



Efficient production of recombinant PP2A at a low temperature using a baculovirus expression system



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ABSTRACT

Protein phosphatase 2A (PP2A) is an enzyme useful for detecting several natural toxins represented by okadaic acid and microcystins. We found that the production of the recombinant human PP2A catalytic subunit (rhPP2Ac) in High Five insect cells could markedly increase when the cells were cultured at 19 °C instead of 27 °C used under conventional conditions. The yield and purity of the enzyme increased four- and three-folds, respectively. The benefit of the altered culturing temperature was observed with the recombinant human protein phosphatase 2B but not 2C α . The different responses among the enzymes suggest the involvement of an enzyme-specific mechanism that leads to the catalytic subunit overexpression. This is the first report to produce rhPP2Ac at a temperature lower than that used under conventional culture conditions (27 °C) used in the baculovirus expression system with High Five insect cells.

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Reversible protein phosphorylation controlled by protein kinases and phosphatases is a major regulation mechanism in all eukaryotic cells. The protein phosphatases are classified into the two classes: 1) the serine/threonine phosphatases and 2) the tyrosine phosphatases. Protein phosphatase 2A (PP2A) is one of the four major classes (PP1, PP2A, PP2B, and PP2C) of eukaryotic serine/threonine phosphoprotein phosphatases [1,2]. PP2A plays pivotal roles in many critical aspects of cellular activities such as metabolism, transcription, translation, the cell cycle, and signal transduction. PP2A exists as a number of holoenzymes. The core enzyme is a dimer consisting of a highly-conserved 36-kDa catalytic subunit and a 65-kDa structural subunit. PP2A is inhibited by okadaic acid (OA) responsible for diarrhetic shellfish poisoning (DSP) and microcystins (MCs), which are cyclic heptapeptide hepatotoxins produced by cyanobacteria [3–6]. Several other natural toxins and antibiotics including calyculin-A [7], tautomycin [8], nodularin [9], cantharidin [10], and fostriecin [11], also inhibit PP2A, suggesting that PP2A would be a valuable tool in assays for the detection of these toxins. Based on the inhibitory action, an assay method was proposed to determine OA using *p*-nitrophenyl

phosphate (*p*-NPP) as a substrate [12], and PP2A inhibition assays for MCs and OA used native PP2A extracted from hepatocytes, human red blood cells, or rabbit skeletal muscles [13–17]. However, the purity and stability of the enzyme at the time did not meet the sufficient quality standards required for use in kits. Thus, it is crucial that a PP2A product of high purity and good stability is available at large quantities to practically utilize a PP2A assay.

Aiming at usage in assay kits, we recently produced the recombinant catalytic subunit of human PP2A (rhPP2Ac) by genetic engineering techniques using the baculovirus expression system with High Five insect cells [18]. Because the high-level expression of recombinant proteins in *Escherichia coli* often results in their cytoplasmic deposition as insoluble inclusion bodies, we chose insect cells for the production of rhPP2Ac. Because culture conditions such as temperature, pH, and nutrient supply would control the partition of the recombinant protein into soluble and insoluble fractions [19], a careful examination is required to establish the optimum culture conditions. In general, insect cells are incubated at 27 °C in the baculovirus expression system; however, we attempted to further optimize the production of rhPP2Ac by changing the incubation temperature of High Five insect cells. To obtain an insight into the mechanism of expression,

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cells engineered for the production of rhPP2B and rhPP2C were also subjected to the same culture conditions.

The experimental conditions for rhPP2Ac production were the same as those of the previous study [18], except for a change in the incubation temperature after the infection of High Five insect cells with recombinant baculoviruses. To examine the expression level of rhPP2Ac, we expressed the rhPP2Ac in High Five insect cells at a temperature range of 11–31 °C for 2–6 days. Expressed His_{8x}-tagged rhPP2Ac was detected by Western blot analysis using anti-polyhistidine-peroxidase conjugate (Sigma), followed by the enhanced chemiluminescence (ECL) system (GE Healthcare). Importantly, the levels of rhPP2Ac in the lysate soluble fractions of the cells significantly increased when a lower temperature (15–23 °C) was employed for incubation instead of the routine culture condition (27 °C). As shown in Fig. 1a, the level of rhPP2Ac was highest at 19 °C for 5 days (relative intensity = 100%), followed by those at 19 °C for 6 days (relative intensity = 95.5 ± 10.6%), and at 19 °C for 4 days (relative intensity = 92.4 ± 4.3%), and was 1.7-fold higher than that at 27 °C for 3 days (relative intensity = 59.0 ± 22.6%). Based on the result, culture conditions were set at 19 °C for 4 days in subsequent examinations. To further evaluate the culture conditions, we compared the levels of expression and quality of rhPP2Ac under two conditions: 1) 4 days at 19 °C and 2) 3 days at 27 °C. The resultant rhPP2Ac was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie Brilliant Blue R staining (Fig. 1b). The visualized images were analyzed using LAS3000 Multi Gauge Ver2.2 software (Fuji photo Film Co. Ltd) to estimate the purity of the protein. The purity values of purified rhPP2Ac expressed at 27 °C and 19 °C were 58.0 ± 6.9 and 87.7 ± 3.0%, respectively (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.btre.2016.07.004). Thus, 1.5-fold higher purity was achieved by lowering the temperature from the conventional 27 °C to 19 °C. The phosphatase activity assay using *p*-NPP as the substrate was

performed as previously described [18]. The specific activities of purified rhPP2Ac expressed at 27 °C and 19 °C were 7.7 ± 3.6 and 33.1 ± 7.5 U/μg protein, respectively (Fig. 1c). Impairment of the stability was unrecognizable (data not shown). These results show that the expression of rhPP2Ac at 19 °C in the system of expression of baculovirus using High Five insect cells can produce the recombinant protein with a higher activity and in a larger quantity than in the incubation conducted at a conventional temperature of 27 °C.

To examine the effects of the low temperature expression on other phosphatases, we expressed human PP2B and PP2C in High Five insect cells. PP2B is active as a heterodimer containing a 60-kDa catalytic subunit and a 19-kDa Ca²⁺-binding regulatory subunit. PP2C is a monomer. The cDNAs encoding the human PP2B catalytic subunit α isoform (hPP2B-α), the human PP2B regulatory subunit α isoform (hPP2B-β), and the human PP2C α isoform (hPP2Cα) were amplified using polymerase chain reaction (PCR) using the human cDNA libraries. The sequences of the PCR primers used to generate the recombinant hPP2B-α isoform with a polyhistidine-tag (His_{8x}) at the N-terminal are shown in Supplementary Fig. S2 (in the online version at DOI: 10.1016/j.btre.2016.07.004). The PCR products were cloned into the pENTR/SD/D-TOPO vector using the pENTR Directional TOPO cloning kit (Invitrogen). To construct the recombinant donor plasmid, the cloned DNA fragment was inserted into the Gateway pDEST8 vector (Invitrogen) using the baculovirus expression system with a Gateway Technology Kit (Invitrogen). Recombinant baculoviruses encoding respectively His_{8x}-tagged hPP2B-α, FLAG-tagged hPP2B-β, and His_{8x}-tagged hPP2Cα were produced using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's protocols.

Recombinant baculoviruses were produced and amplified using Sf9 cells, following which we expressed these recombinant phosphatases (hPP2B dimer and hPP2C) in High Five insect cells

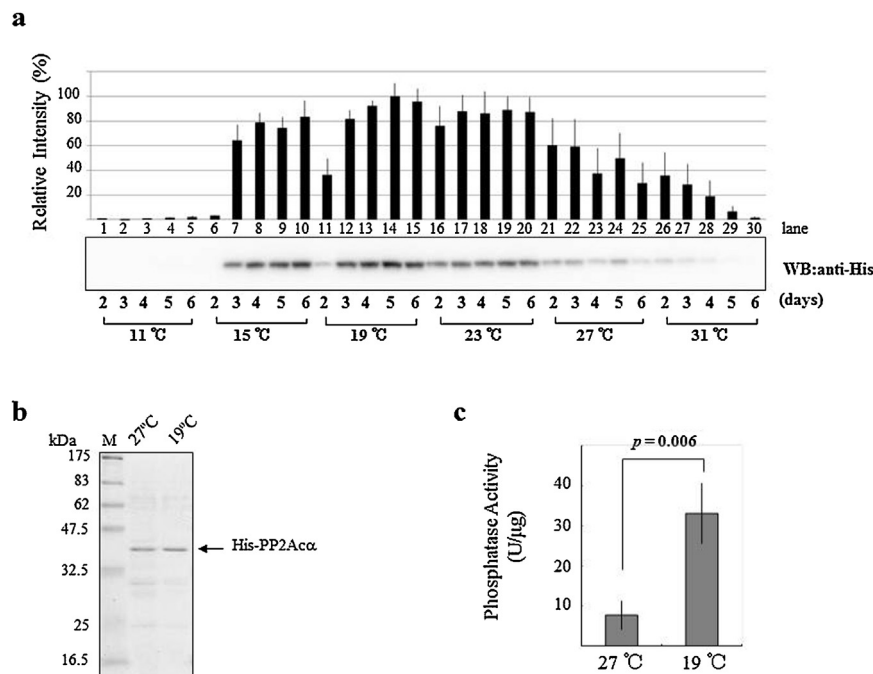


Fig. 1. The comparison of expression, purification, and phosphatase activity of recombinant protein phosphatase 2A (PP2A). (a) His_{8x}-tagged PP2Acα was expressed in High Five insect cells at a temperature range of 11–31 °C for 2–6 days using a baculovirus expression system. The lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting using anti-polyhistidine peroxidase conjugate. The blots are representative of three independent experiments. The quantified graph of the blots are presented as means (n = 3) ± SD. (b) After purification, 0.2 μg of each protein was subjected to 12% SDS-PAGE and visualized using Coomassie Brilliant Blue R staining. (c) The activities of the recombinant proteins were assayed against *p*-NPP as substrate. All assays were performed in triplicate (unit, nmol/min). The *p*-value of two-tailed Student's *t*-test is indicated.

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