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Characterization and Activity Determination of the Human Protein Phosphatase 2A Catalytic Subunit α Expressed in Insect Larvae

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Abstract Protein phosphatase 2A is the major enzyme that dephosphorylates the serine/threonine residues of proteins in the cytoplasm of animal cells. This phosphatase is most strongly inhibited by okadaic acid. Besides okadaic acid, several other toxins and antibiotics have been shown to inhibit protein phosphatase 2A, including microsystin-LR, calyculin-A, tautomycib, nodularin, cantharidine, and fostriecin. This makes protein phosphatase 2A a valuable tool for detecting and assaying these toxins. High-scale production of active protein phosphatase 2A requires processing kilograms of animal tissue and involves several chromatographic steps. To avoid this, in this work we report the recombinant expression and characterization of the active catalytic subunit α of the protein phosphatase 2A in *Trichoplusia ni* insect larvae. Larvae were infected with baculovirus carrying the coding sequence for the catalytic subunit α of protein phosphatase 2A under the control of the polyhedrin promoter and containing a poly-His tag in the carboxyl end. The catalytic subunit was identified in the infected larvae extracts, and it was calculated to be present at 250 μg per gram of infected larvae, by western blot. Affinity chromatography was used for protein purification. Protein purity was determined by western blot. The activity of the enzyme, determined by the *p*-nitrophenyl phosphate method, was 94 μmol/min/mg of purified protein. The catalytic subunit was further characterized by inhibition with okadaic acid and dinophysis toxin 2. The results presented in this work show that this method allows the production of large quantities of the active enzyme cost-effectively. Also, the enzyme activity was stable up to 2 months at −20 °C.

Keywords Recombinant protein phosphatase 2A · Trichoplusia ni · Okadaic acid · Protein phosphatase 2A inhibition · Enzyme stability

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Introduction

Four major serine/threonine-specific protein phosphatase (PP) catalytic subunits are present in the cytoplasm of animal cells: PP1, PP2A, PP2B, and PP2C. The phosphatases 1, 2A, and 2B, are members of the same gene family; PP2C belongs to a different one. In eukaryotic cells, PP2A is the major enzyme that dephosphorylates the serine/threonine residues of proteins [1]. It is a trimmer consisting of a 36-kDa catalytic subunit, PP2AC, and two regulatory subunits, A and B. The core enzyme is a dimmer (PP2AD) consisting of the catalytic subunit and the regulatory subunit A. In mammals, two different A isoforms exist, α and β, which share 86 % sequence identity and are ubiquitously expressed. The regulatory subunit B, with several isoforms known, can associate to the core enzyme and regulates the enzyme localization and specific activity of the different holoenzymes [2]. As with the regulatory subunit A, there are two PP2AC isoforms, α and β, which share 97 % sequence identity [2, 3]. Both isoforms are ubiquitously expressed, but PP2ACα is 10 times more abundant than PP2ACβ. This could be explained by the fact that these isoforms are encoded by different genes, and the promoter for PP2ACα is 7–10 times stronger than the promoter for PP2ACβ, as shown by their respective mRNA levels [4, 5].

The polyether okadaic acid (OA) is one of the most thoroughly studied phosphatase inhibitors. This molecule is produced by marine dinoflagellates, and it accumulates in shellfish [6, 7]. Okadaic acid inhibits PP1, PP2A, and PP2B to different extents, having no effect on PP2C. PP2A is the most strongly inhibited phosphatase, followed by PP1 and PP2B. This toxin, together with its derivatives, dinophysis toxin-1, -2, and -3 (DTX-1, -2, and -3) are responsible of the diarrhetic shellfish poisoning, which affects public health and the shellfish industry [7]. Besides OA and its derivatives, several other toxins and antibiotics have been shown to inhibit PP2A including microsystin-LR [8], calyculin-A [9], tautomycib [10], nodularin [11], cantharidine [12], and fostriecin [13]. This makes PP2A a valuable tool for detecting and assaying these toxins.

One method of producing large quantities of active PP2A involves isolation from animal tissue. Unfortunately, this approach requires the use of large amounts of tissue, in the order of kilograms, and requires up to six chromatographic steps for protein purification [14]. Alternatively, this enzyme can be produced, at different quantities, as a recombinant product in yeast [15, 16], insect [17–19], and mammalian cells [20].

Biologically active recombinant PP2A has been expressed in insect cells in the dimeric (PP2AD) and the catalytic (PP2AC) forms. The recombinant catalytic subunit was shown to be more stable than the dimeric enzyme [17]. Also, when both catalytic isoforms were expressed in insect Sf21 cells, they showed the same activity. Since the catalytic subunit is the most stable form of PP2A, and isoforms α and β have the same activity in vitro, we assayed PP2ACα expressed in insect larvae to determine if a more abundant and cost-effective source of active PP2ACα could be obtained.

Materials and Methods

Pp2acα Expression in Insect Larvae

Human PPP2ACα (GenBank accession no. NM_002715.2) was obtained from Origene as cloned cDNA in the plasmid pCMV6-AC. The sequence was verified by partial sequencing using primers VP1.5 (5′-GGACTTTCCAAAATGTCG-3′) and XL39

(5′-ATTAGGACAAGGCTGGTGGG-3′). After sequence confirmation, the PPP2ACα cDNA was amplified and cloned in the pFasTBacHis plasmid in order to obtain the cloned sequence in phase with a 6 histidine (6 His) tag at the carboxyl end of the cDNA. The resulting plasmid was transformed in competent DH10B *Escherichia coli* cells which contained the receptor bacmid bMON14272. Transposition generated a recombinant baculovirus which carried the PPP2ACα sequence flanked by a polyhedrin promoter, for insect-driven expression, and the poly-His tag. *E. coli* cells positive for transposition (no β-galactosidase activity) were selected. Bacmid DNA from these cells was extracted and transfected in SF21 (Invitrogen) insect cells with cellfectin (Invitrogen). From these cells, the first progeny of recombinant baculovirus was collected. The baculovirus was amplified by infection of SF21 cells, obtaining a baculovirus concentration of 9×10^8 plaque forming units per milliliter (pfu/ml). *Trichoplusia ni* larvae were manipulated and maintained as previously described [21–23]. Fourth-instar laboratory-reared larvae were infected by injection with 1×10^5 pfu of recombinant baculovirus. At 72 h post infection, they were collected, mechanically disrupted in PBS, and lyophilized for storage (Algenex).

Recombinant PP2ACα Purification

Larvae lysates were diluted in 15 ml PBS at 4 °C, containing a protease inhibitors cocktail (Sigma) and centrifuged at 15000 × *g* at 4 °C for 10 min. The soluble fraction was recovered and loaded in a Bio-Scale™ Mini Profinity™ IMAC (Bio-Rad) chromatographic column. The recombinant protein retained, by the Ni^{2+} in the column, was washed and recovered following the column manufacturer's instructions. After elution, PP2ACα was desalted and concentrated with a 10,000-Da cutoff Millipore filtering unit, by three cycles of centrifugation with 50 mM Tris-HCl. The purified protein solution was stored at −20 °C.

Protein Electrophoresis and Western Blot

Soluble protein samples (40 μg) were mixed with sample buffer and loaded in 10 % acrylamide precast gels (Bio-Rad). After protein separation, the gels were stained with Coomasie blue, destained, and photographed with a VersaDoc™ (Bio-Rad) system.

For western blotting, after electrophoresis, gels were transferred to PVDF membranes (Millipore) at 100 V during 1 h. Membrane blocking and antibody incubations were performed with the Snap ID system (Millipore). The primary antibodies used were as follows: anti-poly-His 1:1,000, anti-PP2A 1:1,000 (Millipore), and anti-methyl PP2A 1:1,000 (Millipore). The secondary antibody (Sigma) was used at a 1:5,000 concentration. After antibody incubations and washing, the membranes were reveled with ECLplus West Pico (Thermo), and image acquisition was made with a VersaDoc™ system (Bio-Rad).

The larvae-produced recombinant protein was quantified by western blot, using purified His-tagged recombinant human-secreted phosphoprotein 1 (SPP1, CD Biosciences, Inc) to obtain a concentration curve. SPP1 is a 36-kDa protein and was selected to avoid differences in transference efficiencies due to protein size. Along with the larvae extract, six concentrations of SPP1 were included in the electrophoresis. After transference and antibody incubations, image acquisition was performed with a VersaDoc™ system (Bio-Rad) and quantification with the GeneTools™ software (Bio-Rad).

PP2ACα Activity Determination and Inhibition Assays

PP2ACα activity was determined with the 4-morpholine umbelliferone (4-MUP) and the *p*-nitrophenyl phosphate (*p*-NPP) methods, as previously described [24, 25]. Enzyme activity was calculated from the results obtained by the *p*-NPP method, considering that 1 U of enzymatic activity is defined as the quantity needed to hydrolyze 1 μmol of phosphate from *p*-NPP, per minute, at 30 °C. For the *p*-NPP assay, the sample buffer contained 40 mM Tris–HCl at pH 8.1, 20 mM KCl, 30 mM MgCl2, 2 mM DTT, and 0.05 mg/ml BSA. The enzyme was diluted in sample buffer, and the reaction was started by the addition of *p*-NPP at a 25-mM final concentration. Absorption was measured, in a Biotek plate reader, at 400 nm with a reference wave length of 500 nm at 30 °C. When the 4-MUP method was used, the reaction mixture contained 30 mM Tris–HCl at pH 7, 60 μM Ca^{2+}, 2 mM Ni^{2+}, and 0.25 mg/ml BSA. To this buffer, 10 μl of the enzyme was added, and the reaction was started with 120 μl of 0.117 μM 4-MUP. In order to determine the PP2ACA inhibitory effect of OA and DTX-2, the toxins were added to the reaction mixture containing the enzyme and incubated for 10 min at 37 °C before adding the 4-MUP. Fluorescence was measured, in a Biotek microplate reader, with an excitation wavelength of 360 nm and an emission wavelength of 440 nm at 30 °C. Each condition was assayed by quintuplicate, and three experiments were done.

Statistical Analysis

The results were analyzed using the software SigmaPlot©. One way ANOVA was employed for comparison of significant differences among groups. Comparisons between groups were made by the Holm–Sidak multiple range test. A value of $p<0.05$ ($n\geq3$) was considered significant.

Results

In order to obtain biologically active PP2ACα, the catalytic subunit was expressed in *T. ni* insect larvae. Although the recombinant protein expression was not evident in baculovirus-infected larvae after SDS PAGE and coomasie staining (Fig. 1a), 6 His-PP2ACα was detected by western blot with an anti-poly-histidine antibody (Fig. 1b). The approximate amount of recombinant protein obtained was 250 μg per gram of infected larvae (Fig. 1c).

After purification, the eluted protein was analyzed by electrophoresis and coomasie staining, and a single band of approximately 36 kDa was detected (Fig. 2a). PP2ACα was identified by western blot with an anti-PP2AC (Fig. 2b). Also, because the leucine residue at position 309 (Leu^{309}) of the catalytic subunit is methylated in vivo, we determined the PP2ACα methylation by western blot using an anti-methyl PP2A antibody (Fig. 2b).

After identifying the protein, the activity was studied by fluorescence and absorbance methods with 4-MUP and *p*-NPP, respectively. Both methods produced similar results for serial dilutions of the recombinant enzyme (Fig. 3). Based on the results of the activity determined by the *p*-NPP method, the enzymatic activity was estimated to be 94±7.1 μmol/min/mg of purified protein.

In order to further study the recombinant enzyme, inhibition studies with OA and DTX-2 were performed. Enzymatic activity was determined by the 4-MUP method, and 0.025 U of

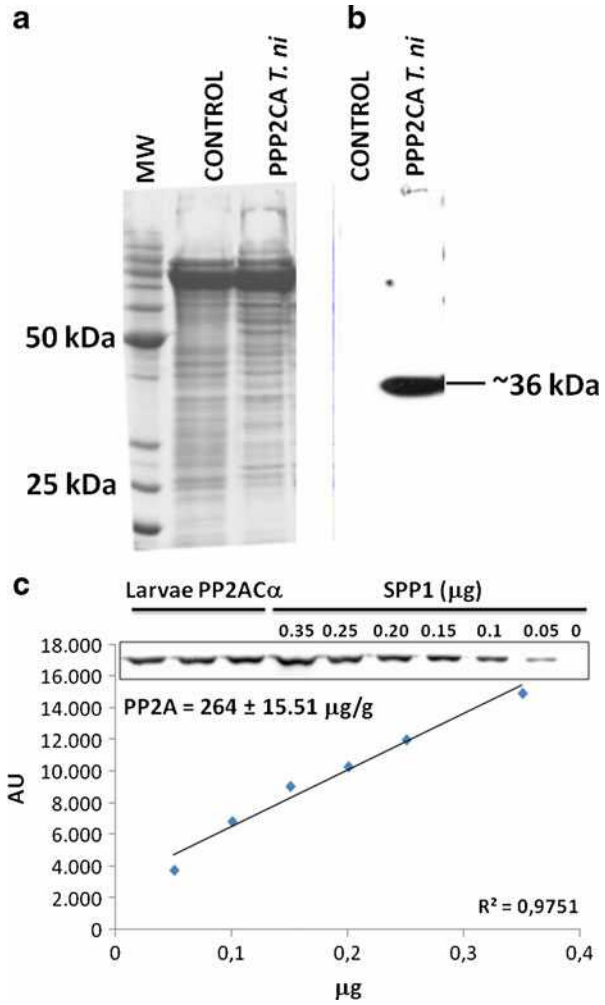

Fig. 1 **a** SDS PAGE of control and baculovirus-infected larvae protein extract. The recombinant PP2ACα cannot be detected when comparing control and baculovirus-infected extracts which carry the coding sequence for this enzymatic subunit (PPP2CA). **b** Western blot of control and infected larvae soluble protein extracts using an anti-poly-histidine antibody. A protein of approximately 36 kDa, which is the molecular weight of the PP2ACα, was detected. **c** Quantification of the recombinant protein in baculovirus-infected larvae. Six concentrations of purified His-tagged SPP1 were used as standards to calculate the recombinant protein concentration in larvae lysates

enzyme was used. OA and DTX-2 were incubated in the presence of the enzyme at concentrations ranging from 0.001 to 10 nM for OA and from 0.0125 to 10 nM for DTX-2. When the enzymatic activity was determined, IC_{50}s of 0.28 and 0.41 nM were obtained for OA and DTX-2, respectively (Fig. 4).

In order to assess the stability of the recombinant enzyme, it was stored for 1 or 2 months at −20, 4 °C, or room temperature (~25 °C). The enzyme was dissolved in 50-mM Tris at pH 7.2 and 0.05 % sodium azide or 50-mM Tris at pH 7.2, 0.05 % sodium azide, and glycerol 50 %. After 1 or 2 months, 0.025 U of the enzyme, as

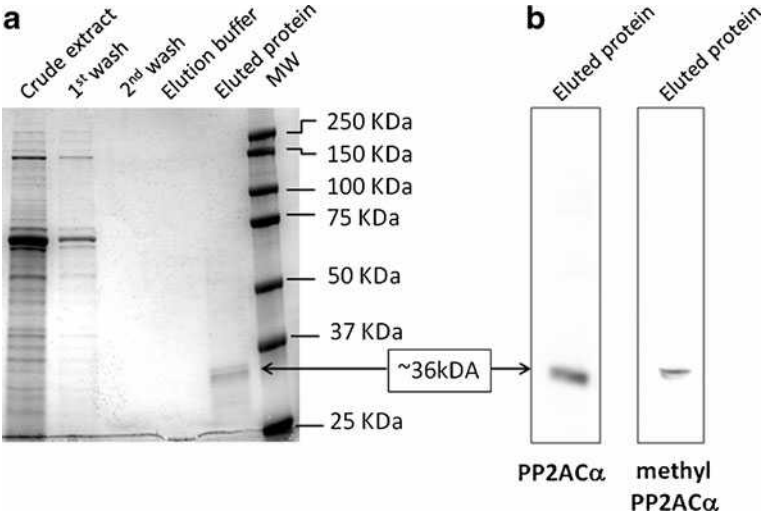

Fig. 2 **a** Polyacrylamide electrophoresis of baculovirus-infected larvae protein extract, washes of the purification column, and eluted protein. An approximately 36-kDa protein was observed without contamination of proteins with other molecular weights. **b** Western blot of the eluted protein using anti-PP2AC and anti-methyl-PP2AC. In both cases, an approximately 36-kDa protein was detected without unspecific bands

determined after purification, was used to determine the activity by the 4-MUP method. The enzyme retained 100 % of the activity observed after purification when it was stored in Tris at pH 7.2 and 0.05 % sodium azide at −20 °C while it lost 44 and 53 % of the activity when it was in Tris at pH 7.2, 0.05 % sodium azide, and glycerol 50 % at −20 °C for 1 and 2 months, respectively. It lost approximately 90 % of the activity when stored in Tris–HCl and 0.05 % sodium azide at 4 °C or room temperature after 1 month. Finally, no activity was detected in the enzyme diluted in Tris–HCl and 0.05 % sodium azide after 2 months at room temperature, or in Tris–HCl, 0.05 % sodium azide, and glycerol 50 % after 1 month when it was stored at room temperature (Table 1).

Discussion

In this study, we show that biologically active human recombinant PP2ACα can be produced in high quantities in *T. ni* insect larvae. The expression of the recombinant protein was confirmed by western blot using an antibody specific for the poly-His tag in larvae lysates without purification. After purification from larvae lysates, it was identified as PP2ACα by western blot using antibodies specific for this subunit and its methylated form. This last experiment indicates that the recombinant protein is methylated in the insect larvae in the same manner as in mammalian cells. The purified protein was active as determined by the 4-MUP and the *p*-NPP methods. This implies that the addition of the poly-histidine tag at the carboxyl end of the recombinant protein does not interfere with the protein activity. The enzymatic activity, as determined by the *p*-NPP method, was 94±7.1 μmol/min/mg. This activity is similar to that obtained for the PP2A purified from rabbit skeletal muscle, which has been reported to be 120 μmol/min/mg using the same method [24]. Also, it is

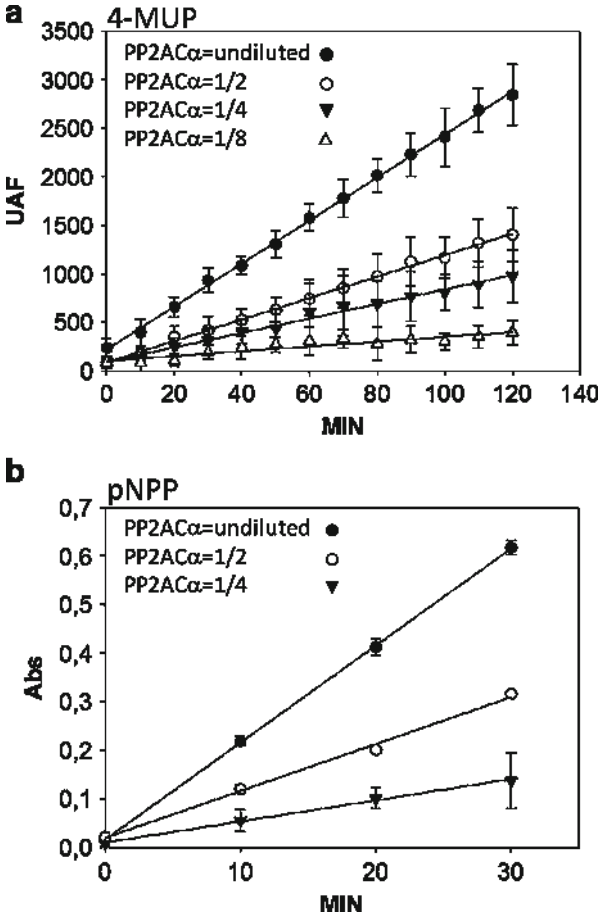

Fig. 3 PP2ACα activity determination by the 4-MUP and *p*-NPP methods (**a**,**b**). Activity was determined for the undiluted purified protein and for its serial dilutions. In both determinations, the enzyme had an activity according to the dilutions used. Each enzyme concentration was assayed with $n=5$, and the SD was calculated with a 99 % confidence level

similar to the activities observed for PP2AC purified from Sf21 cells, which were 83.7 and 81.8 μmol/min/mg for PP2ACα and β, respectively [17]. When the quantity of recombinant protein produced by larvae is compared with the quantity produced in SF21 insect cells informed by other authors (1.25 and 1–2 μg per million cells) [17, 19], up to 2.5×10^8 SF21 cells would be needed to obtain the same mass of PP2ACα that is obtained from 1 g of insect larvae.

PP2A inhibition assays for monitoring natural toxins have been reported that used native enzyme extracted from human or rabbit tissues [25, 26]. However, the assay method employing native PP2A has not been widely used due to fluctuations in enzyme quality. Relying on tissues as a source of PP2A has other drawbacks, including the need of several purification steps to obtain the native PP2A and the high cost of the process. In order to avoid these problems, recombinant expression of PP2A has been applied to obtain a reliable source of this enzyme. Recombinant PP2ACα expressed in Sf21 insect cells has been used to design an assay for the

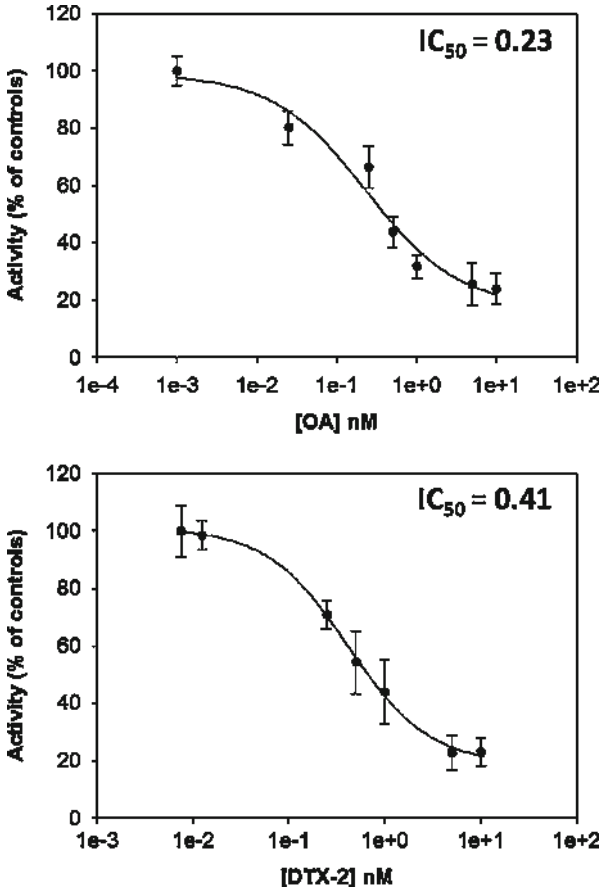

Fig. 4 PP2ACα inhibition by OA and DTX-2. 0.025 U of the enzyme was used in the presence of OA at concentrations ranging from 0.001 to 10 nM or DTX-2 at concentrations ranging from 0.0125 to 10 nM. Enzyme activity was determined by the 4-MUP method. As expected, the inhibitory effect of OA was higher compared to that of DTX-2. Each enzyme concentration was assayed with $n=5$, and the SD was calculated with a 99 % confidence level

detection of microcystins [27], indicating that the recombinant protein is a valuable tool for this type of assays. Based on the results we show in this work, the sensitivity of the recombinant PP2ACα purified from insect larvae to OA is similar to that of native PP2A [25, 26], and the difference in inhibitory potency between OA and DTX-2 can be appreciated as in a previously reported work [28]. This implies that the recombinant enzyme will be useful in assays to detect toxins instead of native PP2A purified from tissues.

As previously mentioned, the production of PP2ACα in insect Sf21 cells has also been reported as a source of high quantities of PP2ACα [17], but this approach is more expensive and requires a laboratory with the technology for maintaining and growing this type of cells. This makes the production of recombinant PP2ACα from larvae a better system because it is cheaper, considering the baculovirus-infected larvae can be reared with an artificial wheat germ diet without the need of a high-tech culture system, and only one purification step is required to obtain the active

Table 1 Percentage of the enzymatic activity retained after 1 and 2 months of enzyme storage in various conditions compared to the enzyme activity after purification

Storage conditions	Storage time	
	1 Month	2 Months
50 mM Tris–HCl, pH 7.2, −20 °C	99,318±16,962	99,699±8,795
50 mM Tris–HCl, pH 7.2, 50 % glycerol, −20 °C	56,545±11,757*	47,755±4,353*
50 mM Tris–HCl, pH 7.2, 4 °C	10,630±3,545*	7,235±2,423*
50 mM Tris–HCl, pH 7.2, 50 % glycerol, 4 °C	9,265±3,859*	7,044±1,615*
50 mM Tris–HCl, pH 7.2, RT	11,011±2,468*	1,713±0,628*
50 mM Tris–HCl, pH 7.2, 50 % glycerol RT	−6,759±7,135*	−4,950±5,923*

*$p \leq 0.01$ (Significant differences with respect to the activity observed after purification, $n=3$)

recombinant protein. Furthermore, the expression of the recombinant protein in larvae allows a very easy and cost-effective scalability of the production process by increasing the number of larvae infected. Also, because only one chromatographic purification step is needed, and that these chromatographic columns can be loaded with high quantities of lysates and can also be serially attached one to another, the scaling-up would not require further purification steps.

When we studied the enzyme stability after 1 or 2 months of storage at different temperature and solvent conditions, we observed that the recombinant protein retained 100 % of its activity only when it was stored at −20 °C in 50-mM Tris–HCl at pH 7.2 and 0.05 % sodium azide. At the same temperature but dissolved in 50-mM Tris, 0.05 % sodium azide, and 50 % glycerol, the enzyme lost activity progressively after the first and second months. At 4 °C in both solvents, there was a loss of approximately 90 % of the enzymatic activity after 1 month of storage and near 100 % of the activity was lost after the second month. At room temperature, after 2 months of storage, the activity was completely lost in both solvents. This indicates that the purified enzyme can be produced in high quantities and stored at −20 °C for long periods without losing activity. We are working in establishing better storage conditions to asses if the protein can be stored at 4 °C without a significant activity decrease.

Conclusions

Because high purity, stability, and sensitivity are crucial for an enzyme to be used in a microplate assay, biologically active human recombinant PP2ACα was obtained from baculovirus-infected *T. ni* insect larvae. This provides a stable and cheap source for the enzyme. It also allows scaling up the process much easier than in previously reported systems for the purification of this enzyme.

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