IMMOBILIZATION OF PHOSPHOTRANSFERASES OBTAINED FROM RESISTANT BACTERIA

Sir:

If an enzyme is successfully immobilized, then the enzyme reaction can be carried out by passing substrates through a column of the immobilized enzyme and the reaction products are easily purified. Moreover, there is a possibility that the immobilization may give a stable preparation of the enzyme. Thus, we attempted immobilization of kanamycin phosphotransferases I, II (neomycin-kanamycin phosphotransferases I, II) and streptomycin phosphotransferase, and confirmed the advantages described above. Moreover, two or more than two enzymes which were present in a 100,000 *g* centrifugation supernatant of disrupted cells of resistant bacteria were successfully immobilized into an insoluble form. Using a column thus prepared, the presence of various enzymes in the supernatant can easily be identified.

The resistant strains, *Escherichia coli* K12 J5 R11-2, *Escherichia coli* JR66/W677 and *Pseudomonas aeruginosa* TI-13, were cultured in a medium which contained 1 % peptone, 0.5 % meat extract and 0.3 % sodium chloride (pH 7.4) at 37°C for 6 hours. The cells were harvested by centrifugation, washed with 20 mM phosphate buffer (pH 7.2, buffer A) which contained 10 mM magnesium acetate, 60 mM potassium chloride and 10 mM 1,4-dithiothreitol, and suspended in the same buffer. The cell suspension was passed through a French pressure cell (1,200 kg/cm^2), and the disrupted cell suspension was ultracentrifuged at 100,000 *g* for 90 minutes. The supernatant thus obtained was designated S-100. To S-100, ammonium sulfate was added to 75 % saturation, and the precipitated protein was dissolved in buffer A and dialyzed against the same buffer.

Enzymes in the dialyzed solution were coupled with cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) or cyanogen bromide-activated cellulose[1,2] under gentle stirring at 4°C for 16 hours, and the solids which bound with enzymes were washed with 1.0 M monoethanolamine, 0.1 M acetate buffer (pH 4.0) in 1.0 M sodium chloride and 0.1 M borate buffer (pH 8.0) in 1.0 M sodium chloride, successively.

From S-100 (1 ml, 16 mg protein) of *Escherichia coli* K12 J5 R11-2 which contained kanamycin phosphotransferase I[3] and 125 mg (dry) of cyanogen bromide-activated Sepharose 4B, 1,300 mg (wet) of immobilized kanamycin phosphotransferase I was obtained. The enzymatic activity was determined by the following procedure: The reaction mixture (1 ml) which contained 0.05 μmole of an aminoglycosidic antibiotic, 4 μmoles of disodium adenosine triphosphate, 10 μmoles of magnesium acetate, 60 μmoles of potassium chloride, 10 μmoles of 1,4-dithiothreitol and the immobilized enzyme in 100 mM potassium phosphate buffer (pH 7.8) was incubated at 37°C under shaking for 15, 30, 60 or 120 minutes. After heating the reaction mixture at 80°C for 5 minutes, the residual antibiotic activity was determined by the disc-plate method using *Bacillus subtilis* PCI 219 as the test organism. The enzymatic activity was as follows: 22 mg (wet) of the immobilized enzyme phosphorylated 0.05 μmole of lividomycin A in 15 minutes (9.09 μmoles/wet g/hr) and 0.046 μmole of kanamycin in 30 minutes (4.18 μmoles/wet g/hr).

From S-100 (1 ml, 23 mg protein) of *Pseudomonas aeruginosa* TI-13[4] which contained kanamycin phoshotransferase (I type) and streptomycin phosphotransferase, and 125 mg (dry) of cyanogen bromide-activated Sepharose 4B, 1,500 mg (wet) of the immobilized material which had both enzymatic activities was obtained. The activities were as follows: 22 mg (wet) of the preparation phosphorylated 0.033 μmole of lividomycin A (3.00 μmoles/wet g/hr) and 0.019 μmole of kanamycin in 30 minutes (1.73 μmoles/wet g/hr), and 78 mg of the preparation phosphorylated 0.009 μmole of streptomycin in 60 minutes (0.12 μmole/wet g/hr).

From S-100 (1 ml, 70 mg protein) of *Escherichia coli* JR66/W677 which contained kanamycin phosphotransferase II[5] and streptomycin phosphotransferase[6], and 125 mg (dry) of cyanogen bromide-activated Sepharose 4B, 1,870 mg (wet) of the immobilized material which had both enzymatic activities was obtained. The activities were was follows: 3 mg (wet) of the preparation phosphorylated

0.05 μmole of butirosin A (33.33 μmoles/wet g/hr) and 0.03 μmole of kanamycin in 30 minutes (20.00 μmoles/wet g/hr), and 14 mg of the preparation phosphorylated 0.023 μmole of streptomycin in 60 minutes (1.64 μmoles/wet g/hr).

From S-100 (10 ml, 700 mg protein) of *Escherichia coli* JR66/W677 and 10 g (dry) of cellulose (Whatman CF-11) activated by cyanogen bromide, 23.3 g (wet) of the immobilized material which had activities of kanamycin phosphotransferase II and streptomycin phosphostransferase was obtained. The enzymatic activities were as follows: 30 mg (wet) of the preparation phosphorylated 0.036 μmole of butirosin A (1.20 μmoles/wet g/hr) and 0.0275 μmole of kanamycin in 60 minutes (0.92 μmole/wet g/hr), and 134 mg of the preparation phosphorylated 0.0265 μmole of streptomycin in 120 minutes (0.10 μmole/wet g/hr).

Next, we attempted to couple the purified phosphotransferases with cyanogen bromide-activated Sepharose 4B. Kanamycin phosphotransferase II and streptomycin phosphotransferase in S-100 of *Escherichia coli* JR66/W677 were purified by affinity chromatography on 3′, 4′-dideoxykanamycin B-Sepharose 4B with a gradient of sodium chloride from 0 to 0.8 M[6]. Streptomycin phosphotransferase was eluted with 0.18 M and kanamycin phosphotransferase II with 0.58 M. These phosphotransferases in the respective fractions were precipitated by saturation with ammonium sulfate, and the precipitated phosphotransferases were dissolved in buffer A and dialyzed against the same buffer. The purified kanamycin phosphotransferase II (40 ml, 160 mg protein) was coupled with 750 mg (dry) of cyanogen bromide-activated Sepharose 4B by the procedure described above, and 15.9 g (wet) of the purified kanamycin phosphotransferase II-Sepharose 4B was obtained. The enzymatic activity was as follows: 2.5 mg (wet) of the immobilized enzyme phosphorylated 0.0275 μmole of kanamycin in 30 minutes (22.00 μmoles/wet g/hr). As already reported[5], kanamycin phosphotransferase II phosphorylates kanamycins, ribostamycin and butirosin A but does not phosphorylate lividomycin A. The immobilized enzyme had the same substrate specificity.

The purified streptomycin phosphotransferase (1 ml, 24 mg protein) was coupled with 125 mg (dry) of cyanogen bromide-activated Sepharose 4B, and 1,500 mg (wet) of the purified streptomycin phosphotransferase-Sepharose 4B was obtained. The enzymatic activity was as follows: 3.5 mg (wet) of the preparation phosphorylated 0.025 μmole of streptomycin in 60 minutes (7.14 μmoles/wet g/hr).

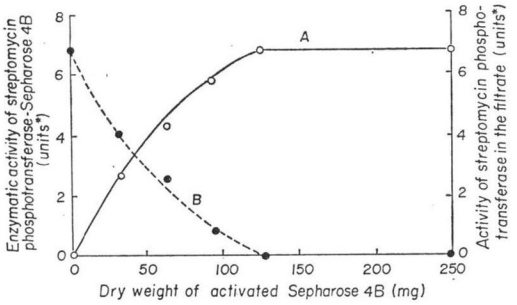
Continuous phosphorylation of kanamycin B and dihydrostreptomycin were carried out on a column of the immobilized phosphotransferases. Purified kanamycin phosphotransferase II-Sepharose 4B (1 g, wet) described above was packed into a column (0.6×4.4 cm) and a solution (250 ml) which contained 121 mg (250 μmoles) of kanamycin B, 756 mg (1 mmole) of disodium adenosine triphosphate trihydrate, 2.5 mmoles of magnesium acetate and 15 mmoles of potassium chloride in 20 mM phosphate buffer (pH 7.8) was passed through the column at the rate of 4 ml/hr at 37°C. During the passage kanamycin B was completely phosphorylated. The phosphorylated product (115 mg, mp 251~258°C, dec.) was isolated by application of column chromatography on Amberlite CG-50 resin (NH_4^+ form) and elution with 0.1 N ammonia. It was identified to be kanamycin B 3′-phosphate by highvoltage paper electrophoresis, IR and PMR studies. Dihydrostreptomycin (116 mg, 180 μmoles) was phosphorylated with disodium adenosine triphosphate trihydrate (544 mg, 720 μmoles) by passage through a column (0.6×4.4 cm) of the purified streptomycin phosphotransferase-Sepharose 4B (1.2 g, wet), and 86 mg of phosphorylated product was obtained as colorless crystals. The product was identified to be dihydrostreptomycin 3″-phosphate[7].

Capacity of cyanogen bromide-activated Sepharose 4B to couple with the purified streptomycin phosphotransferase was examined. As shown in Fig. 1, 6.75 units* of streptomycin phosphotransferase (24 mg protein) saturated the coupling capacity of 125 mg (dry) of cyanogen bromide-activated Sepharose 4B.

Enzymatic activity of these phosphotransferases was maintained stably in the immobi-

* One unit: The enzymatic activity which phosphorylates 1 μmole of an antibiotic in 60 minutes.

Fig. 1. Binding capacity of cyanogen bromide-activated Sepharose 4B with purified streptomycin phosphotransferase.



To a suspension of cyanogen bromide-activated Sepharose 4B (31, 63, 94, 125 and 250 mg, dry) in 2 ml of 0.1 M $NaHCO_3$-0.5 M NaCl, 1 ml of the purified streptomycin phosphotransferase (24 mg protein, 6.75 units*) was added under gentle stirring at 4°C for 16 hours. After filtration, the solid was treated by the method described in the preparation method of immobilized enzymes (see text), and the enzymatic activity of the solid was determined (A). The enzyme activity which remained after the reaction with cyanogen bromide-activated Sepharose 4B was determined (B) by measuring the activity in the filtrate.

lized state. Immobilized streptomycin phosphotransferase was more resistant to heating than the soluble streptomycin phosphotransferase; the purified streptomycin phosphotransferase-Sepharose 4B preserved 72 % of the activity when it was kept at 45°C for 12 minutes, while the purified enzyme solution lost its activity completely.

Moreover, the immobilized enzymes could be used repeatedly. The column of the purified kanamycin phosphotransferase II-Sepharose 4B used in the above-mentioned experiment was successfully reused to transphosphorylate kanamycin B from disodium adenosine triphosphate by the same condition described above, after washing the column with 20 ml of 20 mM phosphate buffer (pH 7.2). Further repeated use of the same column was confirmed.

As described above, immobilization of enzymes which are involved in mechanism of resistance was shown to have many advantages.

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