Phytochemistry, 1977, Vol. 16, pp. 1875-1879. Pergamon Press. Printed in England.

SOME MOLECULAR PROPERTIES OF PLANT STARCH PHOSPHORYLASES AND THE LEVELS OF ACTIVITY OF THE MULTIPLE FORMS

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(*Received* 20 *April* 1977)

Key Word Index—*Pisum*; *Phaseolus*; Leguminosae; *Solanum*; Solanaceae; starch phosphorylases; seed germination; molecular weights.

Abstract—A number of plant tissues have two phosphorylase fractions (I and II) that can be separated by DEAE-cellulose chromatography. When only one is detectable, it corresponds to enzyme II. Peas differed from other legumes in showing an increase in enzyme I during seed germination. Examination of the I type enzymes, by sodium dodecyl sulphate polyacrylamide gel electrophoresis, sector cell ultracentrifugation and gel filtration, indicated that these were dimers composed of similar sub-units of MW near 90000. When phosphorylase II enzymes were examined, the sub-unit MW was found to be higher, near 110000 and, whereas ultracentrifugation techniques indicated a dimer of similar sub-units for the native enzyme, gel filtration gave higher MW values. Phosphorylase II from Victory Freezer peas differed from the other samples, in being able to form mixtures of dimer and tetramer.

INTRODUCTION

The majority of MW determinations on plant starch phosphorylases (EC 2.4.1.1) have used potato preparations. Lee [1] found an $S_{20°,w}$ of 7.9S and from the diffusion coefficient calculated a MW of 207000. Other values have been $S_{20°,w}$ 6.5S at a concentration of 10 mg/ml [2], $S_{20°,w}$ 8.4S [3] and $S_{20°,w}$ 8.0S [4]. The $S_{20°,w}$ value of this enzyme from sweet potato was found to be 8.4S at a concentration of 8.0 mg/ml [5]. Sedimentation equilibrium measurements have given a MW of 187000 for potato [4] and 200000 for the major fraction from hybrid maize seed [6]. Estimates of both sedimentation coefficients and MW have been made using swinging bucket rotors with a sucrose gradient [7]. Using this technique with potato phosphorylase, a sedimentation coefficient of 8.4S was estimated [8] and with sweet corn phosphorylase [9], a value of 8S was obtained and the MW estimated as 160000. The mentation coefficients of samples measured by these methods have been near to 8S and the MW values in the region of 200000. The dimeric forms of glycogen phosphorylase have MW values near 200000 and sedimentation coefficients of about 8S, whereas the values for the tetramer are about 13S [10, 11].

MW determinations of starch phosphorylase, using gel filtration, have been more extensive and the column materials have included cross-linked dextran, agarose and polyacrylamide. A much wider range of MW values have been found, including some significantly higher than 200000. Potato phosphorylase values have varied. One study, using Sephadex G-200, Biogel P-200 and P-300 and Sepharose 6B gave values near to 200000 with all these materials [4]. However, higher values have been reported. Gerbrandy [12] found a single, symmetrical peak of phosphorylase activity in a potato juice extract and the estimated MW at the apex of the peak was 320000. A fraction that was apparently a degraded form had a lower MW [8]. Both of these, when examined by sucrose density gradient ultracentrifugation, gave the same sedimentation coefficient [8.4S) and a MW of 200000 was determined for the undegraded fraction by gradient gel electrophoresis. A fraction, present in smaller quantity in potato extracts and separated by chromatography on DEAE cellulose, had a MW of 180000 as determined by gel filtration on G-200 [13]. Sweet potato phosphorylase has given a value of 210000 on G-200 chromatography [5]. A MW of 315000 was estimated by chromatography on G-200 of sweet corn phosphorylase [9] and this was the same preparation for which a sedimentation coefficient of 8 and a MW of 160000 was estimated by density gradient ultracentrifugation. There is also a report of a value of 320000 by gel filtration on G-200 for the enzyme from hybrid maize, but the value estimated by ultra-centrifugation was 200000 [14]. The MW of the two fractions from peas, separated by DEAE-cellulose chromatography, were estimated by gel filtration on G-200 and Sepharose 6B-CL respectively to be 135000 and 490000.

Multiple forms of starch phosphorylase have been detected by different separation techniques in a number of plant tissues and, in some of these, two or sometimes three fractions have been obtained by chromatography on DEAE-cellulose, e.g. maize [14, 16], potatoes [13], bananas [17], barley [18], mistletoe leaves [19] and peas [15]. In this paper, extracts from a number of plant organs have been chromatographed on DEAE-cellulose and the comparative amounts of the fractions estimated as well as the MW and sub-unit MW.

RESULTS AND DISCUSSION

A number of mainly starch-containing plant tissues, were extracted and the solutions fractionated on DEAE-

cellulose with a gradient to 60 mM citrate, followed by a second gradient to 0.5 M NaCl in 60 mM citrate [15]. Tube fractions were assayed for release of inorganic phosphate and the production of iodine-staining glucan in the presence of Glc-1-P and a glucan primer. Tube fractions in a single elution peak were combined and the total fractions labelled according to their order of elution. Two phosphorylases were found in all sources examined (Table 1), except for mung and green beans. Both of these lacked a fraction that eluted at an ionic concentration equivalent to that of phosphorylase I from peas and other plants. The only fraction isolated was labelled II, as it eluted at a similar ionic strength to this fraction from peas. In rice and bananas, fraction I was not bound to the column. In all other tissues, the ionic concentrations required for elution were similar to those for peas. Some values in Table 1 were previously quoted in the text of ref. [15] p. 888 but were given for 10 g wet weight (at maturity for seeds). The combined activities of phosphorylases I and II were very high in germinating Victory Freezer pea cotyledons and generally high in other developing, mature and germinating seeds, except for rice. Levels in potato tubers and bananas were also high but they were low in leaf and root tissues and also in developing rice seeds at a time when starch synthesis was occurring. Apart from germinating pea cotyledons, whenever phosphorylase levels were high this was mainly due to the amounts of enzyme II.

In germinating seeds of two varieties of peas [15], there was an increase in combined phosphorylase activity in cotyledons on germination and this increase was due to phosphorylase I. This contrasted with a lesser increase on seed development, when the increase was associated with phosphorylase II. Some other legume seeds were assayed for changes in phosphorylase activity on germination and the results are shown in Table 2. The values for mung beans and lupin seeds were determined on unfractionated extracts by estimating phosphate released from Glc-1-P in the presence of NaF [20] but those for broad and green beans were made after DEAE-cellulose chromatography. When the extracts from lupin seeds, which contain no starch at maturity, were chromatographed on DEAE-cellulose, fractions were obtained that could synthesize iodine-staining glucan. In contrast to peas, in the cotyledons of these four seeds there was no significant increase in combined phosphorylase activity on germination. In the extracts of the two starch-storing seeds, on which DEAE-cellulose chromatography had been performed, the amounts of phosphorylase II remained at the same level during germination as they were at maturity but there was no increase in phosphorylase I in broad beans. In green beans, no fraction corresponding to phosphorylase I could be detected at any time of sampling. One factor in the differences may be that some of the seeds are hypogeal whereas others are epigeal and the cotyledons function for a time as photosynthesizing organs.

From the results of gel chromatography of phosphorylase I from Victory Freezer pea cotyledons on Sephadex G-200 and phosphorylase II on Sepharose 6B-CL, combined with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), it was suggested that the I form may be a monomer and the II form a tetramer [15]. The MW values of the enzymes from peas and other plants have been further studied by a number of techniques and the results are shown in Table 3. In all cases where SDS-PAGE was carried out, the enzymes were further purified, after DEAE-cellulose chromatography, by affinity chromatography on a Sepharose 4B-soluble starch column. The starch used for elution was removed by again chromatographing on DEAE-cellulose, when starch was unbound. This final step could not be used for enzyme I from bananas as it did not bind and here the eluate from the affinity column was treated with salivary α-amylase and the phosphorylase separated by chromatography on Sepharose 6B-CL. When samples were

Table 1. Amounts of activity of phosphorylase fractions from DEAE-cellulose chromatography of some plant tissues

Plant	Organ	Activity in fraction (nkat/g fr. wt)		
		I	II	Total
Peas	developing seeds	0.5	18	19
(Victory Freezer)	mature seeds	3.9	33	37
	germinating cotyledons	96	21	117
	expanding leaves	2.1	2	4.1
	fully expanded leaves	0.7	1.9	2.6
	roots	0.7	0.6	1.3
(Alaska)	developing seeds	0.5	13	14
	mature seeds	3.2	11	13
	germinating cotyledons	27	5.5	33
Broad beans	developing seeds	0.31	6	6.3
	mature seeds	3.7	27	31
Green beans	mature seeds	u.d.*	20.5	21
Mung beans	mature seeds	u.d.	66	66
Potatoes	mature tubers	0.6	25	26
Tobacco	expanding leaves	0.8	2.6	3.4
Rice	developing seeds	0.1	0.3	0.4
Bananas	ripe fruits	1	14	15

*Undetectable, < 0.05.

Table 2. Changes in levels of phosphorylases in germinating legume seeds (nkat/seed)

Time from imbibition (days)	Mung beans	Lupin seeds	Broad beans		Green beans
			Fraction I	Fraction II	Fraction II
0		0.52	4.2	67	
1	13				19.2
2		0.65			
3					17
4	10.3				
5					23.5
6	12.9				
7		0.74			21.8
9			4	47	
11			3.4	60	
14		0.79	1.5	48	
Wt of one ungerminated seed (g)	0.04	0.14	1.03		0.49

further purified by these procedures, all other measurements were made on this material.

The sub-unit MW values, which were estimated by SDS-PAGE [21], separated into two groups. Phosphorylase I enzymes gave smaller sub-unit sizes, in the region of 90000, than phosphorylase II enzymes, which were in the region 110–120000. Within an enzyme type the differences were significant, as separate bands could be detected with mixtures that differed in sub-unit MW. Also, when the native enzymes were examined by PAGE, sometimes the protein bands could be separated. For example, mixtures of the phosphorylase II enzymes from Alaska pea and banana separated as did those from potato and Victory Freezer pea but mixtures of Alaska pea and broad bean on the one hand and banana and Victory Freezer pea on the other were not separated.

Ultra-centrifugation measurements, using a sucrose density gradient in a swing-out head [7] and also in a sector cell, as well as gel chromatography, were performed in 0.1 mM Tris, pH 7.0, 0.1 M NaCl except for those values marked in Table 3 and these were carried out in 0.05 mM Tris. Ultracentrifugation in a density gradient gave sedimentation coefficients that were reasonably close (8.4–8.9) for all the samples of enzymes I and II that were studied. These were estimated by sedimenting in the same tube two enzymes with known coefficients, one of which was higher and the other lower than the unknown [7]. The phosphorylases produced single peaks that were essentially symmetrical. The MW values, estimated by the same procedure, were all in the region 170000 to 190000 for both enzymic forms. These sedimentation constants are similar to those derived for glycogen phosphorylases [10, 11] and other starch phosphorylases [1–5, 8, 9] when MW values near 200000 have been estimated. In conjunction with the sub-unit MW data where only one band was detected, this indicates dimeric structures for both phosphorylases I and II, probably of identical sub-units in each case.

When sedimentation coefficients of the activities from some of the sources (Table 3) were measured in a sector cell, values for the sedimentation coefficients also near to 8S were found, except for phosphorylase II from Victory Freezer peas. In this sample two peaks were observed, one of which had a coefficient near 8S but the other was higher, near 16S (Fig. 1). The latter value is more consistent with a tetramer or higher structure, with a MW in the region 4×10^5 or greater. In different

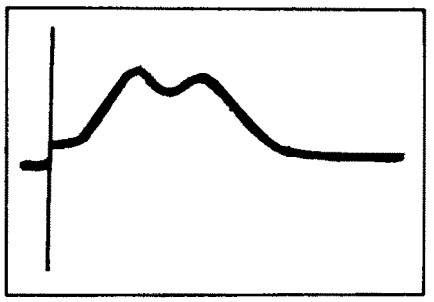

Fig. 1. Ultra-centrifugation pattern in a sector cell of Victory Freezer pea phosphorylase (bar angle 70°, 40 000 rpm, 25 min, protein conc 19.5 mg/ml).

preparations of the enzyme on ultracentrifugation, the properties of the two fractions varied considerably, although the cause of the variation was not apparent. A part of the smaller MW fraction was separated from the mixture using a sector cell with a fixed partition. The specific activity was similar to both the original solution and the heavier fraction from the fixed partition cell, indicating that neither of the MW species was a non-phosphorolytic contaminant. When density gradient ultra-centrifugation in a swing-out head was performed with Victory Freezer pea phosphorylase II in a solution of 0.1 M NaCl, the sedimentation coefficient of the active peak increased and the pattern of activity down the tube became asymmetric (Fig. 2).

When Victory Freezer pea phosphorylase II was chromatographed on Sephadex G-200 in 0.1 M NaCl, the elution pattern indicated heterogeneity (Fig. 3(i)). The total volume of the column was 120 ml. If the eluate was divided as indicated in Fig. 3(i) and the fractions re-chromatographed, A eluted (Fig. 3(ii)) at 36.5 ml, ahead of *E. coli* β-galactosidase (520 000) at 39.5 ml and B eluted at 45.5 ml (Fig. 3(iii)), indicating a MW of 360000, similar to estimates for the other phosphorylase II enzymes by this technique. When the smaller MW species, separated in a sector cell with a partition, was chromatographed on the same column, it also eluted at 45.5 ml. These results suggest that under certain conditions, possibly increased salt concentration, phosphorylase II from Victory Freezer peas forms a dimer–tetramer mixture. Rabbit muscle glycogen phosphorylase *a* forms a tetramer [22] in strong (2.8 M) NaCl solutions and this

Table 3. MW determinations of phosphorylase fractions

Phosphorylase I						Phosphorylase II					
		Sedimentation coefficient		MW (daltons)				Sedimentation coefficient		MW (daltons)	
Plant and organ	Sub-unit MW by SDS-PAGE	by density gradient (Svedberg)	by sector cell S°20, w (c in mg/ml)	by density gradient	by gel filtration on 6B-CL	Plant and organ	Sub-unit MW by SDS-PAGE	by density gradient (Svedberg)	by sector cell S°20, w (c in mg/ml)	by density gradient	by gel filtration on 6B-CL
Alaska pea cotyledons	93000	8.8†	n d.*	184000	200000	Alaska pea cotyledons	108000	8.9	n.d.	187000	440000
Banana fruit	93000	8 6	n.d.	179000	170000	Banana fruit	115000	8.5	n.d.	176000	360000
Broad bean	n d.	n d	n d	n d.	170000	Broad bean	110000	n.d.	n.d.	n.d.	350000
Tobacco leaves	n.d.	n.d.	n.d.	n.d.	170000	Mung bean	105000	8 6	7 8 (c = 5.6)	179000	430000
Victory Freezer pea cotyledons	90000	8 5†	7.9 (c = 0)	176000	170000	Potato	105000	8.4	7 8 (c = 8)	171000	330000
						Tobacco	n.d.	n.d.	n d	n.d.	390000
						Victory Freezer pea cotyledons	110000	8.5†	8.16 (c = 8)	175000	490000

*Not determined. †Measured in solutions of low ionic strength. All other measurements were made at high ionic strength.

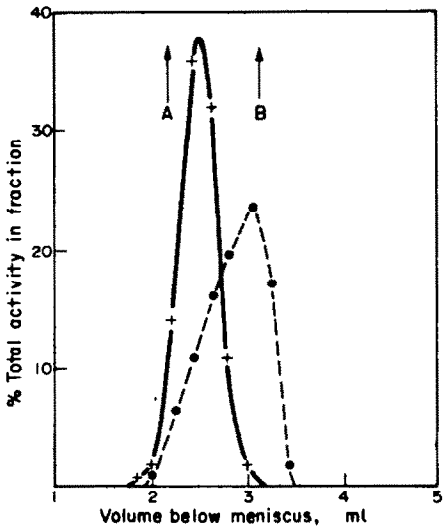

Fig. 2. Sucrose density gradient ultra-centrifugation of phosphorylase II from Victory Freezer peas (– + –, at low salt concentration, – ● –, at high salt concentration. (A) peak of lactic dehydrogenase activity; (B) peak of catalase activity.

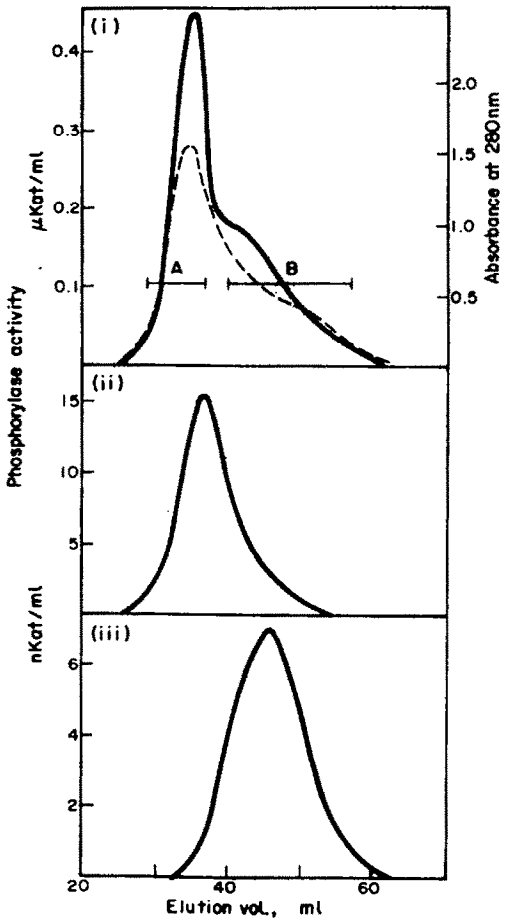

Fig. 3. Elution profiles from gel chromatography of phosphorylase II from Victory Freezer pea seeds. (i) Enzyme preparation, (ii) Fraction A; (iii) Fraction B. (——, phosphorylase activity; – – – protein content).

dissociates into a dimer as the solution is diluted. The degree of dissociation is dependent on protein concentration and pH values above 7.

The elution volumes of the phosphorylase I enzymes on 6B-CL gave estimated MW values in reasonable agreement with the values from ultra-centrifugation and consistent with dimeric structures. They all eluted 8 to 11 ml behind ferritin in a column with a total volume of 123 ml. Previously [15] a lower MW (135000) for phosphorylase I from Victory Freezer peas was estimated by chromatography on Sephadex G-200. On chromatography on a third material, Bio-Gel P-200, this enzyme eluted ahead of lactic dehydrogenase, indicating a MW greater than 150000. On Sephadex G-200 it eluted behind lactic dehydrogenase. The elution volume of another enzyme involved in starch metabolism (*Bacillus subtilis* α-amylase) has been found to be unexpectedly high [23].

The MW values of the phosphorylase II enzymes, estimated from gel chromatography on 6B-CL were much higher than the estimates from ultra-centrifugation and much higher than values calculated for a dimer from SDS-PAGE. They eluted from 3 ml ahead to 2 ml behind ferritin, clearly differentiating them from the phosphorylase I enzymes. The very high value for Victory Freezer pea phosphorylase (490000) would reflect the presence of tetramer. However, on 6B-CL the elution pattern did not show definite assymmetry as did the pattern on G-200.

Contrasting size estimates have previously been obtained for some starch phosphorylases. A preparation of potato gave a MW by gel filtration on G-200 of 320000 but a sedimentation coefficient by density gradient ultra-centrifugation of 8.4S with a calculated MW of 200000 [8, 12, 13]. An enzyme from hybrid maize also gave different values by these procedures [6] as also did a preparation from sweet corn [9] (160000 by density gradient ultra-centrifugation and 315000 by gel filtration on Sephadex G-200). These enzymes were eluted from DEAE-cellulose by relatively high concentrations of salt and probably correspond to the type II enzymes described in this paper. On the other hand there are reports of MW values for potato phosphorylase, determined by gel filtration on Sephadex G-200, of 200000 [4] and 220000 [3]. One explanation of the differences is that these authors studied the I form. Both preparations involved a heat treatment. On gel filtration of the potato phosphorylase of Franken *et al.* [4] on Bio-Gel P-200, the activity eluted behind catalase and a MW of 190000 was calculated. However, in the present study, when a sample of potato phosphorylase II was prepared using DEAE-cellulose chromatography and it was chromatographed on Bio-Gel P-200, the activity eluted ahead of catalase.

In conclusion, a number of plant sources were found to produce two phosphorylases by DEAE-cellulose chromatography under the conditions of elution. Form I, which is eluted first, is in low amount, except in germinating peas; sometimes it is not detectable. Type I enzymes from a number of plants have a lower sub-unit size than type II and ultra-centrifugation and gel filtration both indicate that the native form is a dimer. Ultra-centrifugation data suggest that type II is also a dimer, except for the enzyme from Victory Freezer peas, which can form a mixed dimer–tetramer. However, gel filtration data for type II enzymes indicate a higher MW.

EXPERIMENTAL

Plant material. Plants used were, broad beans (*Vicia faba* cv Coles Dwarf Prolific), green beans (*Phaseolous vulgarus* cv Redland Pioneer), mung beans (*Phaseolus aureus*), *Lupinus luteus* cv Weiko III, *Pisum sativum* cv Alaska and Victory Freezer, *Nicotiana tabacum* (flue cured) and *Oryza sativa* cv Calrose. Potato tubers and bananas were obtained retail. Growth conditions have been described [15].

Extraction and purification of phosphorylases. Extraction, DEAE-cellulose chromatography and assay were performed as in ref. [15]. Some enzymes were further purified by affinity chromatography on a Sepharose 4B-soluble starch column [15], (Procedure A) and DEAE-cellulose chromatography again (B), gel filtration (C) and gel filtration in the presence and absence of amylopectin (D). The enzymes that were further purified, the procedures used and the final specific activities in nkat/mg protein were: Victory Freezer pea phosphorylase I (A, B, C, D—205), banana I (A, B, C—200), Alaska pea I (A, B—220), Alaska pea II (A, B—110), banana II (A, B—280), broad beans II (A, B,—190), mung beans II (A, B—190), potato II (A, B—270), Victory Freezer peas II (A, B, C—190).

Estimation of sub-unit MW by SDS-PAGE and MW by gel chromatography. These procedures have been described [15].

Determination of sedimentation coefficients in a sector cell. These were determined in a 12 mm, 4° cell in an An-D rotor with a Schlieren optical system. The temp. was 20°. Samples were dialysed against 0.1 M Tris buffer pH 7 containing 0.1 M NaCl. For proteins with coefficients less than 10S the rotor speed was 50000 rpm, but when a component with a higher coefficient was present, 40000 rpm. Mechanical separation was performed in an aluminium 12 mm fixed partition cell using 0.55 ml of protein soln.

Estimation of sedimentation coefficients and MW by ultracentrifugation in a sucrose density gradient. In the procedure followed [7] gradients were prepared from 5 × 1 ml aliquots of sucrose soln (20, 16, 12, 8 and 4%). Samples (0.1 ml) were applied in band forming caps. The standards were lactic dehydrogenase (7.18S, 144000) and catalase (11.15S, 250000). Estimates were calculated against each standard and averaged. The head was a Beckman-Spinco SW65K, temp. 15°, speed 64000 rpm and duration 4 hr. Fractions (0.2 ml) were collected for assay.

Acknowledgements—This work was supported by the University of Sydney Research Grant. One of us (R.H.R.) wishes to acknowledge the award of a Henry Bertie and Florence Mabel Gritton Post-graduate Research Scholarship of the University of Sydney. Immature rice seed was collected by the N.S.W. Department of Agriculture Regional Research Centre at Yanco, N.S.W.

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