Protein tyrosine kinase was purified extensively from a 30,000 × g particulate fraction of bovine spleen by a procedure involving four column chromatographies: DEAE-Sepharose, polyamino acids affinity, hydroxylapatite, and Sephacryl S-200 molecular sieving. The purification resulted in more than 3,000-fold enrichment in [Val⁵]angiotensin II phosphorylation activity (specific activity 202 nmol/min/mg). All column chromatography profiles showed single protein tyrosine kinase activity peaks with the exception of that of affinity chromatography, where about 50% of the enzyme activity appeared with the breakthrough fraction; only the bound enzyme was further purified. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography of a purified sample phosphorylated in the presence of [γ-³²P]ATP revealed the presence of a single phosphorylated polypeptide of molecular weight 50,000 which represents about 40% of total protein. Analysis by polyacrylamide gel electrophoresis under nondenaturing conditions showed that protein tyrosine kinase activity co-migrated with the phosphoprotein. Stoichiometry of the phosphorylation of the 50-kDa polypeptide was found to be 1.0 mol/mol. The purified sample did not appear to contain phoshoctxosine protein phosphatase activity. Both casein and histone could be phosphorylated by the purified sample, and the phosphorylation occurred only at tyrosine residue, suggesting that there was no protein serine and threonine kinase contamination.

There were only few studies on the purification of non-receptor-associated protein tyrosine kinases from normal tissues. Swarup and Subramanya (18) purified the protein tyrosine kinase over 200-fold from rat spleen. A protein tyrosine kinase from rat liver microsome fraction has been extensively purified by Wong and Goldberg (19). Recently, Neer and Lok (20) have substantially purified a protein tyrosine kinase from bovine brain as a by-product during the purification of guanine nucleotide-binding proteins. In all these studies, the purity of the enzyme preparation was not reported, and the specific activity of the enzyme preparation appeared to be low.

In the present study, we describe the extensive purification of a protein tyrosine kinase from bovine spleen. The purified sample contains a single phosphotyrosyl peptide which represents about 40% of the total protein. The enzyme preparation is essentially free of protein tyrosine phosphatase and protein serine/threonine kinases. Some molecular and catalytic properties of the purified protein kinase are described.

EXPERIMENTAL PROCEDURES

RESULTS

Purification of Bovine Spleen Protein Tyrosine Kinase—Figs. 1-5 show elution profiles of the chromatographies: DEAE-Sepharose, poly(α-amino acid) affinity, hydroxylapatite, and Sephacryl S-200 columns for the purification of the protein tyrosine kinase. All profiles exhibited single protein tyrosine kinase activity peaks with the exception of that of affinity column chromatography, which showed two activity peaks; one associated with the breakthrough protein fraction and the other retarded (Fig. 2A). Only the latter peak fraction was further purified. The relative amounts of the two peaks of enzyme activity did not change when the applied sample was reduced by half, thus suggesting that there were two different forms of the enzyme. The suggestion is further supported by the lack of binding of the pooled peak 1 (Fig. 2A) fraction to the affinity column when reapplied (Fig. 2B).

For monitoring protein tyrosine kinase activity during the purification, the reaction mixture consisted of 15 μM ATP and 1 mg/ml [Val⁵]angiotensin II. It was, however, found that higher concentration of ATP (0.3 mM) had to be used to maintain the linear time course of the enzyme reaction. Fig.

* Portions of this paper (including "Experimental Procedures" and Figs. 1-6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-797, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
6 shows that the time courses of the enzyme reaction were linear; over long periods of time (at least 20 min) at various stages of the enzyme purification. In addition, the initial reaction of the purified enzyme was linearly related to the protein concentration (Fig. 6B, inset). The dependence of high concentrations of ATP for linear protein tyrosine kinase reaction at early stages of the enzyme purification appeared to be due to the presence of ATPase in the crude enzyme samples. ATPase activity was detected in the DEAE-Sepharose fraction and the first peak of protein tyrosine kinase activity from polyamino acid affinity column (Fig. 2A). However, the purified protein tyrosine kinase did not contain ATPase activity.

Table I summarizes the results of a purification of protein tyrosine kinase from 3 kg of bovine spleen. Each of the column chromatography steps resulted in a significant enhancement in specific activity of the enzyme. The yield of the enzyme activity was about 16%. Considering that about 50% of total protein tyrosine kinase was associated with a different form of the enzyme, which was not further purified (Fig. 2A), the yield for the purified kinase was about 30%. The specific activity of the enzyme at different stages of purification was determined by using 0.3 mM ATP and 3 mg/ml [Va15]angiotensin II. Under this condition, the specific activity of the purified enzyme was about 202 nmol/mg/min. Two procedures were used for the elution of the affinity column (Figs. 2 and 3). The yields and purifications by using the different procedures were about the same. The pooled fractions under the second activity peak of Fig. 3 had a specific protein tyrosine kinase activity of 10,000 units/mg.

While routine assay reactions contained protein tyrosine phosphatase inhibitors, vanadate and p-nitrophenyl phosphate, the purified sample was found to have the same specific activity in the absence of these inhibitors. The result suggests that the purified enzyme preparation was not contaminated with tyrosine protein phosphatase. To test further for the possible contamination of phosphatase, samples of histone or casein phosphorylated on tyrosine residues by the purified protein tyrosine kinase were subjected to the dephosphorylation conditions (see below) to subject to the dephosphorylation conditions in the presence of 2 mM Mn++, 2 mM Mg++, 2 mM Ca++, 2 mM Ni++, 2 mM Ca++ and Ni++ plus calmodulin (40 ng/ml) or 1 mM EDTA, as well as 6 μg/ml of the protein tyrosine kinase preparation. No dephosphorylation of the phosphoprotein was observed under any of the conditions.

Purity of the Bovine Spleen Protein Tyrosine Kinase—Fig. 7A shows the SDS-PAGE pattern of the protein tyrosine kinase samples obtained after the affinity and Sephacryl S-200 column chromatographies. It can be seen that a polypeptide of molecular weight 50,000 was enriched during the purification. Using a larger amount of the protein (5 μg), the SDS-PAGE gel was analyzed by densitometric tracing (Fig. 7B), and the analysis indicated that the 50-kDa polypeptide represented about 40% of the total protein in the Sephacryl S-200 sample.

When the purified sample was subjected to phosphorylation conditions in the presence of [γ-32P]ATP followed by SDS-PAGE and autoradiography, the 50-kDa polypeptide was found to be the only polypeptide significantly phosphorylated (Fig. 8, lane A). Treatment of the gel with an alkaline solution prior to autoradiography did not result in significant decrease in radioactivity of this protein band, suggesting that the phosphorylation occurred only on tyrosine residue(s) (Fig. 8, lane B). The suggestion was substantiated by the direct analysis of phosphoamino acid of the protein sample (Fig. 8, lane C). In order to test whether or not the 50-kDa polypeptide was a protease derivative, the SDS-PAGE analysis of phosphotyrosyl proteins of a freshly prepared bovine spleen particulate fraction was carried out. Autoradiography of the alkaline-treated SDS-PAGE gel shows that the particulate fraction of bovine spleen contained an alkaline-resistant 50-kDa phosphopeptide (Fig. 9). The result suggests that the 50-kDa phosphotyrosyl peptide is not a protease derivative.

The phosphorylation of 50-kDa peptide was time-dependent and plateaued at about 60 min (Fig. 10). A maximum of 0.40 mol of phosphate per 50,000 g of protein could be determined. Since the 50-kDa polypeptide constituted 40% of the total protein (Fig. 7), the stoichiometry of its phosphorylation could be calculated to be 1.0 mol per mol. Foulkes et al. (27) have recently purified Abelson murine leukemia virus tyrosine protein kinase expressed in Escherichia coli to homogeneity. The purified enzyme could be autophosphorylated to 1 mol of phosphate per mol of protein. They also showed that preincubation of the protein under phosphorylation conditions in the presence of unlabeled ATP did not result in blocking of autophosphorylation by labeled ATP, suggesting that the enzyme may catalyze the transfer of γ-phosphate of ATP to ADP. Similarly, the incorporation of [32P]phosphate into the 50-kDa peptide in the present preparation was not blocked by preincubation of the sample with unlabeled ATP under phosphorylation conditions (Fig. 10). To test further the suggestion that the protein tyrosine kinase catalyzes the transfer of phosphate from ATP to ADP, a sample of the enzyme was phosphorylated to close to maximal level with a low concentration of [γ-32P]ATP (50 μM). High concentrations of unlabeled ATP were then added and the protein-bound radioactive phosphate was continuously monitored. Fig. 11 shows that rapid loss in protein-associated radioactivity occurred following the addition of the unlabeled ATP.

To test whether the 50-kDa polypeptide represents protein tyrosine kinase which underwent autophosphorylation or a protein tyrosine kinase substrate, the purified sample was subjected to nondenaturing PAGE, followed by slicing the gel into 5-mm sections and then analyzed for phosphoryl and kinase activity. Fig. 12 shows that the radioactivity and protein tyrosine kinase activity co-migrated on the gel, sug-

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**Table I**

<table>
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<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Units</th>
<th>Protein (mg)</th>
<th>Specificity (units mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
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<tr>
<td>Extraction</td>
<td>1,045</td>
<td>655,000</td>
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<tr>
<td>2. DBAE-Sepharose</td>
<td>190</td>
<td>507,000</td>
<td></td>
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<td>3. Poly (α-amino acid) affinity</td>
<td>258</td>
<td>240,000</td>
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<td></td>
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<td>4. Hydroxylapastate</td>
<td>45</td>
<td>150,000</td>
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<td>5. Sephacryl S-200 superfine</td>
<td>17.5</td>
<td>101,500</td>
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</tbody>
</table>

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2 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PNPP, p-nitrophenyl phosphate disodium; TCA, trichloroacetic acid; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
purified sample electrophoresed with 5 and 3 μg of total protein, respectively; lane C, poly(α-amino acid) affinity column-purified sample (6 μg of total protein). Lower panel, densitometric tracing of the gel in lane A of upper panel; arrow indicates the major protein band. gesticating that the phosphopeptide was the protein kinase. The stained gel showed few minor bands and a major band which was associated with the radioactivity (results not shown).

To test whether the purified protein tyrosine kinase was contaminated with serine/threonine protein kinases, casein and histones, two common substrates of protein kinases, were phosphorylated by the purified sample, and the phosphorylated protein was analyzed for its phosphoamino acid contents. Fig. 13 shows that both proteins were substrates of the purified kinase. The initial rates of casein (3 mg/ml) and histone (2 mg/ml) phosphorylation were 18 and 15 nmol/min/mg, respectively. Phosphoamino acid analysis of the phosphorylated proteins, along with the control sample (containing the purified kinase only), revealed that only tyrosine residues were phosphorylated (Fig. 13, inset). Thus the enzyme preparation appears essentially free of serine and threonine protein kinases.

**Properties of Bovine Spleen Protein Tyrosine Kinase**—Molecular weight of the purified enzyme under non-denaturing conditions was determined by gel filtration (Sephacryl S-200 superfine). Molecular weight calculated on the basis of this determination was 50,000. The similar molecular weight was obtained by SDS-polyacrylamide gel electrophoresis (Fig. 7). Kinetic characterization using a constant concentration of [Val7]angiotensin II (1 mg/ml) with varying ATP concentrations showed that the Km value of ATP was 166 μM. The purified protein tyrosine kinase can be activated by either Mg2+ or Mn2+. Fig. 14 shows that the maximal enzyme activation by Mn2+ was only 20% that of Mg2+ activation. However, much higher concentration of Mg2+ than Mn2+ was required to achieve the maximal activation. Concentrations of Mg2+ and Mn2+ for 50% maximal activation by respective metal ions were 27 and 2 mM, respectively. In the presence of 5 mM Mn2+, the enzyme activity was further activated by Mg2+. Under this condition, the activation was dependent on even higher concentration of Mg2+, and maximal activation was not achieved at 100 mM of Mg2+ (Fig. 14). Thus the spleen protein tyrosine kinase can use either Mg2+ or Mn2+ as metal ion activator. Whereas Mg2+ is a better activator in achieving higher maximal activation, Mn2+ is better in showing higher affinity toward the enzyme. The observation that higher concentrations of Mg2+ were required to achieve maximal activation in the presence of Mn2+ suggests that the two metal ions compete for a common binding site(s).

**DISCUSSION**

We describe in this paper the purification of bovine spleen protein tyrosine kinase to about 40% purity. The success of this purification depended on three previous observations by others. First, Wong and Goldberg (28) found that angiotensin, a small peptide containing a tyrosine residue with no serine or threonine residue, served as a general protein tyrosine kinase substrate. It is therefore possible to assay specifically protein tyrosine kinase activity in the presence of overwhelming protein serine and threonine kinases during the enzyme purification. Second, Swarup et al. (17) showed that rat spleen contained by far the highest protein tyrosine kinase activity among 8 rat tissues surveyed. Thus, mammalian spleen provides a good source of protein tyrosine kinase. Third, Braun et al. (29) found that co-polymers of various tyrosine-containing polypeptides could serve as substrates of protein tyrosine kinase, and polymer of Glu:Ala:Tyr was shown to have high affinity toward a virus-encoded protein tyrosine kinase. We have used a copolymer-Sepharose 4B conjugate as an affinity
FIG. 9. Identification of alkaline resistant spleen membrane phosphoproteins. Freshly prepared bovine spleen particulate fraction (see "Experimental Procedures") was phosphorylated at 30 °C for 2 min in the presence of 50 mM MgCl₂, 7 mg/ml PNPP, 50 μM Na₃VO₄, 0.1% Nonidet P-40, and 50 μM [γ-³²P]ATP. After stopping the reaction with SDS-PAGE sample buffer, the sample was subjected to SDS-PAGE. Stained SDS-PAGE gel was treated with alkaline solution (see "Experimental Procedures") and autoradiographed (lane A). Lane B, purified spleen protein tyrosine kinase autophosphorylated for 40 min with 50 μM [γ-³²P]ATP and then subjected to SDS-PAGE alkaline treatment and autoradiography.

FIG. 10. Time course of autophosphorylation of protein tyrosine kinase. Purified protein tyrosine kinase (4 μg/300 μl) was subjected to autophosphorylation with 0.3 mM of either [γ-³²P]ATP (●—●) or with 0.3 mM unlabeled ATP for 60 min followed by addition of [γ-³²P]ATP trace (○—○). At individual time intervals aliquots of 30 μl were withdrawn and analyzed by SDS-PAGE and autoradiographed. Radioactive protein band was excised from the gel and counted in scintillation counter. Phosphate incorporation was expressed as mol of phosphate per 50,000 g of the protein.

FIG. 11. Effect of radiodilution of ATP on the ³²P-labeled 50-kDa peptide. Two identical samples of purified protein tyrosine kinases were autophosphorylated at 30 °C for 90 min with 50 μM [γ-³²P]ATP. To one sample, unlabeled ATP was added to a final concentration of 300 μM (○—○) whereas the other sample was used as a control (●—●). Aliquots were removed at the indicated time and then analyzed by SDS-PAGE followed by autoradiography. The radioactive protein band was excised and counted.

chromatography resin for spleen protein tyrosine kinase. This affinity chromatography was found to be effective for the enzyme purification.

The purification procedure described in the present study has been used successfully for large-scale preparations of the enzyme (up to three kg). The procedure may be easily scaled up further. The enzyme appears to be very stable. Samples at various stages of purification had been left in the cold room for 2–3 weeks, with little loss in enzyme activity.

The nature of the two protein tyrosine kinases separated by the poly(α-amino acid) affinity column is not known at present. When a rat spleen particulate fraction was phosphorylated, two polypeptides of molecular weight 53,000 and 56,000 were found to be phosphorylated at tyrosine residues (17). It is well known that mammalian spleen contains high amounts of B and T cells. Recently Earp et al. (15, 16) have suggested that membrane fractions from normal B and T cells have at least two different tyrosine-specific kinases. Therefore, it is possible that the poly(α-amino acid) affinity column can separate these protein tyrosine kinases. Purification of the enzyme of the first activity peak (Fig. 2A) is necessary for substantiating this suggestion.

Homogeneous preparations of receptor kinase (30–32) and of protein tyrosine kinases of viral origins (27) have been obtained. None of the non-receptor-associated protein tyrosine kinases of normal tissues has been extensively purified and characterized. Wong and Goldberg (19) purified a protein tyrosine kinase from the liver microsomal fractions and show that it has a molecular weight of 73,000. Neer and Lok (20) have purified a protein tyrosine kinase from bovine brain as a by-product of their guanine nucleotide-binding proteins. From molecular weight and immunological properties they concluded that the enzyme is cellular c³². In both cases, the purity of the enzyme preparation was not reported. Specific enzyme activities of these preparations are much lower than that obtained for the purified bovine spleen enzyme in this study. However, the comparisons between the spleen enzyme and the brain c³² may not be valid, since different substrates were used in the two studies. The bovine spleen enzyme preparation has been shown to be free of protein serine/threonine kinase and protein tyrosine phosphatase activities, and the enzyme protein can be readily identified on an SDS-PAGE gel. The sample is therefore useful for most enzymatic and some molecular characterizations.

The purified spleen protein tyrosine kinase has a specific activity of about 202 nmol/min/mg toward [Val]angiotensin
II. Considering that the preparation is about 40% pure, a homogeneous preparation of the enzyme is expected to have a specific activity of about 500 nmol/min/mg. This specific activity is similar to that of a homogeneous preparation of a protein tyrosine kinase encoded by the Abelson murine leukemia virus (270 nmol/min/mg) (27). The result suggests that non-receptor-associated cellular protein tyrosine kinase and the viral enzyme may have some intrinsic activities. However, since the specific enzyme activities were determined by using the peptide substrate angiotensin II, the possibility that the protein kinases have different activities toward protein substrates, especially physiological protein substrates, has to be investigated.

The spleen protein tyrosine kinase shares some common properties with the Abelson murine leukemia viral enzyme in addition to the similar specific activities. Both enzymes eluted on gel filtration column in buffers containing 0.2 M NaCl as a single molecular species of $M$, about 50,000. When NaCl was omitted from the buffer, both enzymes showed aggrega-
phorylation was a slow process. However, it is possible that the purified enzyme had already contained phosphate and that the observed incorporation of radioactive phosphate represented the exchange reaction rather than net phosphate incorporation. Further studies are needed to elucidate the function of autophosphorylation.

The discovery of viral transforming gene-associated protein tyrosine kinases has brought about intensive studies of this new class of protein kinases. However, physiological function, regulatory properties, and the mechanism of action of protein tyrosine kinases are still largely unknown. The study of non-receptor-associated cellular protein tyrosine kinase is even more hampered, chiefly due to low abundance of these enzymes in the cells and the lack of suitable affinity chromatography procedures for their purification. The purification of bovine spleen protein tyrosine kinase in reasonable amounts with reasonable purity for enzymological and protein analysis should facilitate the study of this enzyme.

Acknowledgments—We wish to thank Dr. T. Suzuki for helpful discussion and C. Y. Wang for proofreading the manuscript.

REFERENCES


Purification of Protein Tyrosine Kinase from Bovine Spleen by Shinya Ushiro and Jerry H. Wang

Materials:

- DMSO (Dimethyl sulfoxide, 99%) 0.5 M solution
- PMSF (Pepstatin A, 1:1000) 0.5 M solution
- Tris buffered saline (TBS) 0.5 M solution

Methods:

1. Purification:
   - Washed spleen tissue was homogenized in TBS and centrifuged at 100,000 × g for 30 min.
   - The supernatant was adjusted to a protein concentration of 1 mg/ml in TBS.

2. Gel electrophoresis and immunoblotting:
   - Polyvinylidene fluoride membranes were cut into 5-mm strips.
   - Membrane strips were soaked in 0.1% SDS and 2-mercaptoethanol for 30 min.
   - Strips were transferred to a Tris-Glycine buffer containing 30% methanol.
   - Membranes were then washed with 30% methanol and 2-mercaptoethanol.
   - Membranes were incubated with anti-phosphotyrosine antibody overnight.
   - Blots were washed with TBS containing 0.1% Tween-20.

3. Autoradiography:
   - Autoradiograms were exposed to X-ray film for 2 days.

Acknowledgments—We wish to thank Dr. T. Suzuki for helpful discussion and C. Y. Wang for proofreading the manuscript.


Purification of Protein Tyrosine Kinase from Bovine Spleen by Shinya Ushiro and Jerry H. Wang
Figure 1: Chromatography of the spleen protein tyrosine kinase on the superdex 200 column. The elution profile was monitored at 280 nm. Fractions 30-40 were collected. The activities of fractions 15-30 were used for protein tyrosine kinase activity determination. Fractions 11-14 were used for protein tyrosine kinase activity determination. Fractions 15-30 were used for protein tyrosine kinase activity determination. Fractions 11-14 were used for protein tyrosine kinase activity determination.