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Research Article

Expression of Protein Tyrosine Kinase c-Src by Means of Protein Tyrosine Phosphatase in *Pichia pastoris*

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Abstract

Active non-receptor Src protein tyrosine kinases are usually expressed with very low yield due to protein toxicity associated with its kinase activity. In this study, Src was expressed by means of Protein Tyrosine Phosphatase 1B (PTP1B) to facilitate the production of active Src by neutralizing their apparent toxicity to cells in *Pichia pastoris*. Several recombinant Src-producing strains were constructed, and they displayed the catalytic activity of protein tyrosine phosphatase. The plasmid with c-Src fused with a his-tag and Green Fluorescent Protein (GFP) was transformed into P. pastoris with/without PTP1B, resulting in five recombinants with different expression levels. The c-Src fusion protein was purified by Ni^{2+} affinity chromatography. Among these recombinant strains, the c-Src co-expressed with PTP1B exhibited higher kinase catalytic activity than those expressing the c-Src alone. Consequently, the c-Src fusion protein expressed in *P. pastoris* might be attractive drug discovering targets against cancer.

Keywords: c-Src; Protein Expression; Protein tyrosine kinase; Protein Tyrosine Phosphatase; *Pichia pastoris*

Introduction

The Src family of protein tyrosine kinases (PTK) comprises a transforming protein v-Src and its cellular proto-oncogene homolog c-Src [1]. Src has been extensively studied for its important role in normal cellular signaling and tumorigenesis [2]. Significant progress has been made in resolution of Src structure, identification of physiological substrates, characterization of Src signaling pathways, and elucidation of its roles in tumorigenesis and osteoporosis [3-5]. Considerable evidence has now demonstrated that Src is an important target for drug discovery against cancer and osteoporosis [6]. However, the questions regarding the detail molecular mechanisms of Src catalysis and regulation still remain unknown. In order to answer the questions, the biochemical and structural studies have to be combined during which the recombinant protein preparation and the site-specific mutagenesis are often demanded. Although the recombinant Src has been successfully expressed in several eukaryotic systems [7,8], such expression systems are inefficient and faultily suited for large-scale mutagenesis studies because of low Src yield and/or just domain expression.

Protein tyrosine phosphorylation plays an important role in cell signaling and many physiological activities, which include tissue differentiation and growth, sugar and fat metabolism, and immune response [9,10]. Protein tyrosine phosphatase 1B (PTP1B), which is the first purified mammalian PTP, is selected as a reference prototype for later PTP studies [11]. The precise regulation of protein tyrosine phosphorylation is determined by the concerted activities of PTKs and PTPs [12]. The recombinant protein tyrosine kinase is indispensable as a target protein

and model molecule in drug screening programs and its preparation is very important in the identification of anti-cancer therapeutics. Because the PTKs can phosphorylate intracellular proteins, disturb cellular homeostasis and result in toxicity to the host cells, the expression of recombinant PTK in P. pastoris cells becomes difficult [13]. However, the successful co-expression of PTKs EGFR-2 and PDGFRβ with PTPs has been reported in our previous study [14].

In this work, the expression of c-Src by means of PTPs in *P. pastoris* was conducted to facilitate the expression of active PTKs by neutralizing their apparent toxicity to cells.

Materials and Methods

Strains and culture conditions

The strains and plasmids used in this study are listed in Table 1. The yeast cells were grown in four different media at 30°C as described previously [14]. *Escherichia coli* cells were routinely grown at 37°C in Luria-Bertani medium (LB; Oxoid, Basingstoke, UK). Antibiotics or other reagents were purchased from Sigma (St. Louis, MO, USA) and selectively added (750 μg ml^{-1} hygromycin, 50 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin, or 300 μg ml^{-1} G418).

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Characteristics	Resources
Strains		
Escherichia coli		
Top10F'	General cloning strain	Invitrogen
DH5α	General expression strain	Sangon Biotech (Shanghai)
Pichia pastoris		
GS115	Wild type, *his4*	Invitrogen
GS115::pAG32S-PTP1B-SKL (abbr. 1Y)	GS115 derivative expressing PTP1B and SKL	Lab collected
GS115::pAG32S-PTP1B (abbr. 2Y)	GS115 derivative expressing PTP1B	Lab collected
GS115::pAG32S-BFP-PTP1B-SKL (abbr. 3Y)	GS115 derivative expressing BFP, PTP1B, and SKL	Lab collected
GS115::pAG32S-BFP-PTP1B (abbr. 4Y)	GS115 derivative expressing BFP and PTP1B	Lab collected
1Y::pPIC3.5-GFP-c-Src (abbr. 1CP)	GS115 derivative expressing PTP1B, SKL, and c-Src	This study
2Y::pPIC3.5-GFP-c-Src (abbr. 2CP)	GS115 derivative expressing PTP1B and c-Src	This study
3Y::pPIC3.5-GFP-c-Src (abbr. 3CP)	GS115 derivative expressing BFP, PTP1B, SKL, and c-Src	This study
4Y::pPIC3.5-GFP-c-Src (abbr. 4CP)	GS115 derivative expressing BFP, PTP1B, and c-Src	This study
GS115::pPIC3.5-GFP-c-Src (abbr. C)	GS115 derivative expressing c-Src	This study
Plasmids		
pPIC3.5K	Ampicillin^R G418^R; P_{AOX1}-based expression vector	Invitrogen
pBluescript SK (+)	Ampicillin^R, cloning vector	Tiangen Ltd (Shanghai)
pDRM054	Ampicillin^R bleomycin^R; vector with the P_{AOX1}-BFP-SKL transfusion gene	Prof. Suresh Subramani, UCSD
pCMV6-XL5	Provided the PTP1B template	OriGene Technologies
pAG32S	Ampicillin^R bleomycin^R; pDRM054 derivative with BFP deletion	Lab collected
pPIC3.5-PTP1B	pPIC3.5K derivative expressing the PTP1B fusion gene	Lab collected
pPIC3.5-PTP1B-SKL	pPIC3.5K derivative expressing the PTP1B and SKL fusion genes	Lab collected
pPIC3.5-BFP-PTP1B	pPIC3.5K derivative expressing the BFP and PTP1B fusion genes	Lab collected
pPIC3.5-BFP-PTP1B-SKL	pPIC3.5K derivative expressing the BFP, PTP1B, and SKL fusion genes	Lab collected
pAG32S-PTP1B	pAG32S derivative expressing the PTP1B fusion gene	Lab collected
pAG32S-PTP1B-SKL	pAG32S derivative expressing the PTP1B and SKL fusion genes	Lab collected
pAG32S-BFP-PTP1B	pAG32S derivative expressing the BFP and PTP1B fusion genes	Lab collected
pAG32S-BFP-PTP1B-SKL	pAG32S derivative expressing the BFP, PTP1B, and SKL fusion genes	Lab collected
pBA3CS	Vector expressing the c-Src gene	SIMM*
pPIC3.5K-His-GFP-SpeI-PDGFRβ-SKL-NotI		Lab collected
pPIC3.5-GFP-c-Src	pPic3.5K-Kozak-His_{10}-linker-GFP-Linker-Thrombin site- c-Src	This study

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Plasmid construction

Standard procedures for the manipulation of plasmid DNA were followed [15], and the plasmids and primers used for this study are listed in Table 1 and Table 2, respectively. The plasmids were constructed and transformed into P. pastoris according to our previous method [14].

Table 2. Primers used in this study.

Primer	Sequence (5'-3')[a]
P1	CGGGATCCACCATGGCTATGGAGATGGAAAAGGAGT
P2-1	GGAATTCCTATGTGTTGCTGTTGAACAGGAAC
P2-2	GGAATTCCTACAACTTGGATGTGTTGCTGTTGAACAGG
A1	AAGGCCTAGATCTAACATCCAAAGACGAAAGG
A2	GACGTCGACAAGCTTGCACAAACGAACTTCTC
AG1	CGTACGCTGCAGGTCGACGGATCCC
AG2	AAAAGGCCTAAGCTTCAGCTGGCGGCCG
AGup	ACATGCATGCATGGAGATGGAAAAGGAGTTCGAGC
AGdown	GGGTACCAGCCATGGTGGATCCTTCGAATAATTA
Bup	CGGTACCATGAGCAAGGGCGAGGAACTGAA
Bdown	ACATGCATGCCAGCTCGTCCATGCCATGT
S1	CATTAACTAGT ACGCGTATGGGTAGCAACAAGAGCAAG
S2	CTATAGCGGCCGC CCTAGGCTATTACTAGAGGTTCTCCCCGGGCTGGTAC

a Restriction enzyme sites used for cloning of PCR products were underlined.

Construction of PTP1B expression strains

PTP1B expression strains 1Y, 2Y, 3Y and 4Y were constructed in our previous study [14].

Construction of c-Src expression strains

To construct PTK expression strains, the gene encoding c-Src was amplified from pBA3CS using primers S1/S2. The fragment was linked with *SpeI/NotI*-digested pPIC3.5K-His-GFP-*SpeI*-PDGFRβ-SKL-NotI plasmids to generate pPIC3.5K-Kozak- His_{10}-linker-GFP-Linker-Thrombin site-c-Src (pPIC3.5-GFP-c-Src). This plasmid was confirmed by PCR with primers S1/S2 (Table 2) and DNA sequencing. The positive plasmid pPIC3.5-GFP-c-Src was linearized by either *SalI* or *PmiI*-digestion and electroporated into *P. pastoris* GS115 and 1Y~4Y to produce strains C and 1CP~4CP, respectively. The obtained five strains were listed in Table 1, and all of them were under the control of an *AOX1* promoter. The *His*-deficient plates and YPD-G418 were used to screen the correct *P. pastoris* transformants. In order to further verify the strains, PCR amplification of strains C and 1CP~4CP was performed with primers S1/S2 and a product of 1648 bp was obtained, which was same as the theoretical value of c-Src.

Preparation of c-Src proteins

Strains 1CP, 2CP, 3CP, 4CP, and C were cultured in shaking flasks. The expression level of GFP in *P. pastoris* was determined by using flow cytometry (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA). The cell extract was prepared as described previously [16]. The concentration of protein was measured according to the method of Bradford (Tiangen Ltd., Shanghai, China). The expression level of c-Src was determined by using Western blotting [16].The c-Src protein was purified by his-tag affinity and ion-exchange chromatography, which were performed on the AKTA Explorer 100 purification system (Pharmacia Bioscience). The c-Src kinase activity and inhibition rate, using poly(Glu_4-Tyr) as the substrate, were assayed by ELISA method with a tyrosine kinase assay kit (Millipore, USA).

Results

Co-expression of BFP-PTP1B and GFP fused c-Src in *P. pastoris*

P. pastoris strains bearing the plasmids pPIC3.5-GFP-c-Src, pAG32S-BFP-PTP1B and pAG32S-BFP-PTP1B-SKL could express GFP, BFP and BFP-SKL which specifically targeted the peroxisome of *P. pastoris* [17]. As expected, when BFP with GFP were co-expressed in *P. pastoris* strains 3CP and 4CP, the blue or green fluorescence was observed by fluorescence microscopy (Figure 1C, D). This result was the evidence that PTP1B can be co-expressed with c-Src in *P. pastoris* cells.

As shown in Figure 1D, both the blue fluorescence and the green fluorescence were nearly widespread in the entire cells in strain 4CP. The BFP-PTP1B was expressed in the cytoplasm without the carboxyl-terminal SKL. By contrast, a typical punctate blue fluorescence was observed in strain 3CP, which suggests that the fusion proteins BFP-PTP1B-SKL was sorted into the peroxisome with the SKL signal, whereas the green fluorescence was nearly widespread in the entire cells (Figure 1C). For control strain C containing pPIC3.5-GFP-c-Src (Figure 1E), strain 1CP containing PTP1B-SKL without BFP (Figure 1A) and strain 2CP containing PTP1B without BFP (Figure 1B), only green fluorescence was observed.

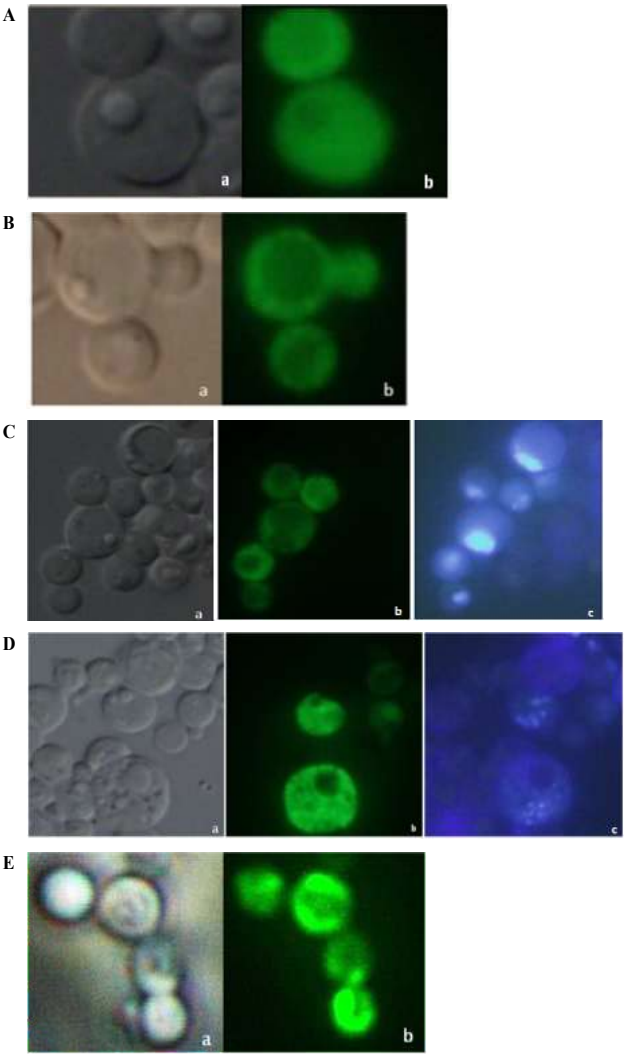

Figure 1. Fluorescence microscopy images of *P. pastoris* cells expressing different proteins. The cultures was induced to express the foriegn proteins with GFP and the cell pellets collected by centrifugation. The resuspended cells in PBS were applied to a slide, and fluorescence scatters of single cell were observed on a laser scanning confocal microscope (LSCM, Leica DMRE) with an excitation wavelength of 488nm. (A) Strain 1CP co-expressing PTP1B-SKL and GFP-c-Src; (B) Strain 2CP co-expressing PTP1B and GFP-c-Src; (C) Strain 3CP co-expressing BFP-PTP1B-SKL and GFP-c-Src; (D) Strain 4CP co-expressing BFP-PTP1B and GFP-c-Src; (E) Strain C expressing GFP-c-Src. (a) differential interference contrast (DIC) image of cells; (b) cells expressing GFP protein; (c) cells expressing BFP-SKL protein in (C) or cells expressing BFP protein in (D) [14].

Expression, purification, and activity analysis of c-Src proteins

Five strains C and 1CP~4CP, were cultured and induced with methanol in shaking flasks and sampled at 24h intervals.

The expression level of c-Src in each strain was determined according to the green fluorescence intensity of the fused GFP protein to select out high yield strains. Under methanol induction, GFP was expressed in strains C and 1CP~4CP, and the maximum was achieved at 24h in strains 3CP and 4CP with a negligible difference (Table 3).

Table 3. Expression and tyrosine kinase activity of c-Src in different *P. pastoris* strains. Cell pellets grown in MGY medium were washed with water and then resuspended in MM medium to an OD600 of 1.0 for methanol induction for 48h in shake flasks.

Strains	Geo-mean GFP fluorescence intensities (GMFI)[a]				Tyrosine kinase activities of c-Src (A_{450})		
	0h	24h	48h	72h	Control	Strain cells lysate	Elute collections of 250 mM imidazole
C	5.2	127	80	45	0.065	0.37	1.35
1CP	5.5	195	158	60	0.074	0.39	2.10
2CP	5.2	188	150	69	0.075	0.37	1.72
3CP	5.4	206	162	59	0.062	0.39	2.27
4CP	4.6	212	155	68	0.077	0.36	1.64

a The Geo-Mean GFP fluorescence intensities were calculated with 104 cells per analysis.

To support the utility of this expression system, we have purified c-Src fusion proteins by using His-tag affinity and ion-exchange chromatography. The c-Src fusion proteins in the cell lysate were determined to be 93 kDa in size by western blotting (Figure 2). The c-Src is a potential target for anti-cancer drug screening. The c-Src kinase activity was measured using an ELISA method (Table 3, Figure 3A). The fraction of c-Src kinase activity was obtained from a His-tag affinity column with a 250 mM imidazole elution. Moreover, the strain 3CP (Table 3) can obtain the highest kinase activity, which was consistent with the green fluorescence intensity in this strain. The BFP-PTP1B-SKL was co-expressed with GFP-c-Src in strain 3CP, and the PTP1B activity was sorted in host peroxisome by targeting signal SKL.

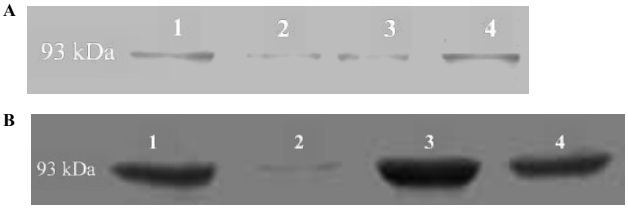

Figure 2. Western blot analysis of cell lysates (A) and their elute collections of Ni^{2+} affinity chromatography (B). Cell pellets grown in MM medium for 24h were disrupted and centrifugated, and the supernatants and their elute collections (electrophoretic purity) of Ni^{2+} affinity chromatography were measured by Western blot analysis using the GFP antibody. Lane 1: 1CP; Lane 2: 2CP; Lane 3: 3CP; Lane 4: 4CP strains expressing c-Src fusion protein in *P. pastoris* [14].

Tyrosine kinase substrate phosphorylation is a complex enzymatic process with double-substrate including poly (Glu4-Tyr) and ATP. However, the molecular model for protein tyrosine phosphatase screening c-Src inhibitors was investigated. The concentration of one substrate would be first determined, and that of another substrate could be then changed to demonstrate its relationship with the phosphorylation reaction. When the ATP was fixed, the substrate phosphorylation significantly increased with ATP concentration (data not shown), if the concentration of ATP was in a range of 1-10 μM. The optimal concentration of poly(Glu_4-phospho-Tyr) and ATP was 10 ng well-1 and 5 μM, respectively. With an increase of c-Src, substrate phosphorylation increased rapidly and reached a plateau when c-Src was 48 ng well-1 (Figure 3). The tyrosine kinase activities in strains 1CP~4CP co-expressing with PTP1B were higher than that in strain C expressing the enzyme alone (Figure 3).

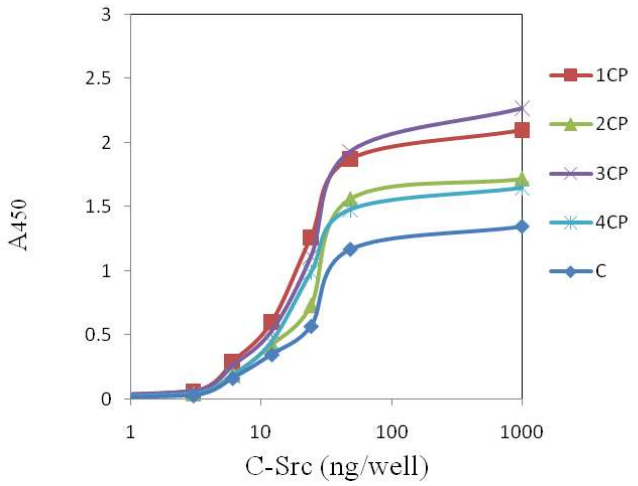

Figure 3. ELISA assay of the phosphorylation activity of c-Src. The relationship between phosphorylation states of substrate and c-Src quantity was illustrated by plot of A450 vs. c-Src quantity. In 96-well plates 10 ng well-1 poly(Glu_4-Tyr) was pre-coated as a substrate. 85 μl of reaction buffer (100 mM Tris-HCl pH 7.4, 50 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mM Na_3VO_4, 0.5 mM DTT, 10 μM ATP) was added to each well. The reactions were performed with the addition of 15 μl purified c-Src fusion protein at varying concentrations [14].

Discussion

c-Src is an important therapeutic target for drug discovery against cancer because of their critical role in osteoporosis. However, the inability to express active c-Src is associated with the c-Src kinase activity, which can phosphorylate intracellular proteins and result in toxicity to host cells [17]. Our previous study demonstrates that the presence of PTP1B can overcome the toxicity of expressing an ac-

tive PTK in *P. pastoris*, and provide practical strategies of recombinantly producing active PTKs in *P. pastoris* [14]. Hence, c-Src was expressed by means of PTP1B in *P. pastoris* to overcome the potential negative effect caused by overexpression of c-Src. The *in vivo* activition of Src kinase is regulated by the phosphorylation status and some factors including protein tyrosine phosphatase 1B [18,19]. Bjorge et al. [20] found that protein-tyrosine phosphatase 1B was capable of dephosphorylating and activating c-Src in several human breast cancer cell lines. However, in our expression system, there was a balance between the dephosphorylation and the c-Src activation by PTP1B, and the dephosphorylation effect of PTP1B might be dominant with respect to the c-Src activation, which caused the balance to tilt to the dephosphorylation. Thus, enhance of c-Src expression could be expected.

P. pastoris usually utilizes the highly efficient methanol-induced *AOX1* promoter (P_{AOX1}) for high-level expression of foreign genes [21]. To decrease the toxicity of PTP1B expression, phosphatase PTP1B with peroxisomal targeting signal 1 (SKL) was expressed and targeted to the peroxisome, which facilitates the separation of toxic metabolic products from the cell components [22]. c-Src was expressed by means of phosphatase PTP1B with/without SKL in *P. pastoris*. The activity of c-Src co-expressed with PTP1B was compared to that without PTP1B in *P. pastoris* after growth in medium containing methanol. The peroxisomal targeting signal 1 (SKL) could influence the expression of c-Src. With SKL co-expression, GFP fused with c-Src was increased (Figure 2B, Lane 1&3). Except BFP, strain 3CP also included the signal peptide SKL, which could specifically target the GFP-c-Src fusion protein to the peroxisomes. Perhaps the location of GFP-c-Src was one of the reasons of higher expression in 3CP. However, enhanced GFP image in 4CP (Figure 2B, Lane 4) could not be reasonably clarified, and might be related with BFP expression in 4CP.

In conclusion, our results showed that the expression of c-Src by means of PTP1B in *P. pastoris* could increase the expression level of c-Src protein. Obviously, for c-Src expression, the kinase activities in strains 1CP~4CP were higher than that in strain C (Figure 3). The GFP fluorescence intensity reflected the expression of c-Src fusion protein. The highest intensities at 24h were achieved by strain 3CP and 4CP (Table 3), which was 62-67% higher than strain C. However, in strain 3CP, the tyrosine kinase activity of purified c-Src product in the elute collections of 250 mM imidazole was the highest among all the recombinant strains.

The kinase activity results indicated that expression of c-Src by means of PTP1B in *P. pastoris* can partly overcome the negative effects associated with overexpression of tyrosine kinases. Meanwhile, the tyrosine phosphatase PTP1B expression sorting at peroxisomes can reinforce the effect. A co-expression strategy has been developed which provides a valuable tool for the production of c-Src. This expression system interferes with the level of protein phosphorylation in host cells, and may prove to be useful for producing other recombinant PTKs as well.

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References

1. Martin GS. The hunting of the Src. Nat Rev Mol Cell Biol. 2001, 2(6): 467-475.

2. Brown MT, Cooper JA. Regulation, substrates and functions of Src. Biochem Biophys Acta. 1996, 1287(2-3): 21-149.

3. Schreiner SJ, Schiavone AP, Smithgall TE. Activation of STAT3 by the Src family kinase Hck requires a functional SH3 domain. J Biol Chem. 2002, 277 (47): 45680-45687.

4. Huang C, Liu J, Haudenschild CC, Zhan X. The role of tyrosine phosphorylation of cortactin in the locomotion of endothelial cells. J Biol Chem. 1998, 273(40): 25770-25776.

5. Ma YC, Huang J, Ali S, Lowry W, Huang XY. Src tyrosine kinase is a novel direct effector of G proteins. Cell. 2000, 102(5): 635-645.

6. Levin VA. Basis and importance of Src as a target in cancer. Cancer Treat Res. 2004, 119: 89-119.

7. Weijland A, Neubauer G, Courtneidge SA, Mann M, Wierenga RK et al. The purification and characterization of the catalytic domain of Src expressed in Schizosaccharomyces pombe comparison of unphosphorylated and tyrosine phosphorylated species. Eur J Biochem. 1996, 240(3): 756-764.

8. Morgan DO, Kaplan JM, Bishop JM, Varmus HE. Production of pp60c-src by baculovirus expression and immunoaffinity purification. Methods Enzymol. 1991, 200: 645-660.

9. Tonks NK. Protein tyrosine phosphatases: from genes, to function, to disease. Nat Rev Mol Cell Biol. 2006, 7: 833-846.

10. Wang WQ, Sun JP, Zhang ZY. An overview of the protein tyrosine phosphatase superfamily. Curr Top Med Chem. 2003, 3(7): 739-748.

11. Lai H, Wang H, Wang J. The function of PTP in plant MAPK path. Chin Bull Bot. 2007, 24: 677-685.

12. Stoker AW. Protein tyrosine phosphatases and signaling. J Endocrinol. 2005, 185(1): 19-33.

13. Wang Y, Xuan YJ, Zhang P, Jiang X, Ni ZH et al. Targeting expression of the catalytic domain of kinase insert domain receptor (KDR) in the peroxisomes of Pichia pastoris. FEMS Yeast Res. 2009, 9(5): 732-741.

14. Tu PN, Wang YM, Cai MH, Zhou XS, Zhang YX. Co-expressions of protein tyrosine kinases EGFR-2 and PDGFRβ with protein tyrosine phosphatase 1B in Pichia pastoris. J Microbiol Biotechnol. 2014, 24(2): 152-159.

15. Frederick M. Short Protocols in Molecular Biology. 3rd Ed., Wiley, New York. 1995.

16. Zhang P, Zhang WW, Zhou XS, Bai P, Cregg JM et al. Catabolite repression of Aox in Pichia pastoris is dependent on hexose transporter PpHxt1 and pexophagy. Appl Environ Microbiol. 2010, 76(18): 6108-6118.

17. Monosov EZ, Wenzel TJ, Lüers GH, Heyman JA, Subramani S. Labeling of peroxisomes with green fluorescent protein in living P. pastoris cells. J Histochem Cytochem. 1996, 44(6): 581-589.

18. Roskoski RJr. Src kinase regulation by phosphorylation and dephosphorylation. Biochem Biophys Res Commun. 2005, 331(1): 1-14.

19. Arias-Romero LE, Saha S, Villamar-Cruz O, Yip SC, Ethier SP et al. Activation of Src by protein tyrosine phosphatase 1B Is required for ErbB2 transformation of human breast epithelial cells. Cancer Res. 2009, 69(11): 4582-4588.

20. Bjorge JD, Pang A, Fujita DJ. Identification of protein-tyrosine phosphatase 1B as the major tyrosine phosphatase activity capable of dephosphorylating and activating c-Src in several human breast cancer cell lines. J Biol Chem. 2000, 275(52): 41439-41446.

21. Cregg JM, Cereghino L, Shi J, Higgins DR. Recombinant protein expression in Pichia pastoris. Mol Biotechnol. 2000, 16(1): 23-52.

22. van der Klei IJ, Yurimoto H, Sakai Y, Veenhuis M. The significance of peroxisomes in methanol metabolism in methylotrophic yeast. Biochim Biophys Acta. 2006, 1763(12): 1453-1462.