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# PURIFICATION OF CHLOROPLAST α-1,4-GLUCAN PHOSPHORYLASE FROM SPINACH LEAVES BY CHROMATOGRAPHY ON SEPHAROSE-BOUND STARCH

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## Summary

Chloroplast α-1,4-glucan phosphorylase (EC 2.4.1.1) has been purified to homogeneity from spinach leaves as revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purification procedure is composed of $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography, and chromatography on Sepharose-bound starch. In order to achieve binding of the chloroplast phosphorylase, a previously described Sepharose-glucan gel (Steup, M. Schachtele, C. and Latzko, E. (1980) Planta 148, 168—173) was modified by introducing hydrophobic groups in addition to the covalently bound starch. The chloroplast phosphorylase exhibited complete binding to this type of gel and could be eluted by a mixture of soluble glucan and NaCl. For the purified chloroplast phosphorylase, sodium dodecyl-sulfate polyacrylamide gel electrophoresis and pyridoxal phosphate determination resulted in a molecular weight estimation of about 110 000 per monomer. The apparent molecular weight of the native enzyme, as determined by polyacrylamide density gradient electrophoresis and gel filtration on Sephadex G-200, was 200000 and 220 000, respectively The data indicate that the chloroplast phosphorylase is a dimer with a molecular weight higher than that of the non-chloroplast phosphorylase.

## Introduction

Although there is considerable evidence that α-1,4-glucan phosphorylase mediates chloroplast starch breakdown [1—3], this enzyme has not been puri-

Abbreviation SDS, sodium dodecyl sulfate, Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

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fied and we know little of its properties. Isolation and characterization of the chloroplast phosphorylase are hampered by the fact that, in leaf extracts, phosphorylase activity often appears to be heterogeneous. In spinach leaves, two forms of phosphorylase are found, one inside and the other outside the chloroplast [4,5]. Recently, a rapid and efficient purification of the non-chloroplast phosphorylase of spinach leaves by chromatography on Sepharose-bound dextrin was described [6]. However, with respect to the purification of the chloroplast phosphorylase, this procedure was ineffective because this enzyme did not bind to the gel, but passed through together with the vast majority of the protein. Therefore, modified Sepharose-glucan gels have been developed in order to achieve binding of the chloroplast phosphorylase as a prerequisite to effective purification.

In the following, a purification procedure for the spinach leaf chloroplast phosphorylase is described. It is based on a modified Sepharose-glucan chromatography. Some molecular properties of the purified enzyme are reported

## Materials and Methods

*Plant material.* For enzyme purification, field-grown spinach (*Spinacia oleracea* L. var. Kasparik) or spinach purchased from the local market was used. Chloroplasts were isolated from expanding leaves of spinach (var Fruremona) grown in water culture [7] under a continuous light-dark regime (10 h light, 200 W · $m^{-2}$, 14 h dark, 15°C).

*Chloroplast isolation.* Intact chloroplasts were isolated and extracted as described elsewhere [6].

*Phosphorylase assay* Phosphorolytic activity was determined according to the method of Bergmeyer et al. [8].

*Polyacrylamide gel electrophoresis* Non-denaturating discontinuous electrophoresis was performed according to Jolley and Allen [9], the total monomer concentration of the separation gel was 8.5% (w/v) Electrophoresis in gradient gels was carried out according to the method of Margolis and Kenrick [10]. For discontinuous electrophoresis under denaturing conditions, the system of Laemmli [11] was used (slab gels of 1.5 mm thickness; 7 or 9% (w/v) total monomer concentration of the separation gel). The samples (containing 8 mM Tris, 0.8 mM EDTA, adjusted with HCl to pH 8.0, 2% (w/v) SDS, 2% (v/v) mercaptoethanol, 9% (v/v) glycerol) were heated for 2 min in a boiling-water bath. After electrophoresis, protein bands were stained as described elsewhere [6].

*Pyridoxal phosphate determination.* The pyridoxal phosphate content of the phosphorylase preparation was determined by the phenylhydrazone method [12].

*Protein determination.* Soluble protein was measured by the biuret method [13] or according to the method of Bradford [14]; ovalbumin served as standard.

*Statistics.* Standard error was determined according to the method of Patau [15].

*Preparation of Sepharose-bound glucan.* Activation of Sepharose and coupling of starch (15 g per 250 g activated Sepharose) were performed as

described elsewhere [6]. After coupling of the starch, the gel (250 g Sepharose) was deactivated by adding 1.25 mol butylamine (dissolved in 50 ml dimethylformamide and 50 ml of 0.1 M $NaHCO_3$, and adjusted to pH 9.5 with HCl) and incubated for a further 2.5 h at room temperature. Following deactivation, the gel was washed with 1 M NaCl (adjusted to pH 12.5 with NaOH), 1 M NaCl (adjusted to pH 2 5 with acetic acid) and 50% (v/v) dioxan and was transferred to the buffer used for chromatography.

The Sepharose-starch preparation was stained with iodine. After exhaustive hydrolysis with amyloglucosidase (from *Aspergillus niger*), 15—25 μmol glucose per g gel were liberated, as determined by the glucose 6-phosphate/hexokinase assay.

*Purification of chloroplast phosphorylase.* 1000 g of spinach leaves were homogenized in 500 ml imidazole hydrochloride buffer, pH 7.0 (0.1 M). The grinding medium as well as the buffers used throughout the purification contained in addition 0.2 mM phenylmethylsulfonyl fluoride [16] and 15 μM thymol [17]. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged for 10 min at 25 000 × *g*. From the supernatant (designated as crude extract; step I in Table I) protein was precipitated between 45 and 60% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in 20 mM citrate, brought to pH 6.5 with NaOH, and then dialyzed against the same buffer for several hours. After centrifugation of the retentate at 40 000 × *g* for 10 min, the supernatant (step II in Table I) was applied to a DEAE-Sephacel column (5.3 $cm^2$ × 15 cm) in order to separate the two phosphorylase forms present in the leaf extract. The column was first washed with 100 ml citrate/NaOH (20 mM, pH 6.5) and then with a linear gradient of 20—60 mM citrate, pH 6.5 (130/130 ml), which eluted the non-chloroplast phosphorylase activity. This eluate was discarded. Chloroplast phosphorylase was recovered from the column by elution with a linear gradient (130/130 μl) of 0—1 2 M NaCl, dissolved in 60 mM citrate (pH 6.5) Fractions containing more than 1 nkat phosphorylase activity per ml were pooled and concentrated by $(NH_4)_2SO_4$ precipitation (70% saturation) The precipitate was dissolved in 10 mM Tris, 1 mM EDTA, adjusted to pH 7.0 with acetic acid (buffer A, step III in Table I) and was then applied to a Sepharose-starch gel which had been activated with butylamine The column (2 $cm^2$ × 10 cm, equilibrated with buffer A) was washed and eluted as indicated in Fig 1 The flow rate during the chromatography on Sepharose-starch was about 25 cm/h. Fractions of the eluate containing phosphorolytic activity were pooled. Dextrin and NaCl were removed from the purified phosphorylase by gel filtration (Sephadex G-100, column 5 3 $cm^2$ × 55 cm, gel equilibrated with 50 mM Hepes, 2 mM EDTA, adjusted to pH 8.0 with NaOH) without any loss in phosphorylase activity (step IV in Table I). Throughout the purification, the temperature was kept at 2°C

## Results and Discussion

The chloroplast phosphorylase of spinach leaves is separable from the non-chloroplast form by ion-exchange chromatography [5]. Following this procedure, the chloroplast phosphorylase preparation was applied to a Sepharose-starch gel which had been deactivated with butylamine. Using this type of gel,

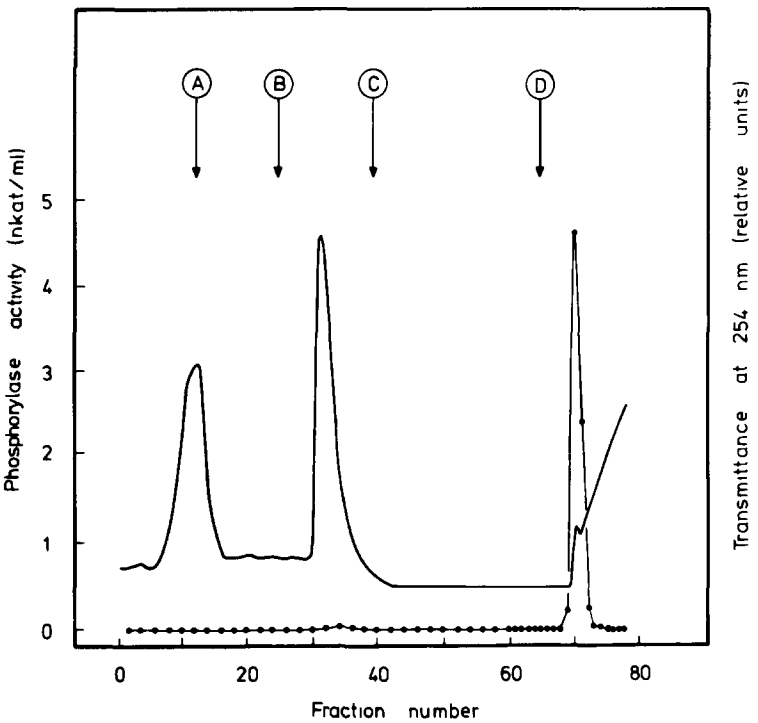


Fig 1 Chromatography of chloroplast phosphorylase from spinach leaves (NaCl eluate of DEAE-Sephacel chromatography) on a Sepharose-starch gel, deactivated with butylamine Elution was with A 100 ml buffer A (see Materials and Methods), B NaCl gradient (0—1 M NaCl dissolved in buffer A, 50/50 ml), C 200 ml of 1 M NaCl dissolved in buffer A, D dextrin gradient (0—12%, w/v, dissolved in 1 M NaCl containing buffer A, 30/30 ml) In the eluate, transmittance at 254 nm (———) and phosphorolytic activity (•———•) were determined

complete binding of the chloroplast phosphorylase was achieved, no activity was removed from the column by washing with buffer (Fig. 1) which eluted a significant amount of protein A second protein peak appeared in the eluate at the beginning of the NaCl washing, together with a tiny amount of phosphorylase activity. However, the phosphorylase activity was recovered from the column as a sharp peak with minimal protein coelution when a dextrin gradient in a buffer containing 1 M NaCl was used as eluant The linear decrease in transmittance was due to unspecific absorbance and was not caused by protein elution.

The fractions containing phosphorolytic activity were pooled. Aliquots of the combined fractions were subjected to non-denaturing polyacrylamide gel electrophoresis For comparison, extracts of isolated intact chloroplasts were run at the same time After electrophoresis, gels were stained for phosphorylase activity or for protein. Phosphorylase eluted from the Sepharose-starch column by dextrin comigrated with the enzyme activity extracted from isolated intact chloroplasts (Fig. 2a) For the purified enzyme, the activity zone coincided with the protein band detectable after staining with Coomassie blue (Fig 2b) Occasionally, in addition to this activity/protein band, one or two weak zones of phosphorylase activity were found near the cathodic

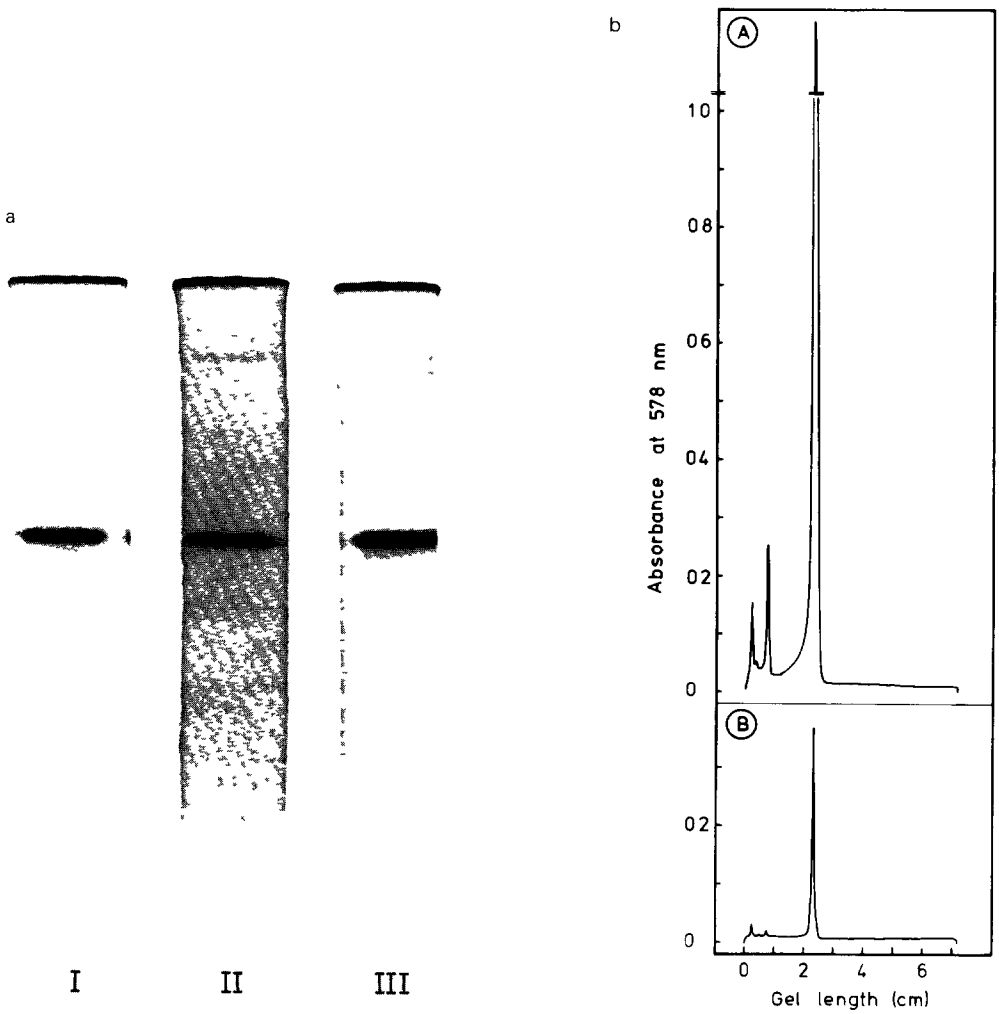


Fig 2 Non-denaturating polyacrylamide gel electrophoresis (a) Electrophoresis was performed with 15 pkat phosphorylase activity (equivalent to less than 0 2 μg protein) of the dextrin eluate from the Sepharose-starch chromatography (I) For comparison, extracts of isolated intact chloroplasts were run simultaneously (II, 3 pkat) As a control, electrophoresis was performed on a mixture of chloroplast extract and dextrin eluate (III, 7 5 pkat purified phosphorylase and 1 5 pkat activity from the chloroplast extract) Electrophoresis was run for 4 5 h at 4°C (20 mA/slab, 260—460 V) Migration direction is from top (cathode) to bottom (anode) After electrophoresis, gels were incubated at 30°C in a mixture of 0 1% (w/v) starch, 20 mM glucose 1-phosphate, 100 mM citrate, brought to pH 6 5 with NaOH Incubation times were 1 h (I), 3 h (II) and 2 h (III) Staining with iodine (b) Protein B and activity A staining of the purified chloroplast phosphorylase Electrophoresis was performed with 400 pkat phosphorylase activity (4 μg protein) Migration direction is from left to right For starch synthesis, the gel was incubated for 1 h Details as in a

end of the gel, these became more visible if relatively large quantities of the purified enzyme were applied to the gel as required for protein detection (Fig. 2b). It is likely that these two minor bands of activity (which corresponded to two faint protein zones) represent oligomers of the chloroplast phosphorylase. After non-denaturating electrophoresis, no protein band lacking a

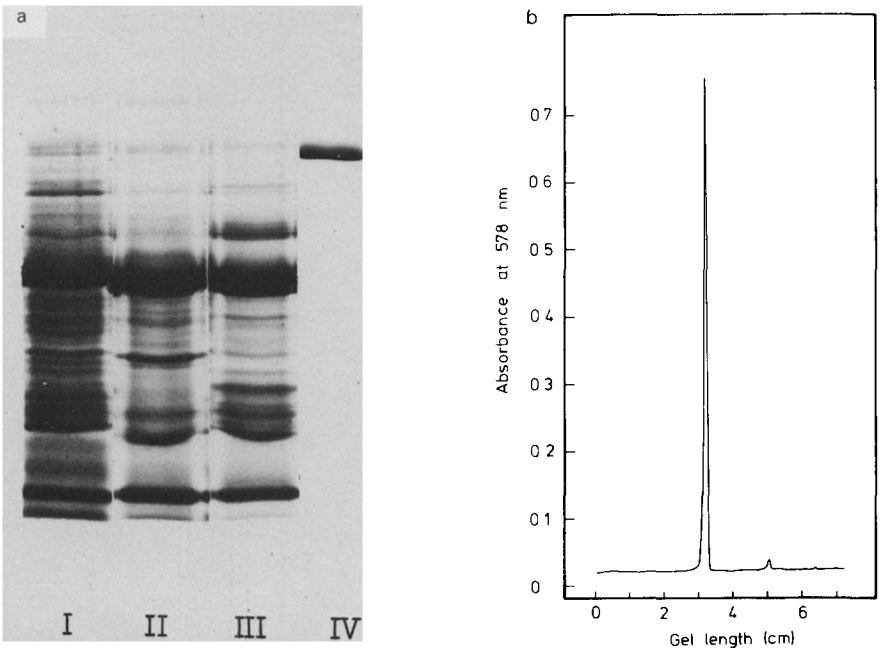


Fig 3 SDS-polyacrylamide gel electrophoresis at various stages of enzyme purification (a) 9% polyacrylamide gel I, crude extract (140 $\mu$g protein), II, precipitate between 45 and 60% saturation with $(NH_4)_2SO_4$ (70 $\mu$g), III, NaCl eluate of DEAE-Sephacel chromatography (70 $\mu$g), IV, dextrin eluate after Sepharose-starch chromatography (8 $\mu$g) Electrophoresis was run for 2 h at 60 V and for 5 h at 120 V constant voltage, temperature was 4°C. Migration direction was from top (cathode) to bottom (anode) (b) Densitogram of a 7% gel after electrophoresis (1 h at 60 V, 5 h at 120 V, 4°C) of the purified chloroplast phosphorylase (8 $\mu$g) Migration direction is from left to right

corresponding phosphorylase activity band was detectable. Denaturating electrophoresis on 7 or 9% polyacrylamide gels indicated that the chloroplast phosphorylase preparation consisted of a single protein (Fig. 3a and b). Traces of one or two other protein bands usually found in the preparation accounted for less than 1% of the phosphorylase protein as judged from densitograms of the stained gels.

Thus, chromatography on Sepharose-bound starch, which contained butyl groups in addition to the covalently bound glucan, allowed an effective purification of the chloroplast phosphorylase from spinach leaves. The complete purification is summarized in Table I. As compared with the crude extract, an approx. 300-fold purification was achieved with a recovery of 6%. However, these values underestimate the effectiveness of the purification procedure, since the phosphorolytic activity in the crude extract is composed of both the chloroplast and the non-chloroplast phosphorylases. Taking this into account, and assuming an approx. 3 · 1 ratio of non-chloroplast to chloroplast phosphorylase activity [5], the actual purification of the chloroplast enzyme was approx. 1000-fold with a recovery of more than 20%. After chromatography on Sepharose-bound starch, the specific activity of the phosphorylase preparation was not increased by further ion-exchange chromatography at pH 7.5 nor by gel filtration on agarose (Bio-gel A 1.5 M; data not shown).

TABLE I

PURIFICATION OF CHLOROPLAST PHOSPHORYLASE

| | Fraction | Volume (ml) | Activity (nkat) | Protein (mg) | Specific activity (nkat/mg protein) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|---|---|
| (I) | Crude extract | 780 | 1762 | 6045 | 0 29 | — | 100 |
| (II) | 45—60% $(NH_4)_2SO_4$ | 410 | 1018 | 1197 | 0 85 | 2 9 | 58 |
| (III) | DEAE-Sephacel | | | | | | |
| | (a) Citrate gradient eluate (discarded) | 120 | 574 | — | — | — | 32 6 |
| | (b) NaCl gradient eluate | 30 | 168 | 99 | 1 7 | 5 8 | 9 5 |
| (IV) | Sepharose-starch (+Sephadex G-100) | 60 | 106 | 1 1 | 96 4 | 332 | 6 |

TABLE II

DETERMINATION OF CHLOROPLAST PHOSPHORYLASE MOLECULAR WEIGHT

For gradient electrophoresis, the polyacrylamide concentration was 4—30%, oligomers of bovine serum albumin served as molecular weight standards Gel filtration on Sephadex G-200 was performed in 50 mM Tris, adjusted to pH 7 5 with HCl, cytochrome *c* (12 400), myoglobin (17 800), chymotrypsinogen A (25 000), ovalbumin (45 000), bovine serum albumin (68 000), immunoglobulin G (150 000) and catalase (240 000) were used as molecular weight standards For SDS-polyacrylamide gel electrophoresis, a 7% separation gel was used, molecular weight markers were RNA polymerase from *Escherichia coli* (165 000, 155 000 and 39 000), phosphorylase from rabbit muscle (94 700), bovine serum albumin (68 000), human immunoglobulin G (50 000 and 23 500), and lactate dehydrogenase from beef heart (36 000) Values in parentheses represent the number of replicates ± S E

| | Native enzyme | Monomer |
|---|---|---|
| Gradient electrophoresis (3) | 198 000 ± 2000 | |
| Gel filtration | | |
| 1st experiment | 220 000 | |
| 2nd experiment | 230 000 | |
| SDS-polyacrylamide gel electrophoresis (6) | | 109 000 ± 1000 |
| Pyridoxal phosphate determination | | |
| 1st experiment | | 113 000 |
| 2nd experiment | | 116 000 |

The purified chloroplast phosphorylase was stable (no detectable loss in activity during 3 weeks) when stored at 4°C as a solution of approx. 0.3 mg protein/ml (e.g., after concentration by Diaflo XM-50 ultrafiltration) in 50 mM Hepes and 2 mM EDTA, adjusted to pH 8.0 with NaOH.

During SDS-polyacrylamide gel electrophoresis the chloroplast phophorylase migrated behind rabbit muscle phosphorylase, indicating a higher apparent molecular weight of the monomer. In addition, the apparent molecular weight of the chloroplast phosphorylase was determined by estimation of the pyridoxal content, by gel filtration on Sephadex G-200, and by polyacrylamide gradient electrophoresis. The data which are summarized in Table II indicate that the chloroplast phosphorylase is composed of a monomer with an apparent molecular weight of approx. 110 000 and with a stoichiometry of one pyridoxal phosphate molecule per monomer. The apparent molecular weight of the monomer is higher than that of the monomer of the non-chloroplast form [6]. Molecular weight determination by gel filtration and gradient electrophoresis suggest a dimeric structure for the native enzyme as observed for other plant phosphorylases [18—20].

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