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Facile preparation of highly active casein kinase 1 using *Escherichia coli* constitutively expressing lambda phosphatase

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ABSTRACT

Casein kinase 1 (CK1) is a widely expressed Ser/Thr kinase in eukaryotic organisms that is involved in various cellular processes (e.g., circadian rhythm and apoptosis). Therefore, preparing highly active CK1 and investigating its properties *in vitro* have important implications for understanding the biological roles of the kinase. However, recombinant CK1 undergoes autoinactivation via autophosphorylation in *Escherichia coli* cells and thus is undesirably prepared as a phosphorylated and inactivated kinase. To circumvent this problem, we established a protein expression system using *E. coli* strain BL21(DE3)pλPP in which λ protein phosphatase (λPPase) is constitutively expressed. Using this system, recombinant CK1 isoforms (α, δ and ε) were readily prepared as unphosphorylated forms. Furthermore, we found that CK1s prepared using BL21(DE3)pλPP showed markedly higher activity than those prepared by the conventional BL21(DE3). Finally, we demonstrated that the kinase activity of CK1δ from BL21(DE3)pλPP was higher than that prepared by a conventional method consisting of troublesome steps such as *in vitro* λPPase treatment. Thus, this simple method using BL21(DE3)pλPP is valuable for preparing highly active CK1s. It may also be applicable to other kinases that are difficult to prepare because of phosphorylation in *E. coli* cells.

Introduction

Casein kinase 1 (CK1) is a widely expressed Ser/Thr kinase in eukaryotic organisms [1–3], and seven mammalian CK1 isoforms (α, β, γ1–3, δ and ε) have been cloned and characterized to date [4–7]. CK1 isoforms (CK1s) play important roles in various cellular processes (e.g., centrosome-associated processes, DNA damage-related signal transduction, circadian rhythm and apoptosis) [8–10]. Therefore, the physiological roles of CK1s and their enzymatic properties have recently received increased attention by researchers in various fields.

The regulatory mechanism of CK1s is of great interest: the kinase activity is inactivated by autophosphorylation, which occurs especially in the C-terminal domain [5,11–14]. In particular, the downregulation mechanism of CK1δ and ε has been well studied, and the autophosphorylated C-terminal domain is considered to act as a pseudo-substrate resulting in inhibition of the kinase. Recombinant CK1s prepared by the *Escherichia coli* protein expression system are highly autophosphorylated, and thus inactivated [11,13,14]. These preparations are unfavorable for enzymatic characterization of *bona fide* kinase activity because CK1s are actively maintained in the dephosphorylated/highly active form by cellular protein phosphatases (PPase) *in vivo* [15].

Therefore, to obtain unphosphorylated and highly active CK1s, bacterially expressed CK1s are needed to be treated with PPase *in vitro*. Conventionally, recombinant CK1s have been dephosphorylated by PPase such as λ protein phosphatase (λPPase) or the catalytic subunit of protein phosphatase 2A (PP2Ac), and then used for *in vitro* kinase assays after overriding the effects of PPase by adding a PPase inhibitor [11,14,16]. C-terminal deletion mutants [CK1s(ΔC)] that lack autophosphorylation and the inhibitory region are commercially available and used for various studies on CK1s *in vitro* and *in vivo* [17–19]. Particularly in recombinant CK1ε(ΔC) prepared from *E. coli*, however, it has been reported that *in vitro* phosphatase treatment led to further activation of the kinase, suggesting that CK1s(ΔC) have additional inhibitory autophosphorylation sites in the catalytic domain [14,16].

Abbreviations: CK1, casein kinase 1; CK1(ΔC), the C-terminal deletion mutant of CK1; λPPase, lambda protein phosphatase; PK, protein kinase; PPase, protein phosphatase; PP2Ac, the catalytic subunit of protein phosphatase 2A

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Thus, CK1s(ΔC) may not be appropriate for evaluation of the full activity of CK1s, especially in *in vitro* studies.

It would be very useful if unphosphorylated CK1s could be prepared using an *E. coli* expression system without the troublesome *in vitro* phosphatase treatment. So far, it has been shown that coexpressed λPPase, a Mn^{2+}-dependent protein phosphatase that can dephosphorylate phospho-Ser/Thr/Tyr [20,21], can suppress the autophosphorylation of overexpressed kinases, such as double-strand RNA-activated protein kinase [22], eukaryotic elongation factor 2 kinase [23] and mitogen-activated protein kinases [24], in *E. coli* cells. In this study, we improved these systems to develop a simple and more efficient method for preparing unphosphorylated protein kinases (PKs) from *E. coli* cells. We established the novel *E. coli* strain, BL21(DE3)pλPP, in which λPPase is constitutively expressed, and applied this system to obtain highly active CK1s.

Here, we selected three CK1 isoforms, α, δ and ε, which are known to be particularly important in various cellular processes and have been well studied regarding the role of autophosphorylation, and used the novel strain to prepare these isoforms. All the CK1 isoforms were readily prepared by a simple protocol as highly active forms, because they were almost completely dephosphorylated by the coexpressed λPPase in *E. coli*. Moreover, the truncated forms, CK1δ(ΔC) and CK1ε(ΔC), were prepared as more active forms than those prepared by conventional BL21(DE3) cells. These results suggest that the use of CK1s and their truncated forms obtained from BL21(DE3)pλPP is favorable for the evaluation of the *bona fide* activity of CK1s in *in vitro* analyses. We also compared the kinase activity of CK1δ and CK1δ(ΔC) prepared using BL21(DE3)pλPP with the activity of those prepared by a conventional method in which CK1s were dephosphorylated by λPPase *in vitro*. These experiments demonstrated that the former had higher specific activity than the latter, indicating that the simple method using BL21(DE3)pλPP is useful for preparation of highly active, unphosphorylated CK1s. Application of this method for preparation of other kinases or proteins is also discussed.

Materials and methods

Materials

[γ-^{32}P]ATP (111 TBq/mmol) was purchased from PerkinElmer. A HiTrap Chelating HP column was purchased from GE Healthcare Bio-Sciences. The plasmids pET23a(+) and pLysE, and *E. coli* BL21(DE3) cells were from Novagen. Anti-His_6 antibody and Phos-tag Acrylamide (AAL-107) were obtained from Wako Pure Chemical Industries. A horseradish peroxidase-labelled anti-mouse IgG + A + M antibody was obtained from Cappel. Sodium orthovanadate (Na_3VO_4) and α-casein from bovine milk were purchased from Sigma-Aldrich.

Establishment of BL21(DE3)pλPP

Plasmid pλPP, which constitutively expresses λPPase, was generated by replacing the T7 lysozyme encoded in pLysE with λPPase cDNA. The primers used for inverse polymerase chain reaction (PCR) to remove the T7 lysozyme cDNA and add the restriction sites, XhoI and KpnI, to the ends were: sense 5′-TTT CTC GAG TTA ATT GAA CTC ACT AAA GGG AGA CCA CAG C-3′ and antisense 5′-TT GGT ACC TAT TTC TTT CCT CCT TTC CTT TTT AAT CTA TCA AAG G-3′. To amplify the cDNA of λPPase, the pETλPPase plasmid [25] and primers (sense 5′-TT GGT ACC ATG CGC TAT TAC GAA AAA ATT GAT GGC-3′ and antisense 5′-TTT CTC GAG TCA TGC GCC TTC TCC CTG TAC C-3′) were used for PCR. The KpnI (double-underlined)-XhoI (underlined) fragment was inserted into the KpnI-XhoI sites of pLysE lacking the T7 lysozyme cDNA. To obtain the *E. coli* strain BL21(DE3)pλPP, the pλPP plasmid was used to transform *E. coli* strain BL21(DE3), which was cultured in LB medium supplemented with 34 μg/ml chloramphenicol.

To confirm the expression of λPPase in *E. coli*, BL21(DE3)pλPP cells were grown at 37 °C to an A_{600} of 0.6–0.8, and then cultured for 6 h at 37 °C. The bacteria (1 ml) were harvested by centrifugation and suspended in 100 μl of buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 40 and 1 mM phenylmethylsulfonyl fluoride). After sonication, cell lysates were divided into the supernatant and the precipitate by centrifugation (20,000 × *g*) at 4 °C for 10 min. The precipitate was resuspended in 100 μl of buffer A by sonication. Aliquots (10 μl each) of the supernatant and the precipitate were analyzed by SDS-PAGE, followed by in-gel phosphatase assay [26] using 4-methylumbelliferyl phosphate (MUP) as a substrate.

Plasmid construction

To generate pETmCK1α, δ and ε, the following primers were used for PCR with the mouse whole brain cDNA library as a template: *CK1α*, sense 5′-GCT AGC ATG GCG AGC AGC AGC GG-3′ and antisense 5′-A CTC GAG GAA ACC TGT GGG GGT TTG GG-3′; *CK1δ*, sense 5′-GCT AGC ATG GAG CTG AGG GTC GGG AA-3′ and antisense 5′-A CTC GAG TCG GTG CAC GAC AGA CTG AAG A-3′; *CK1ε*, sense 5′-GCT AGC ATG GAG TTG CGT GTG GGA AAT AA-3′ and antisense 5′-A CTC GAG TTT CCC AAG ATG GTC AAA TGG C-3′. The PCR fragments were subcloned into pGEM-T easy (Promega), and then ligated into the NheI (bold-underlined) and XhoI (underlined) sites of pET23a(+). The kinase dead (KD) plasmids, pETmCK1α(KD: K46R), δ(KD: K38R) and ε(KD: K38R), were generated by inverse PCR [27] with sense primers [5′-AGA CTA GAA TCC CAG AAG GCC AGG C-3′ for CK1α(KD), 5′-AGG CTT GAA TGT GTC AAA ACC AAA CAT CC-3′ for CK1δ(KD) and 5′-AGG CTC GAA TGT GTA AAG ACG AAA CAT CC-3′ for CK1ε(KD), where dashed underlines indicate the mutation sites], antisense primers [5′-CAC TGC CAC TTC CTC GCC AT-3′ for CK1α(KD), 5′-GAT GGC AAC TTC TTC TCC CGC-3′ for CK1δ(KD) and 5′-GAT GGC TAC TTC CTC ACC AGA GGC-3′ for CK1ε(KD)], and the templates pETmCK1α, δ and ε, respectively. Plasmids pETmCK1α(ΔC: 1–309), δ(ΔC: 1–301) and ε(ΔC: 1–301) were generated by inverse PCR with a sense primer [5′-CTC GAG CAC CAC CAC CAC CA-3′ for all CK1s(ΔC)] and antisense primers [5′-TGC CTG CTG GGC TGC TTT-3′ for CK1α(ΔC), 5′-TGC AGC CCG GCT GGC AC-3′ for CK1δ(ΔC) and 5′-GGG ATT CCG GGC TGC AC-3′ for CK1ε(ΔC)], and the templates pETmCK1s and pETmCK1s(KD), respectively. The 5′-ends of the PCR products were phosphorylated using T4 polynucleotide kinase (Nippon Gene) and self-ligated using T4 DNA ligase (Nippon Gene).

Expression and purification of recombinant CK1s and CK1s(ΔC)

BL21(DE3) carrying pETmCK1s or pETmCK1s(ΔC) were cultured in LB medium supplemented with 100 μg/ml ampicillin. BL21(DE3)pλPP carrying these plasmids were cultured with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The transformed bacteria were grown at 37 °C to an A_{600} of 0.6–0.8, and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. After 20 h at 18 °C, the bacteria were harvested by centrifugation and suspended in buffer A. After sonication, cell debris was removed by centrifugation (20,000 × *g*) at 4 °C for 10 min, and the supernatant was loaded onto a HiTrap Chelating HP column pre-equilibrated with buffer A. The column was washed with buffer A containing 20, 50 and 100 mM imidazole in a graded manner, and then eluted with buffer A containing 200 mM imidazole. The eluates were pooled and dialyzed against 20 mM Tris-HCl, pH 7.5, containing 0.05% Tween 40 and 1 mM 2-mercaptoethanol, distributed as aliquots and stored at −30 °C.

SDS-PAGE, Phos-tag SDS-PAGE and western blotting

SDS-PAGE was performed essentially according to the method of Laemmli [28] on slab gels consisting of a 10% or 12% acrylamide separation gel and a 3% stacking gel. The resolved protein samples were electrophoretically transferred to a nitrocellulose membrane