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# Purification and Properties of Shikimate Kinase II from *Escherichia coli* K-12

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**Shikimate kinase II was purified to near homogeneity from an *Escherichia coli* strain which overproduced the enzyme. The apparent $K_m$ of this isoenzyme for shikimate was 200 μM, and for ATP it was 160 μM. The $K_m$ for shikimate is approximately 100-fold lower than the $K_m$ of shikimate kinase I, suggesting that shikimate kinase II is the isoenzyme normally functioning in aromatic biosynthesis. Shikimate kinase II is dependent on metal ions for activity.**

In an accompanying paper, we reported the cloning in *Escherichia coli* of *aroL*, the gene for shikimate kinase II (2), one of the two isoenzymes which catalyze step 5 in aromatic amino acid biosynthesis (3). In this study, we isolated and purified shikimate kinase II from strain JP1680. This strain contains the *aroL*$^+$ plasmid pMU377 and overproduces the enzyme to levels 50-fold higher than those found in haploid *aroL*$^+$ strains (2). Although JP1680 retains the shikimate kinase I activity, this isoenzyme is present only at the wild-type level and represents less than 0.5% of the total shikimate kinase activity in the strain. The purification is summarized in Table 1.

Strain JP1680 was grown at 37°C in minimal medium (7) supplemented with 0.5% glucose and with thiamine and amino acids as required. The cells were harvested in the exponential phase of growth, suspended in 0.05% M Tris hydrochloride buffer (pH 7.5) containing 10 mM $MgCl_2$, 0.1 M NaCl, and 1 mM dithiothreitol (buffer A), and disrupted by treatment in a French press at 20,000 lb/in$^2$. Crude cell extract was obtained by centrifugation at 25,000 × *g* for 20 min. Nucleic acid was removed from the crude extract by treatment with protamine sulfate as described previously (3). extract from which nucleic acid had been removed was applied to a column (1.6 by 25 cm) of Red A. The column was washed with buffer A until the absorbance of the effluent, monitored continuously at 280 nm, had returned to the base line. Shikimate kinase II activity was then eluted from the column by applying buffer A lacking $MgCl_2$. To stabilize the eluted enzyme, $MgCl_2$ was added to all of the fractions to a final concentration of 10 mM, and those fractions containing significant shikimate kinase activity were pooled and concentrated by ultrafiltration as soon as possible. As shown in the elution profile (Fig. 1), a sharp peak of shikimate kinase activity was released from the column, but very little protein. This step resulted in a 16-fold increase in purity of the enzyme. Other experiments with extracts from the *aroL* strain JP3123 (R. DeFeyter, unpublished results) showed that shikimate kinase I binds to the Red A adsorbent but is not released under the conditions used here, and hence the procedure separated the two isoenzymes.

When analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5), the material eluted from the Red A column was found to contain at least 10 protein species, of

TABLE 1. Summary of the purification of shikimate kinase II from JP1680

| Fraction | Vol (ml) | Amt (mg/ml) of protein[a] | Enzyme activity[b] (mU) | Yield[c] (%) | Sp act (mU/mg) | Purification[c] (fold) |
|---|---|---|---|---|---|---|
| Crude extract | 35.5 | 13.2 | 610 | | 1.38 | 1.0 |
| Protamine sulfate | 34.0 | 6.4 | 637 | 104 | 2.93 | 2.1 |
| Red A | 2.2 | 2.5 | 270 | 44 | 49 | 35 |
| Sephadex G-100 | 1.5 | 0.93 | 140 | 23 | 100 | 73 |

[a] Protein concentrations were measured as described by Lowry et al. (6).
[b] Shikimate kinase assays were performed as described elsewhere (2). Extracts were diluted, when appropriate, in 0.05 M Tris hydrochloride (pH 7.5) containing 0.1 M NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.8% bovine serum albumin. Units of activity are international units (micromoles of product formed per minute).
[c] Yield and purification are relative to crude extract.

At pH 7.5, shikimate kinase II binds tightly to a column of Procion Red HE3B agarose (Amicon Red A) in the presence of 10 mM $MgCl_2$ but does not bind in the absence of this salt. This property was used in the next purification step. Crude which shikimate kinase II appeared to be the smallest. The partially purified material was therefore fractionated by chromatography on a Sephadex G-100 column (2.5 by 80 cm), with buffer A as the eluting buffer at a flow rate of 15 ml/h. A single peak of shikimate kinase activity was eluted; the elution volume corresponded to a molecular weight of 21,400 for the native isoenzyme, which compares well with the previously determined estimate of 20,000 (3). Fractions

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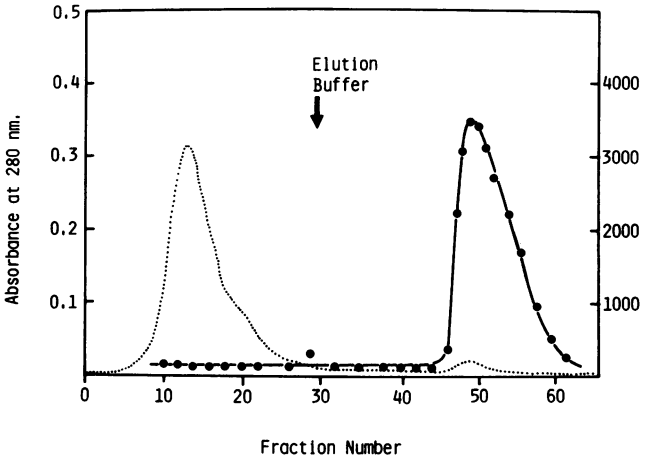


FIG. 1. Procion Red HE3B column chromatography of shikimate kinase II. After the $A_{280}$ of the effluent returned to the base line (arrow), the enzyme was eluted from the column (1.6 by 25 cm) with 0.05 M Tris hydrochloride (pH 7.5) containing 0.1 M NaCl and 1 mM dithiothreitol. Fractions of 4 ml were collected. The shikimate kinase activity (●) and $A_{280}$ (·····) are shown.

containing significant activity were pooled and concentrated. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5), the shikimate kinase II was estimated to be at least 95% pure, yielding an intense band at approximately 17,000 daltons (Fig. 2). Although several faint, contaminating bands were present, further purification steps were not attempted.

Overall, this procedure resulted in a final yield of 23% and a 73-fold increase in purity, suggesting that shikimate kinase II composed between 1 and 5% of the total protein in JP1680 cell extracts. When stored at a concentration of 1 mg of protein per ml in buffer A, the activity decreased by 16% after storage for 1 week at −20°C and by 90% after storage for 2 months.

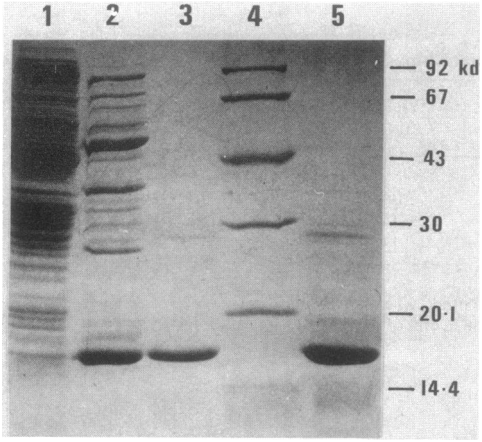


FIG. 2. Analysis of fractions in the purification of shikimate kinase II. Samples taken after the various steps in the purification were electrophoresed on a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate (5) and stained with Coomassie blue. The lanes contain samples after the following: lane 1, protamine sulfate treatment; lane 2, Procion Red column chromatography; lane 3, gel filtration (5 μg of protein); lane 5, gel filtration (15 μg of protein). Lane 4 shows molecular weight standards. kd, Kilodaltons.

TABLE 2. Requirement of shikimate kinase II for cations for activity

| Cation[a] | Concn (mM) | Shikimate kinase activity[b] (%) |
|---|---|---|
| $Mg^{2+}$ | 5 | 100 |
| | 0.5 | 59 |
| | $5 \times 10^{-4}$ | 2 |
| $Mn^{2+}$ | 5 | 28 |
| $Co^{2+}$ | 5 | 19 |
| $Fe^{2+}$ | 5 | 55 |
| $Ca^{2+}$ | 5 | 50 |
| $K^{+}$ | 5 | 1 |

[a] The chloride salts were used, except with $Fe^{2+}$, for which the sulfate salt was used.

[b] Shikimate kinase activity was measured as described elsewhere (2), except that 0.2% bovine serum albumin was added to the reaction mixtures, and is expressed as a percentage of the activity in the presence of 5 mM $MgCl_2$.

Some of the properties of the enzyme were studied. The apparent $K_m$ of shikimate kinase II for shikimate was estimated to be 200 μM at 5 mM ATP, and for ATP it was estimated to be 160 μM at 1 mM shikimate. In assays where the shikimate concentration was increased from 1 to 10 mM, it was observed that the activity of the enzyme decreased by approximately sevenfold, suggesting an inhibition by high levels of the substrate. The basis of this inhibition was not studied further. The enzyme activity was dependent on the presence of a divalent cation as a cofactor. $Mg^{2+}$ was the most effective cation, but significant activity was obtained with all of the other divalent cations tested (Table 2). In this respect, shikimate kinase II activity is similar to the shikimate kinase activity from *Bacillus subtilis* (4).

To test whether the enzyme was inhibited in vitro by any of the aromatic amino acids or vitamins, the activity was measured in the presence of 1 mM tyrosine, phenylalanine, or tryptophan or of 0.1 mM 4-aminobenzoic acid, 4-hydroxybenzoic acid, or 2,3-dihydroxybenzoic acid. However, the enzyme was not markedly inhibited by any of these compounds added singly or in combination, confirming the observations made previously with crude cell extracts (3).

The N-terminal amino acid sequence of shikimate kinase II was determined by G. Tregear at the Howard Florey Institute with an automated gas phase sequencer by the Edman degradative procedure. The eight residues at the N-terminus were found to be $NH_2$-Thr-Gln-Pro-Leu-Phe-Leu-Ile-Gly, a sequence which is in agreement with the prediction from the DNA nucleotide sequence (1) and which indicates that the N-terminal formyl-methionine residue is cleaved from the polypeptide.

In some preliminary studies on shikimate kinase I from *E. coli*, it was noted that the $K_m$ of this isoenzyme for shikimate was greater than 20 mM (unpublished observations), compared with 200 μM for shikimate kinase II. The difference in affinities for the substrate suggests that shikimate kinase II is the isoenzyme that normally functions in aromatic biosynthesis in the cell and that shikimate kinase I functions only when high intracellular levels of shikimate occur, as in strains mutant in *aroL* (2). The very large differences in the $K_m$ values of shikimate II and shikimate kinase I for shikimate, coupled with the failure to isolate mutants lacking shikimate kinase I activity, suggest that this enzyme normally carries out other functions in the cell and that it phosphorylates shikimate only fortuitously.

The polypeptide responsible for shikimate kinase in *B. subtilis* has also been purified (4). In this organism, the

polypeptide is associated with the bifunctional enzyme 3-deoxy-D-arabinoheptulosonate 7-phosphate synthetase–chorismate mutase, forming a trifunctional complex which has shikimate kinase activity and which may represent the key enzyme in the allosteric control of aromatic amino acid synthesis (4). As yet, there is no evidence for any association of shikimate kinase II with other proteins in *E. coli*.

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