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Purification of the Rous sarcoma virus *src* kinase by casein-agarose and tyrosine-agarose affinity chromatography

(oncogene product/tyrosine-specific kinase/calmodulin phosphorylation)

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ABSTRACT **A simple and effective purification method for the *src* kinase, the transforming gene product of Rous sarcoma virus, has been developed by using affinity chromatography on casein-agarose and tyrosine-agarose columns. NaDodSO$_4$/polyacrylamide gel electrophoresis and silver staining analysis showed that the purified kinase preparation was composed of a predominant polypeptide of 60,000-Da. In most of the preparations, however, three minor proteins (54,000, 52,000, and 15,000 Da) were also detected, and they were partially characterized. As one of the exogenous substrates, calmodulin was found to be phosphorylated on tyrosine by the purified *src* kinase.**

The translation product of the *src* gene of Rous sarcoma virus (RSV) is a 60,000-Da phosphoprotein, designated pp60^{src} (1–4). Recent studies revealed that pp60^{src} is a protein kinase that specifically phosphorylates tyrosine residues in the target proteins (5–7), and that the tyrosine phosphorylation is closely associated with the malignant transformation of RSV-infected cells (5–9). To perform biochemical studies on the structure and function of the *src* kinase, it is necessary to obtain a sufficient amount of the purified protein. Purification of the *src* kinase has been reported from several laboratories (10–15). Since conventional methods including ion exchange chromatographies (10–14), give poor yields and, sometimes, degraded *src* kinase because of the long purification period, we were looking for an effective and rapid purification method. Erikson *et al.* (11) and Levinson *et al.* (12) used immunoaffinity columns prepared with tumor-bearing rabbit (TBR) antisera, which contain antibodies against the *src* kinase. The immunoaffinity chromatography may be the most specific and rapid isolation method. However, it requires a large amount of TBR antisera for a large scale preparation, and the drastic elution conditions using SCN salts may cause an unpredictable alteration of the isolated *src* kinase. Moreover, recent study (16) showed that many contaminants can be detected by silver staining in the immunoaffinity-purified sample which has been judged to be pure by [^{35}S]methionine or [^{32}P]phosphate labeling (11, 12). On the other hand, Glossmann *et al.* (15) reported a one-step purification procedure using casein-agarose affinity chromatography. This method provided a substantially purified *src* kinase preparation, although the preparation still contained many other proteins. Recently, we purified a specific tyrosine-*O*-phosphate phosphatase by using L-tyrosine-agarose column chromatography (17). The satisfactory result obtained there prompted us to apply tyrosine-affinity chromatography to the purification of the *src* kinase, which is also expected to interact with tyrosine. This method was used for isolation of the enzyme as described in a preceding paper (18). There, it was only briefly described and this paper is a continuation of the purification. Here, we report more extensively a simple and effective procedure in which the tyrosine-affinity column was used in combination with the casein-affinity column. The *src* kinase thus purified has been shown to be nearly homogeneous by NaDodSO$_4$/polyacrylamide gel electrophoresis and silver staining.

MATERIALS AND METHODS

Materials. RSV (Schmidt–Ruppin A strain)-infected chicken embryo fibroblasts were prepared as described (19). TBR sera were generously provided by H. Hanafusa (The Rockefeller University) and K. Owada (Research Institute for Microbial Diseases, Osaka University). Normal rabbit serum was obtained from Calbiochem. Casein-agarose was purchased from United States Biochemical Corporation (Cleveland, OH) and L-tyrosine-agarose (T-0262) was from Sigma. [γ-^{32}P]ATP (5,000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. L-Tyrosine-*O*-phosphate was synthesized and purified as described (17) according to the method of Mitchell and Lunan (20). L-Serine phosphate and L-threonine phosphate were purchased from Sigma. As exogenous substrates for the *src* kinase, rabbit muscle actin (Sigma) and human erythrocyte calmodulin (Calbiochem) were used.

Purification of the *src* Kinase. Frozen chicken embryo fibroblasts (8 g) transformed by RSV were suspended in 15 ml of buffer A [25 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride/10% (vol/vol) glycerol/0.5% Triton X-100] supplemented with 10 μg of Aprotinin per ml (Sigma), a protease inhibitor. Cells were disrupted in a Dounce homogenizer, and the homogenate was clarified by centrifugation at 100,000 × *g* for 30 min. The supernatant fraction (S100 fraction) was diluted with 2 vol of buffer B (buffer A containing 0.1% Triton X-100) and applied to a casein-agarose column (1.5 × 28 cm) that had been equilibrated with buffer B. After washing the column with buffer B, the *src* kinase was eluted with a linear gradient of NaCl (0.0–0.5 M) in buffer B instead of the stepwise elution described in the original paper (15) to minimize the contamination of casein kinase activity, which was eluted at 0.25 M NaCl. Activity of the *src* kinase was found at 0.05–0.2 M NaCl as a broad peak or, sometimes, a bicorn peak. Peak fractions were pooled and adjusted to 0.8 M $(NH_4)_2SO_4$ and loaded onto a tyrosine-agarose column (1.5 × 19 cm) equilibrated with 0.8 M $(NH_4)_2SO_4$ in buffer B. After washing the column, the *src* kinase was eluted by decreasing the concentration of $(NH_4)_2SO_4$ in buffer B (Fig. 1*A*). Peak fractions were pooled and dialyzed against buffer B overnight, and the dialyzed sample was applied to another tyrosine-agarose column (1.5 × 15 cm). This time, the *src* kinase was loaded in the absence of $(NH_4)_2SO_4$ and then eluted by a linear gradient of $(NH_4)_2SO_4$ (0.0–0.5 M) in buffer B (Fig. 1*B*). The final fraction was concentrated by ultra-

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Abbreviations: RSV, Rous sarcoma virus; TBR, tumor-bearing rabbit.

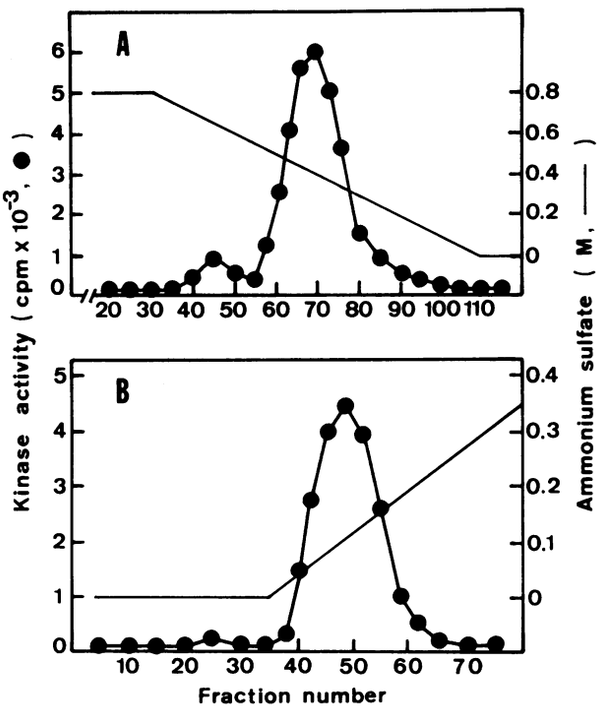

FIG. 1. Tyrosine-affinity column chromatography of *src* kinase. Kinase fraction obtained from the casein-agarose column (fraction 1 in Table 1) was adjusted to 0.8 M $(NH_4)_2SO_4$ and applied to the first tyrosine-agarose column (*A*). Peak fractions (fractions 60–80) were pooled, dialyzed, and applied to the second tyrosine-agarose column (*B*). Elution conditions and assay procedure (0.13 μM [γ-^{32}P]ATP was used) are described in text.

filtration, using a PM10 membrane (Amicon), and dialyzed against stock solution [50 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol/20% (vol/vol) glycerol/0.1% Triton X-100]. The purified *src* kinase was stable for at least 1 yr when stored in liquid nitrogen.

Kinase Assay. Activity of the *src* kinase was assayed according to the method of Collett and Erikson (4). An aliquot (100 μl) of the kinase sample was immunoprecipitated with 5 μl of TBR antiserum diluted 1:10 for 2 hr at 4°C. The immune complex was collected by adsorption onto protein A-containing *Staphylococcus aureus* (Calbiochem) for 20 min at 4°C, washed 3 times with buffer B, and resuspended in 50 μl of the standard kinase assay mixture containing 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/1 mM dithiothreitol/2 μM [γ-^{32}P]ATP (100–500 Ci/mmol). The reaction was started by the addition of ATP and continued for 20 min at 30°C, then it was terminated by adding 400 μl of 10 mM EDTA. The immune complex was washed twice with buffer B, resuspended in $NaDodSO_4$ sample buffer (21), and heated at 100°C for 3 min. The bacteria were pelleted by centrifugation, and the supernatant was subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis according to Laemmli (21). After electrophoresis, gels were briefly stained with 0.25% Coomassie brilliant blue in 35% methanol/10% acetic acid, and destained in the same solution without the dye. Stained bands of immunoglobulin G (IgG) heavy chain were cut out from the wet gel and counted in a liquid scintillation counter. The incorporation was proportional to the amount of the kinase used. In the assay of the crude preparation (S100 fraction), nonspecific incorporation of ^{32}P into serine and threonine residues in normal rabbit IgG was observed. This was subtracted from the ^{32}P incorporation into TBR IgG to estimate the tyrosine-specific phosphorylation. One unit of *src* kinase was defined as the activity that catalyzes transfer of 10 fmol of ^{32}P from [γ-^{32}P]ATP to TBR IgG in 20 min at 30°C.

Table 1. Purification of *src* kinase

Fraction	Protein, mg	Total activity, units	Recovery, %	Specific activity, units/mg
0 Crude supernatant (S100)	601	7200	100	12
1 Casein-agarose chromatography	24.8	6180	85.8	250
2 Tyrosine-agarose chromatography A	0.081	3750	52.1	46,300
3 Tyrosine-agarose chromatography B	0.032	2940	40.8	93,300

One unit of *src* kinase catalyzes transfer of 10 fmol of ^{32}P from [γ-^{32}P]ATP to TBR IgG under standard assay conditions.

Other Methods. Silver staining was performed as described by Oakley *et al.* (22). Phospho amino acid analysis was carried out as described by Hunter and Sefton (5). Protein was measured by the dye-binding method of Bradford (23). On occasion, proteins were concentrated by precipitation with 10% trichloroacetic acid, washed with aqueous acetone, and used for the protein determination.

RESULTS AND DISCUSSION

Purification of the *src* Kinase. The purification procedure is summarized in Table 1. The first step of the procedure must be the fast and effective removal of proteolytic enzymes in the crude supernatant in order to avoid degradation of the *src* kinase (12–14). For this purpose, we used casein-agarose column chromatography, which was originally used

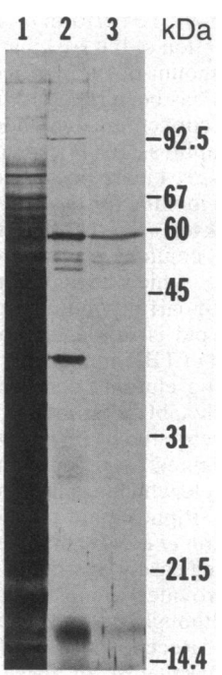

FIG. 2. $NaDodSO_4$ gel electrophoresis of proteins fractionated by affinity chromatography $NaDodSO_4$ gel electrophoresis was carried out as described by Laemmli (21), using a 5.5–15% acrylamide gradient gel. Proteins were visualized by silver staining (22). Samples applied were as follows: lane 1, casein-agarose fraction (fraction 1 in Table 1); lanes 2 and 3, tyrosine-agarose fraction 2 and 3 (see Table 1), respectively. Marker proteins used were as follows: phosphorylase B (92,500 Da), bovine serum albumin (67,000 Da), catalase (60,000 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da), lysozyme (14,400 Da).

for the *src* kinase isolation by Glossmann *et al.* (15). As shown in Table 1, a large amount of proteins were removed from the *src* kinase fraction by this chromatography. It was necessary to hurry through this step for preparation of the intact *src* kinase (60,000 Da). Prolonged handling of the crude supernatant even at 0°C–4°C resulted in the increase of polypeptides at 54,000 and 52,000 Da in the final fraction (see below). Apparent cleavage of the *src* kinase was not observed beyond this step. The successive tyrosine-agarose column chromatographies are shown in Fig. 1. The first tyrosine-agarose chromatography (Fig. 1*A*) was performed at high ionic strength, which facilitates hydrophobic interactions, and the second chromatography (Fig. 1*B*) was carried out at low ionic strength. These procedures are based on the observation that the *src* kinase binds to the tyrosine-agarose at a salt concentration either >0.6 M or <0.05 M in $(NH_4)_2SO_4$. The high salt column was used prior to the low salt column so that the kinase fraction obtained from the casein-agarose column can be applied directly (without dialysis) to the high salt column. The *src* kinase activity was found in the fractions at 0.3–0.5 M $(NH_4)_2SO_4$ in the first tyrosine-agarose column (Fig. 1*A*) and at 0.05–0.15 M $(NH_4)_2SO_4$ in the second tyrosine-agarose column (Fig. 1*B*). Different batches of tyrosine-agarose, however, gave different elution positions, probably because of the difference in tyrosine content per ml of packed agarose beads. Therefore, pilot tests are recommended for each batch of tyrosine-agarose. A small kinase peak (fraction 40–50) in Fig. 1*A* was accompanied by a large amount of Triton X-100 and was not characterized further.

$NaDodSO_4$/polyacrylamide gel electrophoresis of fractions obtained from the chromatography described above is shown in Fig. 2. After three steps of the chromatography, the final *src* kinase preparation was found to be nearly homogeneous. In most of the preparations, however, minor bands at 54,000, 52,000, and 15,000 Da were visible (Fig. 2, lane 3). Contents of these polypeptides varied from preparation to preparation. In some preparations, the amount of the polypeptides at 54,000 and 52,000 Da increased concominantly with the decrease of the main 60,000-Da band, suggesting the degradation of the *src* kinase (12–14).

Protein Phosphorylation by the Purified *src* Kinase. During the purification procedure described above, the *src* kinase fractions were assayed after immunoprecipitation with TBR antiserum. To confirm the kinase activity in a soluble system, various proteins were tested for *in vitro* phosphorylation by the purified *src* kinase, and part of the results are shown in Fig. 3. The purified *src* kinase phosphorylated TBR antiserum IgG (Fig. 3, lane 1) but not normal rabbit serum IgG (Fig. 3, lane 2). As reported by Donner *et al.* (13), actin (42,000 Da) was found to be a good substrate (Fig. 3, lane 3). In addition, we have found that calmodulin, a highly conserved calcium-binding protein found in eukaryotes (24), from various sources is phosphorylated by the *src* kinase. Phosphorylation of human erythrocyte calmodulin (17,000 Da) is shown in Fig. 3 (lane 4). Calmodulin preparations obtained by the method of Kakiuchi *et al.* (25) from bovine brain, chicken brain, chicken erythrocytes, and chicken embryo fibroblasts were also phosphorylated (data not shown). Phospho amino acid analysis (Fig. 4) showed that the phosphorylation of TBR IgG, actin, and calmodulin occurred exclusively on tyrosine residues.

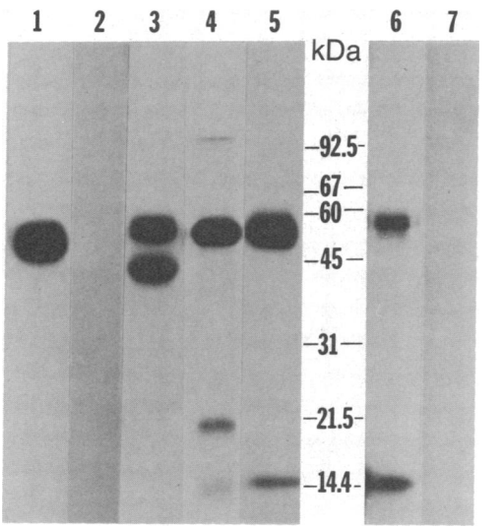

FIG. 3. Protein phosphorylation by purified *src* kinase. Phosphorylation was carried out in the standard kinase assay mixture containing 2 μM [γ-^{32}P]ATP (250 Ci/mmol) and 2 units of purified *src* kinase. Reaction mixture contained 1 μl of TBR antiserum (lane 1), 1 μl of normal rabbit serum (lane 2), 5 μg of rabbit muscle actin (lane 3), 5 μg of EGTA-treated human erythrocyte calmodulin (lane 4), or *src* kinase alone (lane 5). After a 20-min incubation at 30°C, reactions were terminated by addition of $NaDodSO_4$ sample buffer (21) and mixtures were heated at 100°C for 3 min. Phosphorylated proteins were then separated by $NaDodSO_4$/polyacrylamide gel electrophoresis and visualized by autoradiography. In the cases of TBR antiserum and normal rabbit serum, IgG molecules were collected onto protein A-containing bacteria and washed before $NaDodSO_4$ treatment and gel electrophoresis. In lanes 6 and 7, phosphoproteins in the autophosphorylated sample (lane 5) were immunoprecipitated with TBR antiserum (lane 6) or with normal rabbit serum (lane 7) and analyzed similarly. Phosphoproteins of 54,000 and 52,000 Da appeared as a fused thick band. Endogenous phosphorylation of actin or calmodulin alone was not observed.

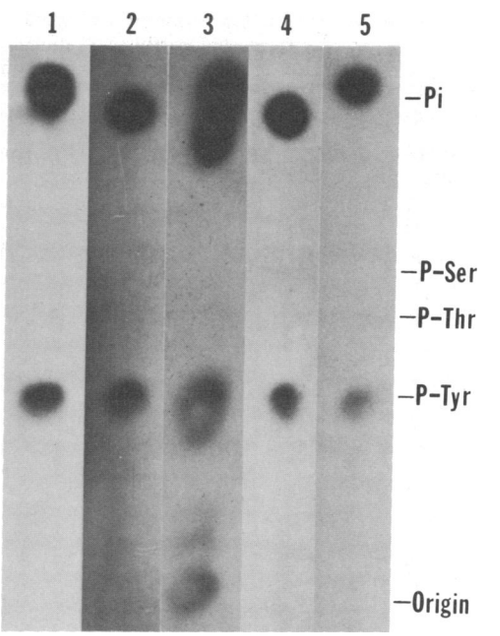

FIG. 4. Phospho amino acid analysis of proteins phosphorylated by purified *src* kinase. Phosphoproteins were separated by $NaDodSO_4$ gel electrophoresis as described in Fig. 3, eluted from the gel, and partially hydrolyzed in 6 M HCl at 110°C for 2 hr. The hydrolysates were lyophilized and subjected to cellulose thin-layer electrophoresis at pH 3.5, as described by Hunter and Sefton (5). Nonradioactive phosphoserine (*P*-Ser), phosphothreonine (*P*-Thr), and phosphotyrosine (*P*-Tyr) were included as internal standards and were located by ninhydrin staining. Phosphoproteins analyzed were as follows: lane 1, phosphorylated TBR IgG; lane 2, phosphorylated actin; lane 3, phosphorylated calmodulin; lane 4, phosphoproteins of 54,000 and 52,000 Da in autophosphorylated *src* kinase preparations; lane 5, phosphorylated protein of 15,000 Da in autophosphorylated *src* kinase preparation.

As shown in Fig. 2, the purified *src* kinase preparation often contained minor proteins of 54,000, 52,000, and 15,000 Da. These proteins were apparently phosphorylated when the kinase preparation was incubated in the presence of [γ-^{32}P]ATP and Mg^{2+} (Fig. 3, lane 5). They were phosphorylated on tyrosine residues (Fig. 4, lanes 4 and 5) and immunoprecipitated by TBR antiserum (Fig. 3, lane 6) but not by normal rabbit serum (lane 7), suggesting that they are associated or related to the *src* kinase. Further characterization of these proteins is under way. It may be noteworthy that the phosphorylation of 15,000-Da protein was preferentially inhibited when actin or calmodulin was used as substrate (Fig. 3, lanes 3 and 4).

We could not detect the autophosphorylation (11, 16) of the 60,000-Da band in the *src* kinase preparation, although *in vivo* ^{32}P-labeled *src* kinase ($pp60^{src}$), which was purified from the extract of ^{32}P-labeled chicken embryo fibroblasts by stepwise elution from casein-agarose and tyrosine-agarose columns, comigrated with the 60,000-Da band on Na-DodSO$_4$ gel electrophoresis (data not shown). The autophosphorylation has been reported by some researchers (11, 13, 16) but was not observed by others (12, 25). The purification method described here may provide a clue to resolve the discrepancy. It may also provide an approach to search for unidentified tyrosine-specific protein kinases, if appropriate substrates for the kinases are available. Since it is easy to scale up the purification system, the method will be especially useful for a large scale preparation of the *src* kinase from materials, such as *Escherichia coli*, expressing a cloned *src* gene (26, 27).

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