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Purification of YPTP1 with Immobilized Phosphonomethylphenylalanine-Containing Peptide as an Affinity Ligand

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A previous study on a yeast protein tyrosine phosphatase, YPTP1, using synthetic phosphotyrosine-containing peptides with various sequences as substrates revealed that DADEpYDA exhibits high affinity (K_m = 4 μM) toward the enzyme. A modified version of this peptide, GDADEpmFDA, immobilized on a resin, was used in this study as an affinity ligand for the purification of YPTP1. Phosphonomethylphenylalanine (pmF) was used as a nonhydrolyzable analog of the phosphotyrosine (pY) residue, with properties similar to pY. A protected form of pmF, Fmoc-pmF(^{t}Bu)$_2$-OH, was chemically synthesized and introduced during solid-phase peptide sythesis. YPTP1 was overexpressed in an *E. coli* strain carrying a plasmid pT7-7-ptp1. Affinity chromatography of the crude lysate afforded PTP1 (39 kDa) of about 50% purity.

Keywords: Dephosphorylation, Phosphonomethylphenylalanine, Phosphotyrosine, Protein tyrosine phosphatase, YPTP1.

Introduction

Protein tyrosine phosphatases (PTPases) catalyze the hydrolysis of the phosphoryl moiety from the phosphotyrosine (pY) residue of cellular proteins (Walton and Dixon, 1993; Zhang and Dixon, 1994a) (Fig. 1). PTPases are widely distributed in eukaryotes, from human to yeasts, and dozens of PTPase genes have been cloned from various organisms (Hunter, 1995). From the unicellular eukaryote, *Saccharomyces cerevisiae*, two distantly related PTPase genes, *PTP1* and *PTP2*, have been cloned (Guan *et al.*, 1991, 1992). The yeast *PTP1* gene

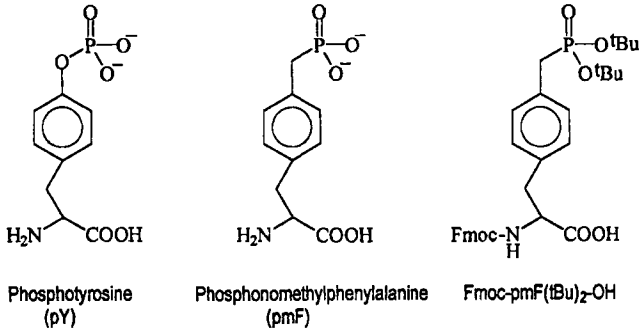

Fig. 1. Structures of phosphotyrosine (pY), phosphonomethylphenylalanine (pmF), and Fmoc-pmF(^{t}Bu)$_2$–OH.

was expressed in *Escherichia coli* and the recombinant protein YPTP1 showed tyrosine-specific phosphohydrolase activities toward phosphorylated protein substrates (Guan *et al.*, 1991).

We previously studied the substrate specificity of the recombinant YPTP1 toward peptide substrates containing a phosphotyrosine residue (pY-peptides) (Kwon *et al.*, 1996). We derived an optimum peptide sequence (DADEpYDA, K_m = 4 μM) recognized by YPTP1 by systematic mutation analysis of phosphotyrosyl-peptide (pY-peptide) substrates. A previous report by Shoelson *et al.* (1991) showed that the substitution of the phosphotyrosine residue with phosphonomethylphenylalanine (pmF) (Fig. 1), a nonhydrolyzable pY analog, does not significantly reduce the affinity of pY-peptides towards PTPases. From these results, it is conceivable that a peptide containing pmF as a replacement for pY in the peptide sequence

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DADEpYDA, when immobilized on a suitable resin, can be used as an affinity matrix for the purification of YPTP1. In this study, we have successfully applied this strategy for the purification of YPTP1 overexpressed in the *E. coli* expression system.

Materials and Methods

Materials Chemicals used for chemical syntheses were from Aldrich (Milwaukee, USA). Fmoc-amino acids were purchased from AnaSpec (San Jose, USA). BOP, HOBT, Wang resin, IPTG, lysozyme, DNase, malachite green, Coomassie blue, buffer materials, and protein molecular weight markers were from Sigma (St. Louis, USA). NovaSyn TG resin was from Calbiochem-Novabiochem (La Jolla, USA). CNBr-activated agarose and N-hydroxysuccinimidyl chloroformate-activated agarose were from Sigma. Epoxy spacer agarose was from Affinity Chromatography Ltd. (Cambridge, UK). BioGel-15 gel was from BioRad (Hercules, USA).

Synthesis of 4-[bis(tert-butoxy)phosphorylmethyl]-N-(fluoren-9-ylmethoxycarbonyl)-DL-phenylalanine (Fmoc-pmF(^{t}Bu)$_2$-OH) Synthesis of Fmoc-pmF(^{t}Bu)$_2$-OH was accomplished with minor modifications of the procedures described by Burke *et al.* (1991).

Synthesis of phosphonomethylphenylalanine(pmF)-containing peptides Solid-phase peptide syntheses were carried out with Fmoc chemistry as described previously for pY-peptides (Cho *et al.*, 1991; Kitas *et al.*, 1991). Briefly, peptides were synthesized manually with BOP, HOBT, and NMM as coupling reagents. Crude peptides were purified by reverse-phase HPLC (C18, 22 mm × 250 mm, Alltech, Deerfield, USA) with a linear gradient from H_2O/0.1% TFA to CH_3CN/0.1% TFA. The purified peptides were characterized by ion-spray mass spectrometry.

The peptide synthesis on NovaSyn TG resin were carried out according to the standard method for solid-phase peptide synthesis (Kitas *et al.*, 1991). The procedures were the same as those for the syntheses of pmF-containing peptides.

Immobilization of pmF-peptides The peptide GDADEpmFDA was immobilized on AffiGel-15 gel (BioRad) according to the manufacturer's instructions. Briefly, AffiGel-15 gel (2 ml, 15 μmol of arms per ml of resin) was washed three times with 10 ml of 50 mM acetate buffer (pH 5.0) and mixed with pmF-peptide (15 μmol) in 1.5ml of the same buffer. It was then incubated at 4°C for 1 wk. Unreacted arms were blocked by treatment with 1 M ethanolamine solution in 50 mM carbonate-bicarbonate buffer adjusted to pH 8.5. The resulting resin was used to prepare an affinity column.

Preparation of cell-free extract containing YPTP1 IPTG addition to exponentially growing cultures of *E. coli* BL21 (DE3) carrying the plasmid pT7-7-YPTP1 induced YPTP1 expression (Kwon *et al.*, 1996). After IPTG addition, the cultures were incubated with shaking at 30°C for 3 h and the cells were disrupted by repeated freezing and thawing in the presence of lysozyme. Crude lysate was prepared as described previously (Tsai *et al.*, 1991).

Affinity purification of YPTP1 The affinity resin (3 ml) prepared as described above, was poured into a plastic column (10 mm × 100 mm) and equilibrated at 4°C with buffer A (30 mM TrisHCl, 2.5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM benzamidine, pH 7.0). The crude lysate containing YPTP1 obtained from 50 ml of *E. coli* cuture was loaded on the affinity column and incubated for 5 min. The column was washed with three column volumes of buffer A and the bound materials were then eluted with 50 mM phosphate buffer (pH 7.0) containing 1 M NaCl. The fractions containing PTPase activity were combined and concentrated (Centricon-30, Amicon, Bevery, USA). It was then analyzed by polyacrylamide gel electrophoresis.

PTPase assay The PTPase assay was carried out as described previously (Cho *et al.*, 1991). Briefly, the PTPase reaction was carried out at 25°C with p-nitrophenyl phosphate as substrate in buffer B (100 mM Hepes, 10 mM DTT, 5 mM EDTA, pH 7.0). After quenching the reaction by addition of 0.5 N NaOH solution, the p-nitrophenol released was quantitated by absorbance measurement at 405 nm.

Results and Discussion

Synthesis of Fmoc-pmF(^{t}Bu)$_2$-OH and the syntheses of phosphonomethylphenyl-alanine(pmF)-containing peptides To incorporate pmF residue in a peptide during solid-phase peptide synthesis, we chemically synthesized pmF with the amine group protected with a fluorenylmethyloxycarbonyl (Fmoc) group and the phosphonic acid protected with *tert*-butyl groups with minor modifications of the procedures described by Burke *et al.* (1991a, b). The identity of the final product was confirmed by ^{1}H-NMR spectroscopy and ion-spray mass spectrometry. The 4-[bis(*tert*-butoxy)phosphorylmethyl]-N-(fluoren-9-ylmethoxycarbonyl)-D,L-phenylalanine, Fmoc-pmF(^{t}Bu)$_2$-OH (Fig. 1), was obtained in gram quantities as a diastereomeric mixture and it was used to introduce pmF residue into the peptide. Solid-phase peptide syntheses were carried out with Fmoc chemistry as described previously for phosphotyrosyl peptides (Kitas *et al.*, 1991). In the case of GDADEpmFDA, the diastereomers were not resolved by reverse-phase HPLC and, therefore, the diastereomeric mixture was used for immobilization.

Immobilization of pmF-peptides Several commercially available resins with activated arms were tested to immobilize pmF-peptides. Under standard reaction conditions neither CNBr-activated agarose, N-hydroxysuccinimidyl chloroformate-activated agarose, nor epoxy spacer agarose were successfully derivatized with the peptide GDADEpmFDA, possibly due to the exceptionally abundant negative charges on the side chains of the peptide. The immobilization was successful only with AffiGel-15 gel (BioRad) which has an N-hydroxysuccinimide ester at

the end of the positively-charged spacer arm. Maximum incorporation of the peptide was 2 μmol/ml gel. Neither a prolonged reaction nor addition of more peptide improved the level of the peptide immobilization. Unreacted arms were blocked by treatment with ethanolamine and the resulting resin was used as an affinity matrix.

Affinity purification of YPTP1 By affinity chromatography of the crude lysate containing overexpressed YPTP1, using the peptide GDADEpmFDA immobilized on BioGel-15 gel, we obtained a 22-fold purification of YPTP1 in a single step. The specific activity of the purified sample was 16 μmol/min/mg towards p-nitrophenyl phosphate as substrate, compared to the value of 0.73 μmol/min/mg of the crude lysate. The purified YPTP1 was determined to be ca. 50% pure based on the activity of the sample. For reference, the samples of YPTP1 purified independently to apparent homogeneity showed specific activities of 31 and 35 μmol/min/mg toward the same substrate (Kwon *et al.*, 1996; Park *et al.*, 1997). The purified YPTP1 was also analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). The affinity resin used in this study contains phosphonic acid moieties which possibly act as cation exchangers and bind unwanted proteins which coelute with YPTP1.

The strategy used in this affinity purification of YPTP1 was to use as an affinity ligand the immobilized pmF-peptide with a sequence which shows a high affinity toward YPTP1. Previously, we obtained the information that DADEpYDA exhibits the best affinity (K_m = 4 μM) among pY-peptides with various sequences examined (Kwon *et al.*, 1996). In this study, we adopted this sequence except that the phosphotyrosine residue was now replaced by pmF and a Gly residue was added at the N-terminal as a spacer. In this way, we successfully purified overexpressed YPTP1 to ca. 50% purity in a single step. In the same condition, YPTP1 did not bind to the peptide acetyl-AAApmFAAA synthesized on NovaSyn resin, providing evidence for the sequence-specifc binding of YPTP1 to the pmF-peptide.

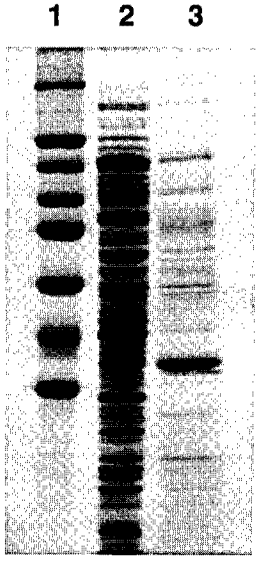

Fig. 2. Purified proteins were analyzed by SDS-PAGE on a 10% gel and visualized with Coomassie brilliant blue. Lane 1, molecular weight standards, from top to bottom: 205, 116, 97, 84, 66, 55, 45, and 36 kDa; lane 2, crude cell lysate; lane 3, affinity column eluate (YPTP1, 39 kDa).

The purification yield of the PTPase, however, was not satisfactory (yield; 12%) and it has yet to be improved. Decreasing the quantity of the crude lysate loaded into the column did not significantly improve the yield. While loading the sample, a significant amount of YPTP1 appeared in the effluent fractions without binding to the column under various conditions tested. The unbound YPTP1, however, was fully recovered in the loading and washing fractions and they could be further purified by application to a freshly equilibrated affinity column.

The method of affinity purification described in this report can be applied to the purification of other PTPases which exhibit preferences toward certain sequences around pY. We and other laboratories have studied the sequence-specific recognition of substrates by PTPases (Cho *et al.*, 1991, 1993; Chatterjee *et al.*, 1992,; Hippen *et al.*, 1993; Ruzzene *et al.*, 1993, Zhang *et al.*, 1994b, 1993, Kwon *et al.*, 1996). In those studies, the amino acid sequences favorably recognized by the enzymes were derived for several PTPases. These sequences can be used for the design of affinity ligands to purify PTPases. Above all, the strategy used in this study might be extended for the purification of the PTPase(s) that recognizes and dephosphorylates a certain peptide sequence of a protein, a possible substrate of the PTPase.

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