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Expression, purification and characterization of *Solanum tuberosum* recombinant cytosolic pyruvate kinase

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ABSTRACT

The cDNA encoding for a *Solanum tuberosum* cytosolic pyruvate kinase 1 (PKc1) highly expressed in tuber tissue was cloned in the bacterial expression vector pProEX HTc. The construct carried a hexahistidine tag in N-terminal position to facilitate purification of the recombinant protein. Production of high levels of soluble recombinant PKc1 in *Escherichia coli* was only possible when using a co-expression strategy with the chaperones GroES-GroEL. Purification of the protein by Ni^{2+} chelation chromatography yielded a single protein with an apparent molecular mass of 58 kDa and a specific activity of 34 units mg^{-1} protein. The recombinant enzyme had an optimum pH between 6 and 7. It was relatively heat stable as it retained 80% of its activity after 2 min at 75 °C. Hyperbolic saturation kinetics were observed with ADP and UDP whereas sigmoidal saturation was observed during analysis of phospho*enol*pyruvate binding. Among possible effectors tested, aspartate and glutamate had no effect on enzyme activity, whereas α-ketoglutarate and citrate were the most potent inhibitors. When tested on phospho*enol*pyruvate saturation kinetics, these latter compounds increased $S_{0.5}$. These findings suggest that *S. tuberosum* PKc1 is subject to a strong control by respiratory metabolism exerted via citrate and other tricarboxylic acid cycle intermediates.

Introduction

Pyruvate kinase (PK[1], EC 2.7.1.40) catalyzes the irreversible transfer of a phosphoryl group from phospho*enol*pyruvate (PEP) to ADP, thereby generating pyruvate and ATP. It is therefore involved in the provision of pyruvate used as substrate for aerobic respiration, the substrate-level synthesis of ATP and in the control of the metabolism of the important glycolytic intermediate PEP. In plants, PK exists as tissue-specific isozymes that are present in the cytosol (PKc) and the plastid (PKp) [1]. Plant PKs are encoded by a relatively large gene family. For instance, the genome of the model plant *Arabidopsis thaliana* contains 10 PKc genes, 3 PKp genes and 1 PK-like gene [2,3]. In *Solanum tuberosum*, 5 PKcs and 4 PKps have been identified [3]. In plants, PKc is important in sink-source relationship as well as for the control of carbon and respiratory metabolism [3–5]. PKp activity is needed for the catabolism of storage compounds in germinating seeds [6] and the biosynthesis of seed oil [2]. The kinetic properties of native plant PKs purified from a variety of sources have been the subject of extensive characterization. From these studies, it appears that the regulatory properties of PKs isozymes are often species-, tissue- and developmental stage-specific, suggesting a high level of specialization in the regulation of PKs depending on particular organismal and metabolic contexts [1]. Plant PKc allosteric regulation by amino acids Asp and Glu as well as intermediates of the glycolytic pathway and the tricarboxylic acid (TCA) cycle was demonstrated in a number of cases [7–9]. Strong sensitivity of the enzyme to pH has also been reported [7–9]. Additional PKc regulatory mechanisms have been described [10]. These include evidence for the involvement of proteolytic processing at the C-terminus as well as phosphorylation and ubiquitination in the regulation of PKc activity and *in vivo* steady-state levels. However, the importance of post-translational modifications in the control of PKc is still not completely elucidated. Further studies of the enzymes involved in PKc phosphorylation and proteolytic processing would benefit from the availability of the enzyme in recombinant form. However, despite the general importance of PKs in plant central metabolism, only two reports describe the successful expression of plant PKs in heterologous systems and little information is available on the properties of the recombinant enzymes [2,10]. We wanted to expand the range of available recombinant PKs by attempting the production of high yields of recombinant PKc as this would greatly facilitate fur-

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[1] *Abbreviations used:* DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactoside; LDH, lactate dehydrogenase; Ni–NTA, Ni^{2+}–nitrilotriacetic acid; PEP, phospho*enol*pyruvate; PEG, polyethylene glycol; PK, pyruvate kinase; PKc, cytosolic pyruvate kinase; PKp, plastidic pyruvate kinase; TCA, tricarboxylic acid.

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ther studies of this enzyme, in particular the molecular characterization of protein kinase(s) and the proteolytic process putatively involved in posttranslational regulation of this enzyme [10].

The primary goal of the present study was therefore the purification and the characterization of a recombinant PKc produced in *Escherichia coli*. To this end, we used the sequence of the first cloned PKc cDNA [11]. This sequence is encoded by the *S. tuberosum* (potato) *PKCYT1* gene, which appears to be highly expressed in tubers [3]. The production of high yields of the recombinant protein in *E. coli* was only possible with the co-expression of the GroES-GroEL chaperone complex encoded by the pGro7 plasmid. We report on some of the kinetic properties of the recombinant enzyme, including substrate saturation behavior and identify several intermediates of the TCA cycle as effectors.

Materials and methods

Materials and chemicals

Buffers, chemicals, metabolites and reagents used were of analytical grade and purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Nepean, ON, Canada). Restriction enzymes and reagents used for recombinant DNA work were purchased from Fermentas (Burlington, ON, Canada). The pGro7 plasmid was from Takara Bio Inc. (Mountain View, CA). Sequencing primers were from Sigma Genosys (The Woodlands, TX). The pProEX HTc expression vector and Ni^{2+}–nitrilotriacetic acid (Ni–NTA) agarose were supplied by Invitrogen (Burlington, ON, Canada).

Construction for recombinant PKc expression in E. coli

S. tuberosum PKc cDNA in pBluescript SK (−) (StPKc) [11] was digested with *Msc*I and *Sac*I, producing a 1577 bp DNA fragment with a blunt 5′ end and cohesive 3′ extremity. The 5′ extremity of this fragment corresponds to the fourth nucleotide of the PKc coding sequence. The fragment also contained the stop codon of the StPKc sequence. This fragment was cloned into the pProEX HTc expression vector previously digested with *Stu*I and *Sac*I. The generated construct encoded a 541 amino acid recombinant protein with a theoretical molecular weight of 58,922.3 Da. The N-terminal extension of its sequence contained a hexahistidine (6xHis) tag and the encoded protein was named (6xHis)StPKc1. The ligated plasmid was used to transform different competent *E. coli* cells (strains DH5α, BL21(DE3), Rosetta™(DE3), HB101 and HB101 carrying the pGro7 plasmid encoding for GroES-GroEL) in various attempts to optimize production of recombinant protein in the soluble fraction. The identity of the construct was confirmed by diagnostic digestions and sequencing.

Production of (6xHis)StPKc1

E. coli carrying the construct encoding (6xHis)StPKc1 was grown in Luria–Bertani broth medium (200 mL total volume) at 37 °C to an A_{600} of 0.5 with the appropriate antibiotics. Isopropyl ß-D-thiogalactoside (IPTG) was then added to the culture at a final concentration of 0.6 mM and bacteria were grown with agitation (250 rpm) for 18 h at 18 °C. For the production of control (non-induced) cultures, the cultures were grown similarly, but in absence of IPTG. For the production of (6xHis)StPKc1 in HB101 carrying the pGro7 plasmid, cells were grown and induced as described above, except that they were inoculated in the presence of L-arabinose (0.5 mg mL^{-1}). Cells were centrifuged for 10 min at 5000×*g* at 4 °C. Cell pellets were resuspended in 10 mL of extraction buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 0.1% (w/v) Triton X-100, 1 mM phenylmethylsulphonylfluoride, adjusted to pH 8.0 with NaOH. Cells were disrupted at 4 °C twice using a French press (18,000 psi) and the resulting extract was centrifuged for 30 min at 10,000×*g*. The supernatant was filtered over a cellulose acetate filter (0.22 μm pore size). At this step, in some experiments, aliquots of the pellet and the supernatant were subjected to electrophoretic analysis. To purify soluble (6xHis)StPKc1, the supernatant was incubated with Ni–NTA resin (0.5 mL settled bed volume) previously equilibrated in extraction buffer. The protein was allowed to bind to the resin for 1 h at 4 °C. Subsequent steps in the purification were performed at room temperature. The slurry was then poured into a 0.5 cm-diameter column. All the extract was allowed to pass through the column. Subsequently, the column was washed using 30 volumes of new extraction buffer followed by 60 volumes of extraction buffer containing 20 mM imidazole. The column was then washed with 60 volumes of a solution buffer containing 50 mM NaH_2PO_4, 5 mM ATP, 10 mM $MgCl_2$ and 300 mM KCl, adjusted to pH 8.0 with NaOH (ATP wash buffer). Elution of the bound proteins from the column was achieved with 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, adjusted to pH 8.0 with NaOH. Ten 0.5 mL fractions were collected. Fractions containing the peak of (6xHis)StPKc1 (typically 1.5 mL total) were identified by SDS–PAGE stained by Coomassie blue and activity assays. These fractions were dialyzed against a buffer containing 50 mM Tris–Cl, pH 7.5 and 1 mM dithiotreitol (DTT). The dialyzed enzyme preparation was centrifuged for 10 min at 10,000×*g* and the supernatant stored at −20 °C in 50% (v/v) glycerol until used.

Pyruvate kinase activity assay and protein determination

To assay recombinant PKc activity, the PK reaction was coupled to that of lactate dehydrogenase (LDH, EC 1.1.1.27) [12]. Spectrophotometric assays were carried out at 30 °C and monitored at 340 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). One unit (U) of activity corresponds to the production of 1 μmol of pyruvate per minute. Except when mentioned otherwise, reactions were done in a solution containing 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, 4 mM PEP, 4 mM MgADP, 0.32 mM NADH and 0.4 U LDH in a final volume of 200 μL. Reaction rates were linear with time and proportional to the quantity of (6xHis)StPKc added. Enzyme activity measurements at different pHs during the determination of optimum pH, substrate saturation kinetic and effector studies were done by substituting the 50 mM Tris buffer in the assay described above by a buffer capable of maintaining ionic strength (0.05 M acetic acid, 0.05 M 2-(*N*-morpholino)ethanesulfonic acid and 0.1 M Tris) [13]. In this case, pH was adjusted by adding NaOH or HCl. Enzyme assays and kinetic analyses were carried out in triplicate on a minimum of two independent enzyme preparations. Protein was determined using the method of Bradford [14] with bovine serum albumin as a standard.

Electrophoresis and immunoblot analysis

Analysis of proteins by electrophoresis in polyacrylamide gels under denaturing conditions in the presence of SDS (SDS–PAGE) was performed using 10% (w/v) acrylamide gels. Gels were stained with Coomassie R-250 or transferred to nitrocellulose [15]. Immunoblot analysis of PKc was performed with anti-PKc polyclonal immune serum (1/5000 dilution) directed against the *Brassica napus* native enzyme [7]. The polyhistidine tag was detected using the Penta·His mouse monoclonal antibody (1/2000 dilution) from Qiagen (Toronto, ON, Canada). The GroEL protein was detected using the anti-*E. coli* GroEL mouse monoclonal antibody clone 9A1/2 (1/10,000 dilution) from One World Lab (San Diego, CA). Detection of antigen/antibody complexes was done using alkaline phosphatase-tagged secondary antibodies (Promega, Nepean, ON, Canada) [15].