] [mM]	V_{max} [c] [mmol]
soluble starch	6.7	5.6	8.0	4.2
amylose	14.3	3.3	20.0	2.7
glycogen	22.2	2.6	40.0	0.7

[a] Concentration of the primer: 3.0 μmol of anhydrous glucose per ml.
[b] K_m-values: expressed in mg molecule (mM) per l.
[c] V_{max}-values: expressed in mmol of product formed per min per mg of enzyme per l.

Table IV. Affinity of the potato phosphorylase isozymes for various primers.

Primer [a]	Slow		Fast	
	Affinity [b] [mM]	V_{max} [c] [mmol]	Affinity [b] [mM]	V_{max} [c] [mmol]
soluble starch	0.5	3.2	1.0	2.8
amylose	2.6	2.1	3.8	1.9
glycogen	6.2	1.6	7.7	1.3

[a] Concentration of the primer: 0.3 to 9.2 μmol of anhydrous glucose per ml.
[b] Affinity: values expressed in mg molecule (mM) per l.
[c] V_{max}: values expressed in mmol of inorganic phosphate formed per mg of enzyme per l.

Table V. Kinetic constants of degradation for potato phosphorylase isozymes.

Substrate [a]	Slow		Fast	
	K_m [b] [mM]	V_{max} [c] [mmol]	K_m [b] [mM]	V_{max} [c] [mmol]
soluble starch (0.1–10.0)	0.8	1.0	0.5	2.9
amylose (0.1–10.0)	3.1	0.9	1.1	1.4
glycogen (1.0–80.0)	6.5	0.2	1.3	0.3

[a] Concentration of substrate: values expressed in mmol of anhydrous glucose per l.
[b] K_m-values: expressed in mg molecule (mM) per l.
[c] V_{max}-values: expressed in mmol of product formed per min per mg of enzyme per l.

Discussion

In crude extracts of potato, phosphorylase has been shown to exist in multiple forms [6, 8–10, 19, 20, 40]. However, to determine their physiological role in the tuber it is necessary to isolate the enzymes and to study the individual properties. Various attempts have been made to purify the phosphorylase isozymes from different plant sources [2–6, 12] but little was done to study their properties [6, 11]. This may be due to the very high lability of the purified isozymes.

We could obtain electrophoretically homogenous slow and fast potato phosphorylase isozymes after four steps (Table I, Fig. 3). The several minor protein bands which were not separated by column chromatography have been completely removed by preparative PAA-gel electrophoresis according to [28], Fig. 3, compare column b and c.

Therefore a combination of column chromatography and preparative PAA-gel electrophoresis is the method of choice for the isolation of enzymes in a highly purified state.

The molecular weight of the slow enzyme, which is 209000 ± 10000, agrees well with other reports [13, 17, 40, 41]. We assume, that the slow isozyme isolated by us is identical with the phosphorylase isolated by others. Therefore, we have compared our values from slow isozyme with the literature values wherever possible.

Kamogawa *et al.* [15] have obtained crystalline potato phosphorylase by column chromatographic procedure with DEAE-Cellulose and DEAE-Sephadex after absorbing the enzyme to starch. The synthetic activity of their enzyme was about ten times higher than that of ours. However, they have used soluble starch concentration twenty fold higher. Furthermore the assay was done at pH 6.0.

The slight variations in respect to optimal pH values and temperature among the "isozymes in gel" and *in vitro* is expected due to different microenvironment in the gel [42]. The optimal temperature and pH of the isozymes *in vitro* differ with various primers due to structural differences of the accepter used. The relation between the phosphorylase activity and structure of polyglucan used has been extensively discussed elsewhere [13]. Lavintman *et al.* [43] have also observed a shift in pH-optima of corn phosphorylase to alkaline side when phytoglycogen is used as an acceptor instead of amylopectin.

The molecular weight determination according to Andrews [30] method showed the molecular weight to be 209000 ± 10000 and 165000 ± 5000 for the slow and fast isozymes respectively. By porosity (6 – 24%) gradient PAA gel [44] the values obtained for the slow isozyme is 200000 and for the fast isozyme is 162000 which agrees well with this report. Molecular weights obtained by sedimentation studies [17, 41] and by gel filtration [40] are between 200000 and 220000. Recently, Itawa and Fukui [40] have reported a molecular weight of 108000 by SDS-gel electrophoresis for freshly prepared potato phosphorylase. Further these authors have observed on prolonged storage at 4 °C four bands with a molecular weight of 40000 to 60000. Our values for subunits of slow and fast isozymes would correspond to this findings.

The values obtained after electrofocusing with Ampholine pH 3 – 10 were pH 5 and 5.5 for the slow and fast isozymes respectively are comparable to the values obtained by Gerbrandy and Doorgeest [11] with Ampholine at pH 3 – 10 in a LKB 8101 column.

Hg^{2+} and Ag^{+} ions as well as p-CMB were inhibitory to both phosphorylase isozymes is in accordance with others [45, 46]. In contrast to our findings Cu^{2+} [45, 46] and Zn^{2+} [45] did inhibit the potato enzyme. We have seen that Fe^{2+} ions inhibit both the isozymes. The inhibition varies between 40 to 50% depending on the acceptor used for the polyglucan synthesis. About 50% of inhibition of maize phosphorylase isozyme I in presence of Fe^{2+} ions was observed by Tsai and Nelson [47]. They also have reported that Mn^{2+} and Mg^{2+} ions are activators. Kinetic results suggested that the fast and slow isozymes have different specificities. For the same substrate the phosphorolytic activity of the fast isozyme was always higher than of the slow isozyme. The synthetic activity of the slow isozyme with G-1-P in presence of the same primer was higher than the fast isozyme. The fast isozyme degraded soluble starch 2.9 times faster than the slow isozyme. The synthetic activity of the same isozyme was only 75% of the slow isozyme. When the synthetic activity of the slow isozyme with G-1-P in presence of soluble starch was arbitrarily considered as 100, in presence of amylose and glycogen it was only 59 and 46 respectively. This relation for the fast isozyme was 100:64:17. As a substrate and primer the soluble starch was superior to amylose and glycogen was inferior to amylose. For plant phosphorylases [43, 48, 49] including potato [45] the glycogen has been found to be a poor substrate and primer.

The K_m value of the slow isozyme for phosphorylation of the soluble starch at 30 °C and pH 6.5 was 0.8 mM. Under the same conditions the affinity of the slow isozyme for soluble starch was 0.5 mM. Lee [41] has reported 3.7 mM as K_m value for G-1-P in presence of potato amylopectin for the potato enzyme. The affinity of the same polyglucan was found to be 0.8 mM at 30 °C and pH 6.3 for the potato phosphorylase by the same author. For phosphorolysis in the K_m value for the soluble starch at 30 °C is 0.8 mM in contrast to [50] where 2.3 mM was found (Table V).

Our present studies show that the partially purified fast isozyme changes into the slow form in presence of citrate ions and the slow form changes into the fast form in presence of phosphate ions. We have formerly observed in the potato tubers fast isozyme changes into the slow form after incubation in citrate buffer [18]. Lerner *et al.* [51] also have recently

reported that the fast moving grape catechol oxidases are transformed into the slow form in presence of citrate ions. Gerbrandy *et al.* [44] have observed that the potato phosphorylase slow isozyme *in vitro* is changed into the fast form by the action of proteases. A similar observation was done by Iwata and Fukui [40] after the action of proteases on potato phosphorylase isozyme with a molecular weight of 215000 by gel filtration. Under the light of these previous reports we presume that phosphate ions influence the release of proteases which convert the slow isozyme with a higher molecular weight into a low molecular form in the sprouting tubers when the degradation of the polyglucans takes place. In presence of citrate ions the release of such proteases is most probably inhibited and the enzyme remains a "slow" isozyme. However it is not clear how the faster isozyme is converted into the slow form in presence of citrate ions. This type of conversion from slow to fast form and vice versa is performed only in using a partially purified enzyme and not with a highly purified enzyme preparation. The role of yet unknown factors is obvious.

Phosphorylase isozymes have been studied from different plants [4–12, 43, 47–49, 52] and algae [2, 3, 53–55]. It has been reported that changes in amounts of phosphorylase and other enzymes in connection with seasonal cell development have been shown to occur [9, 56, 57]. Siepmann and Stegemann [8] have reported that in a young and freshly harvested tuber the slow isozyme is replaced by the faster isozyme during sprouting. Gerbrandy and Verleur [6] have reported that the electrophoretically slow moving phosphorylase isozymes are active in young and mature tubers whereas fast moving isozymes are active in older tubers. They suggested that the slow isozymes are mainly concerned with starch synthesis, whereas the fast moving isozymes are mainly concerned with degradation of polyglucans. As it was shown [44] that the slow isozyme *in vitro* is converted into the fast form by the action of proteases, it is suggested that very specific proteases are released during sprouting when degradation of the polyglucans is essential for the growing sprouts. The kinetic data show that the isozyme with higher molecular weight which is abundant in young and freshly harvested potato tubers is more active in the direction of synthesis. The fast moving isozyme from older and sprouting tubers (frozen) can degrade the polyglucans at a faster rate than the slow isozyme. The changes in the relative concentrations of slow and fast phosphorylase isozymes could be part of an *in vivo* control of the polyglucan synthesis and degradation in potato tubers.

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