PURIFICATION OF AMINOGLYCOSIDE 3′-PHOSPHOTRANSFERASE II

Sir:

In 1967, UMEZAWA *et al.*[1] found a kanamycin-neomycin phosphotransferase in *Escherichia coli* carrying R factor and proved that the resistance is directly related to the formation of this enzyme.[2] In 1972, YAGISAWA *et al.*[3] found another phosphotransferase in *E. coli* carrying a different R factor. These enzymes were different in their substrate specificities. The former which is now called aminoglycoside phosphotransferase I (APH(3′)-I) transfers phosphate from ATP to the 3′-hydroxyl group (the 3-hydroxyl group of the 6-amino-6-deoxy-D-glucose moiety) of kanamycin and also to the 5″-hydroxyl group (the 5-hydroxyl group of the D-ribose moiety) in lividomycin A but not to the 3′-hydroxyl group of butirosin A.[4] The latter which is called aminoglycoside phosphotransferase II transfers phosphate to the 3′-hydroxyl group of kanamycin and butirosin A but not to the 5″-hydroxyl group of lividomycin A. Similar enzymes which are found in *Pseudomonas aeruginosa* have been purified by affinity chromatography[5] and the presence of three types of APH(3′)-I was confirmed by the finding of differences in chromatographic behavior, molecular weight, and optimal pH and *Km* values. However, APH(3′)-II obtained from pseudomonas had the same characteristics as that obtained from *E. coli* carrying R factor (*E. coli* JR66/W677) and was found to be much more stable than APH(3′)-I. Therefore, in order to study the chemical and biological properties of the enzymes in more detail, we attempted to prepare pure APH(3″)-II. In this paper, we report the complete purification of APH(3′)-II and its properties.

E. coli JR66/W677 was grown at 37°C for 6 hours in a 570-liter fermentor containing 300 liters of a nutrient medium (1.0 % peptone, 0.5 % meat extract, 0.3 % sodium chloride, pH 7.4). The cells were collected by centrifugation and washed with deionized water. The wet packed cells (970 g) were frozen and stored at −20°C. All purification procedures were carried out at 4°C. The enzymatic activity was determined by the method described in a previous paper[5] determining the residual antibiotic and 1 unit (U) was defined as the amount of enzyme which phosphorylated 1 μmole of butirosin A per hour. Protein content was estimated by the method of LOWRY *et al.*[6]

The frozen cells (370 g) were suspended in 400 ml of buffer A which was 60 mM potassium chloride and 10 mM magnesium acetate in 20 mM sodium phosphate buffer (pH 7.0) and disrupted by passing through a French pressure cell (1,200 kg/cm²). The disrupted-cell suspension was centrifuged at 10,000 *g* for 60 minutes. The precipitate suspended with buffer A (300 ml) was again centrifuged. The crude extract (800 ml) was chromatographed on a column (2.3×48.0 cm) of DEAE-Sephadex A-50 equilibrated with buffer A. After the column was washed with 1,500 ml of buffer A, the enzyme was eluted with buffer A containing sodium chloride in a linear gradient from 0 to 0.5 M. The combined active fractions (2,100 ml) were dialyzed for 18 hours against 40 liters of buffer B which contained 60 mM potassium chloride and 10 mM magnesium acetate in 5 mM sodium phosphate buffer (pH 7.0). The insoluble material in the inner solution was removed by centrifugation at 10,000 *g* for 15 minutes and the supernatant was chromatographed on a column (3.0×41.0 cm) of hydroxylapatite which was equilibrated with buffer B. The elution was carried out successively with 5, 10, 20, 50 and 100 mM sodium phosphate buffer (pH 7.0) containing 60 mM potassium chloride and 10 mM magnesium acetate. The enzyme was eluted with 50 mM buffer. The combined active fractions (850 ml) were diluted to 2,100 ml with a solution containing 60 mM potassium chloride and 10 mM magnesium acetate and subjected to affinity chromatography using a kanamycin-Sepharose 4B[5] column (3.0×34.0 cm) equilibrated with buffer A. After the column was washed with buffer A (1,500 ml), the enzyme was eluted by a linear gradient made from 1,500 ml of buffer A and 1,500 ml of buffer A containing 1 M sodium chloride as shown in Fig. 1. The active fractions were combined (900 ml) and concentrated by Diaflo ultrafiltration (UM-10, Amicon Corporation). After the insoluble material in the concentrated solution was removed by centrifugation at 10,000 *g* for 15 minutes, the enzyme solution

Fig. 1. Affinity chromatography of APH(3′)-II (Kanamycin-Sepharose 4B column)

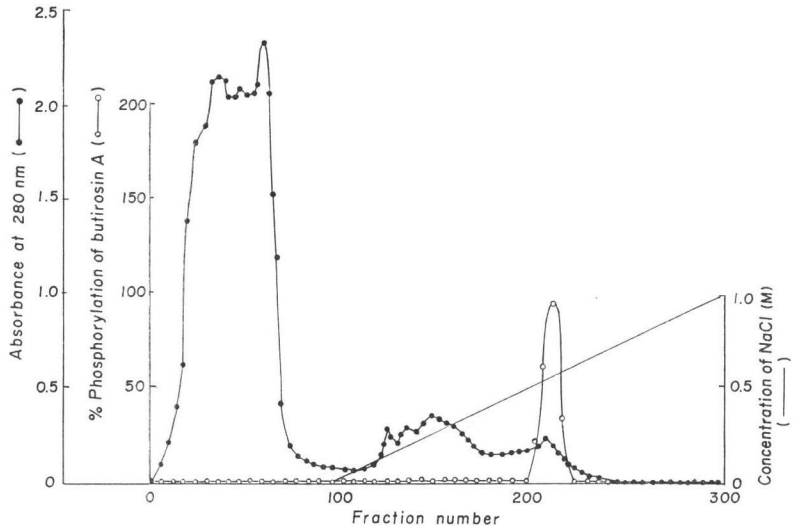
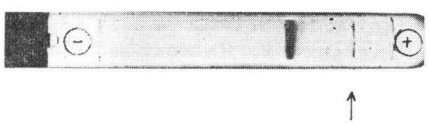


Table 1. Purification of APH(3′)-II

Purification step	Volume (ml)	Protein content (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	800	19,680	2.0	100
DEAE-Sephadex A-50 column	2,100	6,200	4.6	72.5
Dialysis and hydroxylapatite column	850	1,774	9.1	41.0
Kanamycin-Sepharose 4B column	900	79	85.2	17.1
Diaflo and Sephadex G-100 column	30	26	114.7	7.6

Fig. 2. Polyacrylamide gel electrophoresis of APH(3′)-II

The purified enzyme (10 μg as protein) was applied to a 7.5% polyacrylamide gel (pH 8.9). The arrow indicated the position of the reference dye (bromophenol blue). Enzyme was stained with Coomassie brilliant blue.



(20 ml) was applied to a column (2.8×70.0 cm) of Sephadex G-100 equilibrated with buffer A. On this column, trace contaminants which appeared in earlier fractions were separated from the enzyme and the enzyme activity appeared in a single peak as shown by UV absorption at 280 nm. The enzyme solution (30 ml) thus obtained showed a high specific activity (114.7 U/mg protein). The purified enzyme solution in buffer A was stored at

Fig. 3. Estimation of molecular weight of APH (3′)-II by electrophoresis

The purified enzyme (10 μg) was applied to polyacrylamide gel containing 0.1% SDS and 8 M urea. The arrow indicated the mobility of the APH(3′)-II. Reference proteins used were: bovine serum albumin (BSA), 67,000; ovalbumin (Oval), 45,000; α-chymotrypsinogen A (α-Chy), 25,000; myoglobin (Myo), 18,000; and cytochrome C (Cyt C), 12,500.

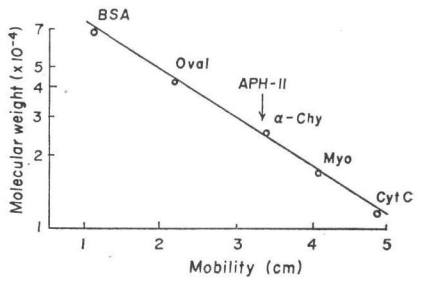


Table 2. Amino acid composition of APH(3′)-II

Amino acid	Mole per cent	Amino acid	Mole per cent
Lysine	1.8	Alanine	14.7
Histidine	2.8	Cystine	0.0
Arginine	7.5	Valine	3.6
Aspartic acid	11.4	Methionine	2.3
Threonine	3.6	Isoleucine	4.0
Serine	3.5	Leucine	13.0
Glutamic acid	12.0	Tyrosine	1.9
Proline	5.2	Phenylalanine	4.4
Glycine	8.3		

The enzyme (2.0 mg) which was obtained from the purified enzyme solution in buffer A by dialysis against water for 24 hours was hydrolyzed in 6 N HCl at 105°C for 30 hours in a sealed tube. Amino acid analysis was performed on the amino acid analyzer model JLC-5AH (Japan Electron Optics Laboratory Co., Ltd.).

−20°C without any loss of activity for a month. Purity of the enzyme in each step is shown in Table 1. The overall purification was 56.5-fold with a yield of 7.6%.

The purified enzyme showed a single band on electrophoresis in 7.5% polyacrylamide gel at pH 8.9 as shown in Fig. 2. The gel was cut into 2 mm slices and extracted with buffer A. The enzymatic activity was found only in the stained band. The purified enzyme also gave a single band on polyacrylamide gel electrophoresis at pH 7.0 and 8.9, even when the gel concentration was varied from 10 to 15%. The purified enzyme was homogeneous as judged by these experiments and by Sephadex G-100 gel filtration. When the enzyme was subjected to electrophoresis on polyacrylamide gel containing 0.1% sodium dodecyl sulfate and 8 M urea,[7] it showed a single entity, with a molecular weight of 25,000 by comparison with reference proteins as shown in Fig. 3. The value thus obtained was close to that 27,000 obtained by Sephadex G-100 gel filtration. The amino acid composition is shown in Table 2.

The homogeneity of the purified enzyme was also confirmed by an immunological method. Rabbit antiserum to the purified enzyme was prepared by the method of HARTMAN and UDENFRIEND[8] and when tested by agar diffusion method,[9] it produced a single continuous precipitin line against the purified enzyme and also against the 105,000 *g* supernatant of *E. coli* JR66/W677. In another test, the enzymatic activity was decreased by addition of the antiserum. The immunological studies will be described in a later paper.

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