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Note

A NEW METHOD FOR THE ISOLATION AND PURIFICATION OF CREATINE KINASE

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Recently, Gurne et al. (1) reported on an elegant method for isolating and purifying porphobilinogen synthase subunits. The reported technique took advantage of the ability of the enzyme subunits to associate and dissociate from each other. The enzyme was immobilized on Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, New Jersey 08854), and dissociated in 4 M urea. The remaining coupled subunits were then used to bind subunits from enzyme in solution. The major advantages of this technique is specificity, which permits the isolation and purification of the enzyme from a mixture of proteins.

We have successfully applied this new technique to the isolation and purification of another type of subunit enzyme, creatine kinase (EC 2.7.3.2) from bovine skeletal muscle.

Purified bovine skeletal muscle creatine kinase (CK) was immobilized directly onto Sepharose 4B using the cyanogen bromide method of March and co-workers (2). Creatine kinase activity was assayed at 30°C by a modification of the procedures of Oliver (3) and Rosalki (4) using Statzyme *n-l* (Worthington Biochemical Corp., Freehold, New Jersey 07728). Protein bound to Sepharose 4B was determined by the method of Kabat and Mayer (5). Solutions containing protein were assayed by the procedure of Lowry et al. (6).

Dissociation of CK subunits from immobilized derivative was accomplished by twice washing the CK-Sepharose 4B with 8 M urea containing 0.1 M 2-mercaptoethanol in 0.02 M Tris-HCl, pH 8.0. The first wash was filtered off and the gel resuspended in a twofold volume of the urea-Tris buffer and again filtered. Finally, the gel was suspended in just enough buffer to fluidize the beads and allowed to incubate for 1 h at room temperature. Crude CK was obtained by homogenizing 10 g of beef

skeletal muscle in 20 ml of 0.02 M Tris-HCl, pH 8.0, containing 1×10^{-3} M EDTA Na_2 and 1×10^{-3} M 2-mercaptoethanol. The disrupted tissue was centrifuged at $15{,}000 \times g$ for 1 h to pelletize solid material. The supernatant was assayed for protein and CK activity. Mercaptoethanol was added to a final concentration of 0.1 M, after which solid urea was added to a final concentration of 8 M. The solution was allowed to incubate for 1 h at room temperature, thus ensuring subunit dissociation.

Purification of muscle-type CK was accomplished by first filtering off the dissociating buffer from the CK-Sepharose 4B on a coarse, sintered glass funnel and washing with 2 volumes of additional dissociating buffer. The crude dissociated CK extract (10 ml) was added to 5 ml of the "stripped" Sepharose 4B, and 45 ml of the homogenization buffer was added to dilute the urea to 2 M. This suspension was stirred occasionally over a 1-h period at room temperature followed by filtration. The gel was then washed with 10 volumes of homogenization buffer and finally with 0.03 M phosphate-buffered saline, pH 7.4. Next, the reassociated gel was washed once with dissociating buffer, filtered, resuspended in a small volume of the same buffer, and incubated for 1 h at room temperature. After incubation, the suspension was filtered and the filtrate diluted to 2 M urea concentration with homogenization buffer. It was then dialyzed against three changes of the buffer for 2 days to remove urea. The dialyzed filtrate was then assayed for protein and CK activity with electrophoresis on a Corning Medical agarose system. Gels were scanned densitometrically and fluorometrically on a Corning Medical Model 720 densitometer/fluorometer.

Results from three experiments are shown in Table 1. As can be seen, we were able to isolate only 6–15% of the total crude enzyme reacted with the CK-Sepharose 4B. In the first two experiments (Table 1), 90–100% of the total units offered were recovered. In experiment 3, purified CK-MM was used, and inactivation may have occurred due to lack of excess protein. This effect has been demonstrated by Dawson et al. (7). Muscle-type CK is

TABLE 1. **Enzyme Activity and Recovery**

Experiment	Units applied	Units purified	Units recovered	% purified	% recovered	Specific activity of purified enzyme (IU/mg)
1	6,445	710	5,735	11	89	122
2	13,734	1,994	13,764	15	100	283
3[a]	2,000	120	673	6	34	

[a] Used purified CKMM (specific activity 382 IU/mg).

TABLE 2. **Protein Loading on CK-Sepharose 4B**

Experiment	mg protein bound/ ml Sepharose 4B[a]	mg protein reacted/ml gel	Coupling efficiency (%)
1	3.8	5.0	75
2 and 3	9.1	10.0	91

[a] Protein was determined by 6 N HCl hydrolysis followed by ninhydrin determination of the amino acid content.

a relatively stable enzyme in the presence of other proteins, and can be protected by albumin (4). By electrophoresis on agarose gels, one protein band was observed in the γ-globulin region when stained in Amido Black 10B. This corresponded to a single fluorescent band from CK activity as detected by overlaying the gel with concentrated Statzyme according to Corning Medical procedure.

The amount of enzyme bound to the Sepharose 4B in each experiment is shown in Table 2. In each case, 75–91% of the protein reacted was bound to the CNBr-activated gel. Enzyme on the gel was demonstrated by suspending some gel beads in CPK-Statzyme assay solution and monitoring the change in absorbance at 340 nm. After stripping with 8 M urea, enzyme activity on the gel was reduced but not completely removed, indicating that not all the bound CK was stripped of subunits. Completely dissociated CK is not enzymatically active. Apparently, the enzyme activity observed was due to the high degree of CNBr coupling. Lower degrees of CNBr activation and greater dissociation of subunits should increase observed recovery of CK enzyme from crude extracts. However, we have shown that this technique can be used to isolate and purify CK enzyme.

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