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THE RAPID PURIFICATION OF A PHOSPHOTRANSFERASE FROM WHEAT SHOOTS

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Summary

A phosphotransferase from wheat shoots which specifically phosphorylates the 5′-position of nucleosides has been purified by a simple procedure involving chromatography on Matrex Gel Blue A (Cibacron Blue F3GA).

Phosphotransferases from carrots [1] or wheat shoots [2] have been used for a number of years for the specific 5′-phosphorylation of nucleosides. However, the enzyme preparations described are usually impure and contain large amounts of phosphatase, thus yields of nucleoside 5′-phosphate are often low especially with poor substrates. We now report a simple method for the purification of the phosphotransferase from wheat shoots by affinity chromatography on Matrex Gel Blue A (Amicon Ltd, Woking, Surrey). This is an agarose-based support containing immobilised Cibacron Blue F3GA and the enzyme is eluted from the support either by a pulse of substrate (uridine) or by changing the pH or ionic strength of the eluant buffer.

Wheat shoots were grown in the dark on moist vermiculite and were harvested after 7—10 days. The shoots were ground in a pestle and mortar with 3 vols. of 0.2 M sodium acetate buffer, pH 6, and the pulp was either filtered through muslin or centrifuged at low speed. The filtrate/supernatant was adjusted to pH 5.5 with acetic acid and diluted to 10 vols. with water. The enzyme solution was applied to a TEAE-cellulose column (1.2 × 24 cm) which was eluted with 0.5 M sodium acetate buffer, pH 5. Those fractions which contained phosphotransferase activity were pooled and solid $(NH_4)_2SO_4$ was added until a 90% saturated solution was obtained. The

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precipitate which formed at 4° C was removed by centrifugation (17 000 × *g*, 20 min) and the pellet suspended in double distilled water (10 ml). The suspension was dialysed for 18 h against several changes of water and finally 0.08 M sodium acetate, pH 4, and then applied to a Matrex Gel Blue A column (0.5 × 20 cm). In a typical experiment the crude phosphotransferase (50—200 U) in sodium acetate buffer pH 4 was applied to the Matrex Blue column which was washed with 2 bed vols. of 0.08 M sodium acetate buffer, pH 4. The enzyme was eluted by one of three procedures:

(a) 0.15 M sodium acetate with a pH gradient 4—5.5
(b) sodium acetate pH 5, with a concentration gradient 0.1—0.25 M
(c) uridine (25 mM) in 0.08 M sodium acetate pH 5.

The fractions containing phosphotransferase activity were pooled and further purified by gel filtration on Sephadex G-100 eluting with 0.1 M sodium acetate, pH 5 (Table I). The enzyme at this stage ran as a single band on SDS-

TABLE I

MATREX GEL BLUE A PURIFICATION OF WHEAT SHOOT PHOSPHOTRANSFERASE
Flow rate through Matrex Gel Blue A column (0.5 × 20 cm) 0.2 ml/min.
4[a] Elution with 0.15 M sodium acetate pH 4—5.5.
4[b] Elution with 25 mM uridine in 0.08 M sodium acetate (pH 4).
4[c] Elution with sodium acetate buffer (pH 5) 0.1—0.25 M.

Step	Volume (ml)	Total activity (U) Phosphatase	Phosphotransferase	Total protein (mg)	Activity ratio**	Specific Activity (U/mg)
1 Crude	80	212	9.5	170	0.045	0.055
2 TEAE-cellulose	105	105	10.5	50	0.10	0.21
3 90% $(NH_4)_2SO_4$	10	58.5	8.6	30.5	0.15	0.28
4[a]	30	3.0	6.0	0.3	2.0	20.0
4[b] Matrex Gel	20	4.0	9.0	0.29	2.25	30.0
4[c] Blue A	25	4.5	7.0	0.32	1.51	22.0
5 Sephadex G-100*	20	0.98	4.2	0.08	4.20	52.5

*Column (1.2 × 24 cm) elution with 0.08 M sodium acetate (pH 4).
**Phosphotransferase/phosphatase. The enzyme can be stored at −20° C in 0.08 M sodium acetate (pH 4), but slowly loses activity and is completely inactivated after 1—2 months. The enzyme is considerably more stable when attached to an insoluble support [4].

polyacrylamide gel electrophoresis at pH 5 (protein visualised with Coomassie blue). The molecular weight of the purified phosphotransferase at pH 5 was estimated to be between 48 000 (SDS-polyacrylamide gel electrophoresis) and 44 000 (gel filtration on Sephadex G-100) by comparison with standard protein markers. The wheat-shoot enzyme was also purified as described for the carrot enzyme [2] and enzyme samples after each purification step was dialysed against 0.08 M sodium acetate pH 4, before being applied to a Matrex Gel Blue A column. No improvement in purification was obtained over our methods described above.

The protein content of samples was determined by the method of Lowry et al. [3]. The phosphotransferase activity was determined as previously described [4] at 37° C and pH 5, using 25 mM adenosine as substrate and 100 mM 4-nitrophenyl phosphate as donor, the amount of 5′-AMP produced was either determined spectrophotometrically after TLC analysis of

the reaction and elution from the plate, or by scintillation counting using [2-^{3}H]adenosine (Radiochemical Centre, Amersham) as substrate. 1 unit of enzyme is defined as that amount of activity which will transfer 1 μmol phosphate from 4-nitrophenyl phosphate to adenosine under standard assay conditions. Different phosphate donors react differently with the enzyme (Table II). Phosphatase activity was estimated by the spectrophotometric determination of 4-nitrophenol released from 4-nitrophenyl phosphate under standard assay conditions with adenosine omitted from the reaction.

TABLE II

RELATIVE YIELDS OF 5'-AMP OBTAINED USING DIFFERENT PHOSPHATE DONORS UNDER STANDARD CONDITIONS

All experiments were carried out at 37°C and pH 5, with wheat shoot enzyme, donor (100 mM) and adenosine (25 mM) as acceptor. The yield of AMP was then determined after a fixed time interval (5 h) when steady conditions had been established.

4-Nitrophenyl phosphate	100
2,4-Dinitrophenyl phosphate	150
Phenyl phosphate	60
4-Chlorophenyl phosphate	50
Ethyl phosphate	0

The binding of the enzyme to the Matrex Gel Blue A depended on pH and ionic strength, maximum binding being obtained at pH 4 and low ionic strength (e.g. 0.08 M buffer). The phosphotransferase could be eluted from the affinity column either by increasing the ionic strength of the buffer or by raising the pH, as there was little binding of the enzyme to the support above pH 6. The best method for recovering the enzyme from the affinity column was to elute with a pulse of substrate, uridine being the most effective as it is the best nucleoside substrate for the enzyme. We find, in agreement with the observations of Brungraber and Chargaff [5], that the purified phosphotransferase always has some phosphatase activity. For the wheat shoot enzyme with 4-nitrophenyl phosphate as donor, the K_m for phosphotransferase (adenosine as acceptor) was 4.0 mM and K_m for phosphatase was 2.8 mM. Using phenyl phosphate as donor Brungraber and Chargaff [5] found the K_m for phosphotransferase (uridine as acceptor) was 3.5 mM and the K_m for phosphatase to be 1.9 mM.

The stability of the Matrex Gel Blue A column was excellent and it was used repeatedly after regeneration with 1 M sodium acetate, pH 5. Provided the column had been washed thoroughly before use, no dye leakage from the column took place. Attempts to purify the enzyme on Matrex Gel Red A, a similar affinity support based on Procion Red HE 3B, were unsuccessful presumably because this dye did not interact with the enzyme.

The purification outlined above is simpler than that previously described for phosphotransferases [2] and the overall recovery of phosphotransferase activity is 65% with 1000-fold increase in specific activity. For comparison, the enzyme after each stage of an adaption of the conventional purification [2] was further purified on a Matrex Gel Blue A column. The purity of enzyme after affinity chromatography was very similar in all cases. Thus, we believe that our procedure is a simple, effective method for purifying this

enzyme which can be used to prepare 5′-phosphates of a variety of nucleosides and oligonucleotides.

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