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Expression, purification, and bioactivity of GST-fused v-Src from a bacterial expression system*

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Abstract: v-Src is a non-receptor protein tyrosine kinase involved in many signal transduction pathways and closely related to the activation and development of cancers. We present here the expression, purification, and bioactivity of a GST (glutathione *S*-transferase)-fused v-Src from a bacterial expression system. Different culture conditions were examined in an isopropyl *β*-D-thiogalactopyranoside (IPTG)-regulated expression, and the fused protein was purified using GSH (glutathione) affinity chromatography. ELISA (enzyme-linked immunosorbent assay) was employed to determine the phosphorylation kinase activity of the GST-fused v-Src. This strategy seems to be more promising than the insect cell system or other eukaryotic systems employed in earlier Src expression.

Key words: v-Src, GST-fusion, Inclusion body, Orthogonalization, Protein tyrosine kinase
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INTRODUCTION

The v-Src gene was first detected in the Rous sarcoma virus (RSV) in 1970, and encodes an oncogenic protein with 527 amino acids (Martin, 2001). The Src family consists of a group of highly homologous proteins with protein tyrosine kinase (PTK) activity. In mammals, eight members have been identified: Src, Lyn, Fyn, Lck, Hck, Fgr, Blk, and Yes. Most members of this family exhibit a broad spectrum of expression in humans, except Hck, Blk, and Lck, which express in a specific organ or tissue (Superti-Furga and Courtneidge, 1995; Brown and Cooper, 1996; Zoller *et al*., 1997). v-Src has an N-myristoylation motif (M) that is required for its association with the plasma membrane, a unique region (U) that is heterologous in the Src family, two Src homology domains (SH2 and SH3), and an SH1 catalytic domain where the phosphorylation site (Tyr416) and ATP-binding site (Lys295) are located (Margaret *et al*., 2002; Fincham *et al*., 1994). SH2 and SH3 play important roles in protein-protein interaction. The former, consisting of about 100 amino acid residues, can recognize and then combine with a multi-peptide containing a phosphorylated tyrosine, while the latter, consisting of about 50 amino acid residues, binds with a protein substrate via a proline and hydrophobic amino acid residues (Margaret *et al*., 2002). In general, because of the lack of a C-terminal region (C), v-Src has an open and loose configuration, while the C-terminal region serves as a regulatory switch for tyrosine phosphorylation in c-Src.

As a non-receptor protein tyrosine kinase, v-Src can autophosphorylate and phosphorylate the selective substrates via the tyrosine residue; also, it does not undergo down-mediation in general. Because of this phosphorylation process, c-Src serves as an important intermediate in signal transduction pathways in different cell types (Okada, 2000; Korade and Corey, 2000), mediating physiological processes such

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as growth, proliferation, differentiation (Margaret, 2002; Ma and Huang, 2002; Ptasznik, 2000; Ram and Lyengarm, 2001), marcescence (Jones *et al*., 2000; Schlessinger, 2000; Susva *et al*., 2000), migration (Fincham *et al*., 1994; Jones *et al*., 2000), and conglutination (Fincham *et al*., 1994). Additionally, as a strong carcinogenic agent, Src has been implicated in the activation and development of several human tumors, including esophageal cancer, stomach cancer, colon cancer, chronic leukemia, and neuroblastoma (Biscardi *et al*., 2002; Aviziente *et al*., 2002; Frame, 2002; Irby and Yeatman, 2002; Dehm *et al*., 2001). Thus, identifying the mechanisms by which Src interacts with specific substrates, determining the aberrant events involved in its activation, and developing specific inhibitors of its tyrosine kinase activity are active areas of recent research.

To date, several members of the Src family had been cloned, expressed, and purified from different expression systems (Gilmer and Erikson, 1981; Lydon *et al*., 1992; Budde *et al*., 1993; Hideyuki *et al*., 1993; Sun and Budde, 1997; Susa *et al*., 2000). Because of its important role in signal transduction pathways and tumor development, efforts to obtain high levels and enhanced activity of Src are ongoing. Here, we report the construction, expression, and purification of v-Src from a bacterial system, using IPTG regulation. In the induced culture, a combination of chemicals, temperature, and time-shift was employed to enhance the yield of the fusion protein. Following purification using the GSH (glutathione) affinity column, the fusion protein showed high purity via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis. Enzyme-linked immunosorbent assay (ELISA) demonstrated that the fused protein showed relatively high enzymatic activity.

MATERIALS AND METHODS

Plasmid construction

The full-length v-Src coding sequence was PCR amplified from plasmid pFastbac HTb, kindly presented by Dr. Miller of Stony Brook Health Sciences Center, and was cloned into plasmid pGEX-KT as a *Bam*HI/*Eco*RI fragment. The constructed expression plasmid, pGEX-KT-v-Src, was screened in DH5α and transformed into BL21(DE_3)pLysS for expression. The sequence of pGEX-KT-v-Src was confirmed by DNA sequencing.

Expression of the fusion protein

The transformed cells were grown in LB (Luria-Bertani) (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) or YT (Yeast Tryptone) (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) medium, respectively, with ampicillin (50 μg/ml) for 2.5 h (22 °C, 250 r/min). After the optical density (OD_{600}) of the culture approached 0.1, IPTG was added to a final concentration of 0.1 mmol/L for the induced expression, followed by 3 h culturing at 22 °C at a shaking rate of 250 r/min. When the OD_{600} of the culture reached 3.0, the bacterial cells where harvested by centrifugation (4 °C, 7700 g, 10 min).

SDS-PAGE and Western blotting

Samples were taken from the harvested cells and analyzed by SDS-PAGE, followed by staining at 37 °C using Coomassie brilliant blue (CBB) R-250. Western blotting was conducted as described (Sambrook and Russell, 2002). The primary antibodies were mouse monoclonal antibodies against GST. The secondary antibodies were mouse anti-mouse IgG linked with horseradish peroxidase (HRP).

Purification of the fusion proteins

The harvested cells were lysed by sonication (250 W, 12 min) in 10 ml phosphate buffer [20 mg/L lysozyme, 5 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethyl-sulfonyl fluoride (PMSF), pH 7.3]. The buffer was stirred for 30 min after Triton X-100 was added to a final concentration of 0.2%, then was washed with 10 mmol/L phosphate buffer (pH 7.3). One-step affinity purification, using Glutathione Sepharose 4B, was carried out as described (Glutathione Sepharose 4B Instructions). The upper solution was reserved, while the pellets were washed with 10× volumes of 10 mmol/L phosphate buffer (2 mol/L urea, pH 8.0) for 8 h, dissolved in 10 mmol/L phosphate buffer (8 mol/L urea, 0.01 mol/L Tris, pH 8.0), and stored at 4 °C for 2 h. Then it was centrifuged (4 °C, 12000 g, 15 min) for the harvesting of the fusion protein in the inclusion bodies.

Protein concentration assay

The concentration of the holoproteins in the

IPTG-induced culture cells was obtained according to Bradford (1976) method. Bovine serum albumin (BSA) standards were used to measure the standard curve of the protein concentration with the Bradford method. The concentration of v-Src protein in the cell extract was obtained following standard methods in the literature (Tyrosine Kinase Activity Assay Kit Instruction).

In vitro kinase assay

The GST (glutathione *S*-transferase)-Src activity was measured by ELISA for the phosphorylation of v-Src with versatile substrates, poly(Glu:Tyr) 4:1. The phosphorylation reaction was performed at 30 °C for 50 min in the protein kinase assay buffer (5-fold ATP/$MgCl_2$, 20 mmol/L Tris-HCl, 1 mmol/L Na_3VO_4, 2 μg/ml polypeptides, pH 7.4) and stopped by EDTA (120 mmol/L, pH 8.0). After storage at 37 °C for 30 min, the reaction system was washed four times in 250 μl washing buffer, and mixed with 200 μl inhibitor buffer. After another period of storage (37 °C, 30 min), 100 μl HRP-based monoclonal antibodies HRP-PY20, specific against phosphorylated Tyr, were added. After shaking at 25 °C for 1 h, the filtration plate was washed four times in 250 μl washing buffer. After color reaction was with Ponceau S substrate solution, it was stopped with 100 μl terminating buffer, and the OD_{450} of the reaction system was measured.

RESULTS

Cloning of v-Src with plasmid pGEX-KT

Plasmid pGEX-KT, which expresses the target protein as a fusion protein with glutathione *S*-transferase (GST), was designed, and the larger *Bam*HI/*Eco*RI fragment was selected for the construction of the expression plasmid. The full-length sequence of the v-Src gene was PCR amplified and ligated with the former fragment. The result was the expression plasmid pGEX-KT/v-Src.

Because the GST-fused portion binds specifically and strongly to glutathione (GSH), one-step affinity chromatography was employed to obtain the fusion protein. In addition, a thrombin cleavage site was included for releasing of the target protein from the fused protein.

Induced expression of v-Src protein

Compared to the non-induced cells (Fig.1, Lane 1), a new band of 86000 was obtained in induced cells (Fig.1, Lanes 2~4 and 5~8) in SDS-PAGE of coproteins of the host cells. Taking the molecular weight of the GST protein (26000) and that of the target protein (60000) into account, the size of the new band fit with what was expected for the protein.

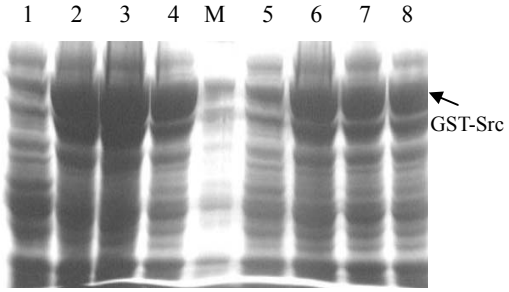

Fig.1 SDS-PAGE analysis for the expression of GST-Src at 30 °C and at 22 °C
Lanes 1~4: the products at 30 °C when the IPTG concentration is 0 (Lane 1); 0.1 mmol/L (Lane 2); 1.0 mmol/L (Lane 3); and 10 mmol/L (Lane 4). Lanes 5~7: the products at 22 °C when the IPTG concentration is 0.1 mmol/L (Lane 5); 1 mmol/L (Lane 6); and 10 mmol/L (Lane 7). Lane M: Molecular protein marker (97400, 66200, 43000, 31000, 20100, and 14400)

In the Western blot (Fig.2), the 86000 band in the induced cells was again present (Lanes 2~4), indicating that this band contained the GST-fused target protein.

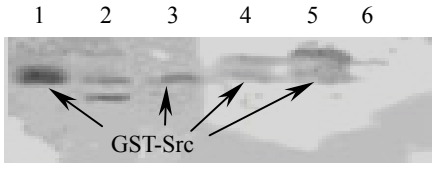

Fig.2 Western blot of pGEX-KT/v-Src
Lane 1: Expression of GST-Src; Lane 2: Molecular marker; Lanes 3~5: Purification[0] of the production of expression of GST-Src; Lane 6: Negative control (without induction)

To maximize the yield of v-Src, we examined the fused product under different culture conditions, including cell lines, concentration of chemicals, culture temperature, and time.

We at first selected DH5α cells for expression because of their high transformation efficiency. But, in view of the virulence of v-Src to the host cells, another *E. coli* cell line, BL21(DE_3)pLysS, was considered and ultimately chosen. Containing the plasmid pLysS which expresses the T7 lysozyme, BL21 cells yield much less host protein. But in comparison to DH5α, BL21 cells show endurance in expressing viral recombinant proteins that are toxic to the host cells. From BL21, we recovered a greater percentage of the IPTG-regulated proteins, which were also more clearly visualized via SDS-PAGE and Western blotting.

The combination of changing concentrations of IPTG, culture temperature, and induction time were analyzed. Results (data not shown) demonstrated that when other conditions remained unchanged, culture temperature most affected the induced protein yield. IPTG concentration affected yield, although not as much, and induction time influenced it the least. (1) A temperature below 30 °C increased yield rapidly. Considering the proliferation of the bacterial cells, 22 °C was chosen; (2) In general, IPTG inhibits the Lac repressor from combining with the Lac promoter, which results in reducing GST-fusion protein yield. But in the induction cells with IPTG, the yield of the fused protein expression decreased after reaching a peak. We concluded that there must be a maximum IPTG concentration for the induced cells, and that if the IPTG concentration is excessive, the cells cannot endure the toxicity and will proliferate slowly, even die. We found that the concentration of 0.1 mmol/L IPTG was optimal for inducing cells; (3) Though the yield increased with induction time, we considered the minor influence of induction time and the exponentially descending curve for the yield and selected a shorter time of 5 h. Thus, these conditions (0.1 mmol/L IPTG, 22 °C, 5 h) were routinely employed.

The protein yield efficiency of YT and LB culture media were compared. When other conditions were unchanged, the yield from the YT culture medium was 3- to 4-fold that from the LB medium. We found that fusion protein from LB was 7%~9% of the coproteins, while data on YT showed 30%.

Purification of the fusion protein

Sonication facilitates extraction of the proteins from the cells and avoids problems associated with freeze-thaw cycles, such as long processing time and the denaturation or inactivation of the extracted proteins. Considering the heat produced by the ultrasonic waves, this process must be performed in ice water (0 °C) at a relatively low power (≤400 W) and with a short processing time (≤10 min) to preserve protein activity. Appropriate amounts of lysozyme (2 mg/ml) and DTT (5 mmol/L) were added to enhance sonication efficiency, and, in the case of DDT, to enhance affinity between GST-Src and the GSH Sepharose 4B gel.

Two strategies for protein extraction from inclusion bodies were compared. In the first one, lysate buffer (0.5% Triton X-100, pH 8.0) was used. But the efficiency of protein purification was much lower than anticipated, though part of miscellaneous proteins were eliminated after the reaction (3 h, 25 °C). Thus, we tried a second strategy. The inclusion bodies were washed in phosphate buffer (2 mol/L urea, pH 8.0) for 8 h (4 °C). Results showed that the purity of the inclusion bodies reached 90% or higher. With the low concentration of denaturing agent, and owing to the low temperature and long reaction time, most of the soluble proteins were almost completely eliminated, ensuring the high purity of inclusion bodies.

A stabilization time of 15 min before elution using GSH washing buffer (pH 7.0) enhanced GSH affinity. It seemed that a lower temperature (4 °C) could reduce the degree of denaturation and inactivation of the fusion protein. After running it through the GSH Sepharose 4B gel, we purified the fusion protein in the upper solution. The purity reached 90% in the collected samples from the 2~4 ml, while miscellaneous proteins were found in the first and fifth milliliters.

Two optical density methods were employed. First, the Bradford method was used to determine the concentration of proteins because of its facility and rapidity (Bradford, 1976). Almost all proteins take up CBB R-250, which has an obvious OD peak at 570 nm. So, by detecting the OD_{570} of every solution, the linkage between the proteins and CBB R-250, which is directly proportional to the concentration of corresponding proteins, could be calculated. Second, in view of the obvious absorption of GST solutions at 280 nm generally, the concentration of GST-Src was measured and calculated as had been previously reported (Sambrook and Russell, 2002).

Finally, we found that by using BL21(DE_3)pLysS bacterial cells, we could obtain 15.5 mg GST-Src protein per liter of YT culture medium. In the whole fusion protein, 13% was found to be soluble protein, with the remaining 87% being inclusion bodies.

In vitro kinase assay

The tyrosine kinase activity of GST-fused Src was determined using ELISA, as described in Fig.3 (Tyrosine Kinase Activity Assay Kit Instruction). The color development reaction, via phosphorylated tyrosine-specific antibodies linked with horseradish peroxidase (HRP-PY20), was applied. When the phosphorylation reaction was complete, the reaction system was transferred to a 96-well multiscreen filtration plate coated with streptavidin (SA), and washed. The nonspecific products were eliminated from the plate, while the phosphorylated poly(Glu:Tyr) remained, adhering to the wells via the stabilized SA.

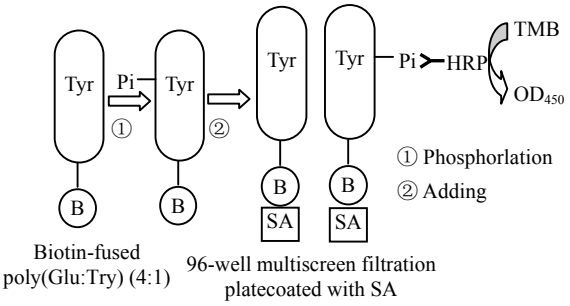

Fig.3 Cartoon demonstration of determining protein kinase activity in ELISA (Tyrosine Kinase Activity Assay Kit Instruction)
TMB: 3,3′5,5′-tetramethyl benzidine

Twenty samples of every 200 μl volume were collected using column affinity (30 °C, pH 7.4). All samples had relatively high tyrosine kinase activity (1.13 μmol/(min·mg) on average), and those of the 16th~20th samples reached the peak.

DISCUSSION

Expression and purification with high efficiency

We show here that a GST-fused Src expressed in a bacterial system has tyrosine kinase activity in vitro. Recombinant Src was abundantly produced and purified efficiently in a simple one-step procedure using a GSH Sepharose 4B gel. The tyrosine kinase activity of purified Src was determined by ELISA with biotin-linked poly(Glu:Tyr) and showed a relatively high value.

Expression of Src family members, full-length or truncated, free or fused, is thought to provide important information on the roles of these proteins in cell physiological biochemistry. v-Src, when compared to the homologous protein c-Src, not only shows an open and loose configuration, but because of the loss of the C-terminal region also shows relatively stable phosphorylation activity. Thus, in vivo or in vitro, v-Src protein plays an important role in carcinogenesis, inducing normal cells to transform into cancer cells. For this reason, we chose v-Src, not c-Src, as a carcinogenic protein source and anticipate application of our results in screening of anti-cancer drugs and of corresponding antibodies.

To identify the molecular mechanisms of protein tyrosine kinases, considerable effort has been devoted to expressing and purifying individual members of this class of molecules in many expression systems. Unfortunately, to date, results have shown a loss or low levels of kinase activity, or the approaches involved highly complex processes. Gilmer and Erikson (1981) obtained a low activity Lac-fused v-Src protein in eukaryotic cells (Gilmer and Erikson, 1981). To improve kinase activity, Lydon *et al.*(1992) and Budde *et al.*(1993) purified soluble pp60c-Src, with activity of 0.125 μmol/(min·mg) and 3.9 μmol/(min·mg), respectively, from the baculovirus system in sf9 insect cells. Research in the area has become more promising as easier and more economical processes are applied. In 1997, Sun and Budde (1997) expressed GST-fused v-Yes in a bacterial system. As reported by Sambrook and Russell (2002), the activity of c-Yes protein from a bacterial system was improved, as was yield efficiency. In our research, though, the value of v-Src was not as high as that of pp60c-Src obtained by Lydon *et al.*(1992). We have established a basis for the potentially high-yielding expression of Src in an easier expression system.

Activity resumption from inactivation

One problem often encountered with bacterial expression systems is that although the induction of IPTG results in high yield, the majority of the re-

combinant proteins aggregate in inclusion bodies, resulting in catalytic inactivation.

IPTG is a strong chemical inducer, promoting the yield of many natural proteins or recombinant proteins, especially protein fused with glutathione *S*-transferase (GST; EC 2.5.1.18). Encoded by the parasitic helminth *Schistosoma japonicum*, GST is a 26000 protein with C terminal of Sj26. GST-fusion proteins being soluble in aqueous solutions and can be easily purified by affinity chromatography on immobilized glutathione. The GST part of fusion protein can be cleaved off by digestion with site-specific proteases, such as thrombin or blood coagulation factor X, followed by abstersion with glutathione-agarose, which can absorb any suitable protein with a GST region via affinity (Smith and Johnson, 1988).

Relatively easy bacterial expression with IPTG induction was used to obtaining an abundant yield of Src. But further efforts must be devoted to enhancing the percent yield of soluble fused protein. So, several strategies were employed to decrease the quantity of aggregated fused protein.

Measurement of Src kinase activity

ELISA is gaining in popularity for use in determining kinase activity, while radioimmunoassay (RIA) had formerly been most widely used. Compared to RIA, in which [γ-^{32}P]ATP or other radioactive isotopes are incubated, ELISA is more convenient and without isotopic contamination. Because it is a rapid, automated, high-throughput approach, we chose ELISA for measuring tyrosine kinase activity of purified v-Src.

We obtained a relatively high activity value for v-Src in vitro. The mechanisms of PTKs in vivo are more complicated, as many cations and/or anions are expected to be involved. Azuma *et al*.(1991) and Susa *et al*.(2000) found that in the presence of Mg^{2+} and Mn^{2+}, the activity of c-Yes increases both in vivo and in vitro. They reported that the maximal activity was higher with Mg^{2+} than with Mn^{2+}, but that for optimum conditions, both of the divalent cations were needed.

Our strategy shows more promise than the insect cell system or other eukaryotic systems because of its convenience, relatively high-level yield, effectiveness, and economy. Further studies will be necessary, including elucidation of more characteristics of the v-Src protein and the development and detection of immunogenic antibodies against v-Src.

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