Purification of a novel pyruvate kinase from a green alga

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A chloroplast pyruvate kinase has been purified to homogeneity from the green alga *Selenastrum minutum*. The subunit composition of the enzyme differs markedly from pyruvate kinases from other sources. Subunit and native M_rs were 210 kDa and 235 kDa, respectively, indicating a monomeric structure. Immunological and peptide mapping analyses suggested structural homology to a bacterial, but not higher plant or mammalian pyruvate kinase.

Pyruvate kinase; Glycolysis; Chloroplast; (*Selenastrum minutum*)

1. INTRODUCTION

Pyruvate kinase (PK, EC 2.7.1.40) is a key regulatory enzyme of glycolysis which has been extensively studied from a wide variety of sources. The kinetic and/or molecular properties of several plant plastid and cytosolic isozymes of PK (PK_p and PK_c, respectively) have been reported [1–6]. Although a PK_c was recently purified to homogeneity [3], this has not been achieved with PK_p. In this report, we describe the purification of PK_p from a green alga, and present evidence suggesting that its subunit structure is different from other PK's.

2. MATERIALS AND METHODS

Rabbit muscle and liver type PKs, and *Bacillus stearothermophilus* PK were obtained from Sigma Chemical Co. Homogenous PK_c and partially purified PK_p from castor bean endosperm were prepared as previously described [3,4]. The green alga, *S. minutum*, was grown and harvested as in [5].

The assay procedure and conditions for PK were as described [5]. One unit of enzyme is defined as the amount of enzyme activity resulting in the production of 1 μmol pyruvate/min at 30°C.

The purification scheme of Lin et al. [5] for PK_p from *S. minutum* was used with the following modifications: (i) 110 g of cells were used, (ii) CDR (2 g) (Whatman) was added with stirring to the dialyzed resuspended $(NH_4)_2SO_4$ pellets and the solution percolated with suction through a layer (7 × 0.5 cm) of CDR pre-equilibrated with dialysis buffer, (iii) the filtrate was diluted to 8 mg/ml prior to anion exchange chromatography on a column (2.6 × 38 cm) of Q-Sepharose; (iv) a larger butyl agarose column was used (1.5 × 8 cm), and (v) a FPLC Superose 6 HR 16/50 (prep grade) column replaced

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Abbreviations: PK, pyruvate kinase; PK_p and PK_c, plastid and cytosolic isozymes of pyruvate kinase, respectively; CDR, cell debris remover; PAGE, polyacrylamide gel electrophoresis

the Superose 6 HR 10/30 (prepacked) column (sample volume = 1.0 ml; flow rate = 0.25 ml/min).

The production of rabbit polyclonal antibodies, affinity purification of rabbit anti-PK_p IgG, SDS-PAGE, enzyme immunoremoval, and Western blotting were performed as described previously [4] with the exception that polyvinylidene difluoride membranes (Whatman) were used in place of nitrocellulose. Subunit and native M_rs were determined as in [4]. Protein concentrations were determined by the method of Bradford [7] using bovine γ-globulin as protein standard. Carboxymethylation was performed according to Lane [8]. Peptide mapping by CNBr fragmentation was carried out as described in [9].

3. RESULTS AND DISCUSSION

Approximately 50% of the total PK activity in the crude extract was PK_c and this was removed following Q-Sepharose anion exchange chromatography. The overall purification procedure of PK_p is summarized in table 1. The final specific activity was 218 units/mg and is similar to the values for homogenous plant and non-plant PKs [3,10,11]. After the final purification step, a single protein staining band was present following SDS-PAGE which corresponded to a subunit M_r of 210 kDa (fig.1A). This was not changed by SDS-PAGE in the presence of 8 M urea, or after carboxymethylation of the protein. The native M_r of the homogenous enzyme was estimated to be 235 kDa on a calibrated FPLC Superose 6 HR 10/30 gel filtration column. This value is almost identical with the value previously reported for the partially purified enzyme [5]. These results suggest that *S. minutum* PK_p is monomeric. In contrast, all PKs studied to date (including *S. minutum* PK_c) have subunit M_rs ranging from 50 to 70 kDa [3–5,10,11]. While most PKs have been found to be homotetrameric, the enzyme from the anaerobic bacterium *Zymomonas mobilis* is a homodimer [10]. *S. minutum* PK_c appears to be homodecameric [5], whereas PK from germinating castor bean endosperm cytosol and mammalian erythrocytes is heterotetrameric

Table 1

Purification of chloroplastic pyruvate from *Selenastrum minutum*

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purifica-tion (−fold)	Yield (%)
I Crude extract	710[a]	3486	0.20	1	100
II (NH₄)₂SO₄, 25–50%	390[a]	2825	0.14	0.7	55
III CDR	439[a]	1720	0.26	1.3	62
IV Q-Sepharose	442	143	3.1	15.5	62
V Butyl agarose	244	8.2	29.8	149	34
VI Superose 6	96	0.44	218	1090	14

[a] Calculated assuming that PK_p represents 50% of the total PK activity present in crude extracts of *S. minutum* [5]

[3,4,11]. The subunit composition of *S. minutum* PK_p is therefore unlike any PK examined to date.

Rabbit anti-(*S. minutum* PK_p) immune serum immunoprecipitated *S. minutum* PK_p (fig.2), but had no effect on the activities of PK_p from developing castor
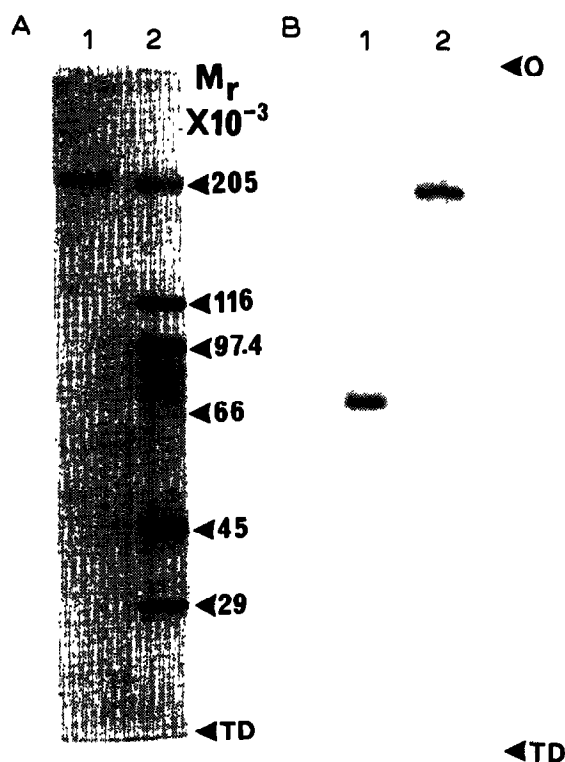

Fig.1. SDS polyacrylamide mini-gel electrophoresis and Western blot analysis. (A) SDS-PAGE (8% (w/v) separating gel) of PK_p purified from *S. minutum*. Lane 1 contains 5 µg of the final preparation, and lane 2 contains 5 µg of various M_r standards. The gel was stained with Coomassie blue R-250. (B) Western blot analysis of *S. minutum* and *Bacillus* PKs. Samples were subjected to SDS-PAGE and blot-transferred to a polyvinylidene difluoride membrane. Western analysis was performed using affinity purified anti-(*S. minutum* PK_p) IgG and antigenic peptides were visualized using an alkaline-phosphatase-tagged secondary antibody as in [4]; phosphatase staining was for 10 min at 30°C. Lane 1 contains 10 ng of purified *Bacillus* PK, and lane 2 contains 10 ng of purified *S. minutum* PK_p. O, origin; TD, tracker dye front.

bean endosperm, PK_c from *S. minutum* or germinating castor bean endosperm, *Bacillus* PK, or rabbit muscle or liver PK. Anti-PK_p IgG was affinity purified using 20 µg of the 210 kDa polypeptide of the final *S. minutum* PK_p preparation which had been excised from a Western blot. The affinity purified anti-PK_p IgG also immunoprecipitated *S. minutum* PK_p activity with 0% activity remaining compared with the control. Immunoremoval of *S. minutum* PK_p by affinity purified anti-PK_p IgG confirms that the protein band migrating with a M_r of 210 kDa following SDS-PAGE of the final preparation (fig.1A) is in fact PK_p.

Western blots of ng amounts of purified *S. minutum* and *Bacillus* PK revealed single immunoreactive polypeptides migrating with M_rs of 210 kDa and 65 kDa, respectively, when probed with the affinity/purified rabbit anti-PK_p IgG (fig.1B). There was no immunological cross-reactivity on Western blots when µg quantities of PK's from the following sources were probed with the same IgG: *S. minutum* PK_c, PK_p and PK_c from castor bean endosperm, rabbit liver and muscle PK. Thus, although PK_cs from *S. minutum* and from castor oil plant endosperm and leaf tissue are im-
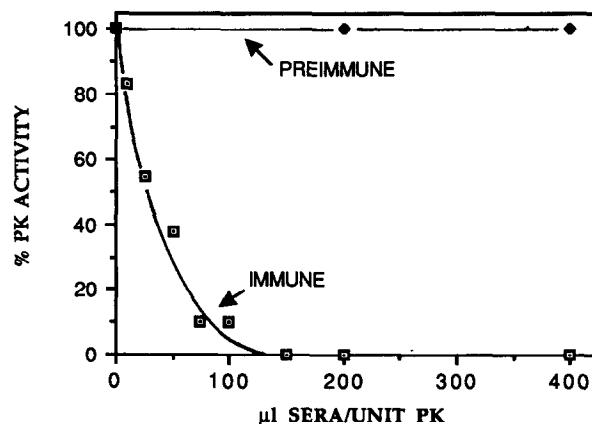

Fig.2. Effect of rabbit anti-(*S. minutum* PK_p) immune serum and preimmune serum on the activity of *S. minutum* PK_p. Immunotitrations were performed using 0.05 unit of PK as previously described [4].
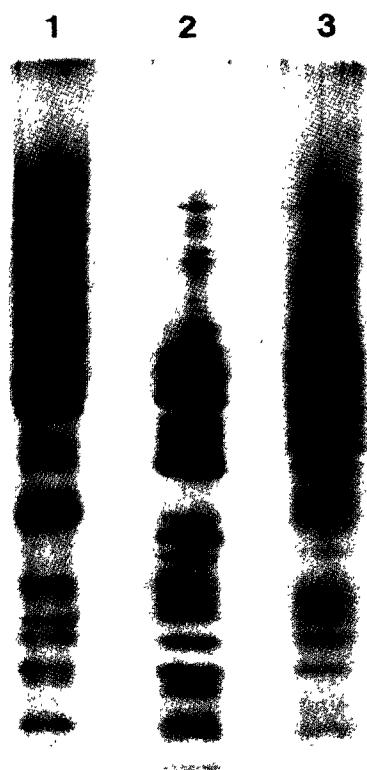
Fig.3. Electrophoretic patterns of CNBr cleavage fragments of mammalian, bacterial, and green algal PK. Peptide fragments generated from gel slices containing 4 μg of each protein were resolved by SDS-PAGE on 14% (w/v) mini-gels and stained with silver as in [9]. The designations are: 1, rabbit muscle PK; 2, *Bacillus* PK; 3, *S. minutum* PK_p.

munologically related [4,5], PK_ps from these two species are unrelated.

The structural relatedness of rabbit muscle PK, *Bacillus* PK, and *S. minutum* PK_p was investigated using peptide mapping of their CNBr fragmentation products. The respective peptide maps were different

(fig.3). However, the smaller fragments generated from CNBr cleavage of the bacterial PK and algal PK_p appeared to have some similarities (lanes 2 and 3, fig.3). This implies that the bacterial and algal polypeptides may contain some regions of homology. Previous work has shown that *Bacillus* and higher plant PK_c CNBr cleavage patterns are quite dissimilar [9].

In summary, Western blot and peptide mapping analyses indicate that, as is the case for many plastid localized proteins [12], the green algal PK_p may be more closely related to its prokaryotic, rather than eukaryotic, counterpart. Studies are in progress to further characterize this novel PK and to determine if higher plant PK_ps also show a distinct subunit structure.

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