## An Improved Procedure for Purification of Alkaline Phosphatase from Rat Kidney

For some time, the work in our laboratory has been concerned with studies of rat kidney alkaline phosphatase[1-3]. Although several procedures for the purification of alkaline phosphatase from different sources have been described[4-13], the enzyme has never been purified from rat kidney. On the other hand, the application to the kidney of the methods employed for the purification of the alkaline phosphatase from other sources did not yield good results. The paper will report the methods that have been developed for purifying alkaline phosphatase from rat kidney.

*Materials and methods.* The animals used were young male rats of the Wistar strain, weighing 100–150 g. Quantitative alkaline phosphatase analysis was performed at 25 °C by a continuous optical method described by MELANI and GUERRITORE[14]. The reaction mixture was as follows: 0.5 ml of 200 m*M* glycine buffer (pH 10.4), 0.1 ml of 1.5 m*M* $MgSO_4$, 0.1 ml of 150 m*M* Na-*p*-nitrophenylphosphate, 0.02 or more enzyme solution and water to 1 ml.

The rate of release of *p*-nitrophenol from Na-*p*-nitrophenylphosphate was determined following the increase in absorbance at 405 nm using an Eppendorf recording photometer, operating with selected spectral lines from a mercury lamp.

One enzyme unit was defined as the amount which hydrolyzes 1 µmole of substrate/min under the conditions used. Starch gel electrophoresis was performed in a horizontal apparatus according to SMITHIES[15] at 10 °C for 16 h using 0.3*M* *Tris*/HCl buffer (pH 8.8) in the electrode compartments; the potential difference between the ends of the gel was 150 V. After the electrophoresis was completed, the gel was sliced horizontally. For visualization of phosphatase activity, one portion of the gel was placed in 100 ml of freshly mixed solution containing 100 mg sodium α-naphthylphosphate, 100 mg Fast Bleu RR, 1 ml of 10 m*M* $MgCl_2$ and 50 ml of 0.2*M* *Tris*/HCl buffer (pH 8.8). The other portion was stained with 1% (w/v) Nigrosin WS dissolved in methanol-acetic acid-water (9:2:9, by vol.) for 1 h. After washing with the same solvent mixture, protein components were seen as blue-black zone.

Protein contents were determined by the biuret method according to BEISENHERTZ et al.[16], except that for the individual column fractions, protein content was estimated from the optical density at 280 nm as suggested by WARBURG and CHRISTIAN[17].

*Results.* A typical preparation of alkaline phosphatase is described below and the results are summarized in the Table. The entire procedure was performed at room temperature. The rats were killed by decapitation. The kidneys were removed as rapidly as possible and homogenized in a Potter apparatus with about 5 volumes of 0.01*M* *Tris* buffer (pH 7.8) containing 0.25*M* sucrose and 0.15 m*M* $ZnSO_4$. Mitochondria, nuclei and cell debris were removed from homogenate by centrifuging for 15 min at 10,000 *g*.

The supernatant was centrifuged 60 min more to separate the microsomes where the enzyme is located. To bring the microsomal proteins into solution, the microsomes were suspended by mechanical stirring in a freshly made ice cold 1% solution of sodium deoxycholate in 0.05*M* *Tris* buffer at pH 7.8. The volume of sodium deoxycholate added was such as to contain about 0.2 mg of deoxycholate/estimated mg of microsomal protein. At this stage purified pancreatic lipase (3 mg/ml of microsomal suspension) was added. After 6 h of incubation at 25 °C with occasional stirring, the mixture was centrifuged at 100,000 *g* for 60 min and the clear supernatant was found to contain over 60% of the original activity of microsomal suspension. This supernatant was dialyzed against 0.05*M* *Tris* buffer (pH 8.1) for 12 h at 5 °C. Aliquots of the dialyzate, corresponding to 30 mg of microsomal protein, were passed through a DEAE-Sephadex A25 column (20 × 2 cm) previously equilibrated with 0.05*M* *Tris* buffer, pH 8.1. The column was eluted with a linear NaCl gradient formed from 100 ml of the buffer used for the equilibration and 100 ml of the same buffer containing 1*M* NaCl. The eluate was collected in fractions of 1.8 ml and each was tested for optical density at 280 nm and for alkaline phosphatase activity. A typical elution profile is given in Figure 1 in which it is clear that all the alkaline phosphatase activity is recoverable in the few initial fractions at sodium chloride concentration ranging from 0.03–0.15*M*. The fractions rich in enzyme were pooled, concentrated, dialyzed against the starting buffer and then rechromatographed in the same way as before. The elution profile is given in Figure 2. There is only a single peak of 280 nm absorbing material which corresponds to

Preparation of alkaline phosphatase from rat kidney

| Fraction | Total units | Specific activity (u/mg protein) | Yield % | Purification (fold) |
|---|---|---|---|---|
| Crude extract | 150 | 0.05 | 100 | |
| Microsomal suspension in deoxycholate | 96 | 0.6 | 64 | 12 |
| Supernatant after lipase digestion | 64 | 1.6 | 43 | 32 |
| First fractionation on DEAE-Sephadex | 36 | 15 | 24 | 300 |
| Second fractionation on DEAE-Sephadex | 34 | 15.8 | 23 | 316 |


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the peak of the alkaline phosphatase without any further important increase of specific activity.

The absorption spectrum of this material is given in Figure 3. The absence of any peak at 260 nm indicates a lack of significant contamination of nucleic acids.

Starch gel electrophoresis showed that the area which stained for protein also gave a positive phosphatase reaction.

*Discussion.* The only purification step which requires further mention is the achievement of the soluble enzyme from microsomes where it is located, closely associated with insoluble material[18].

The methods used vary from simple water extraction[19] to autolysis[20] frequently accompanied by a simultaneous or separate treatment with proteolytic agents[21] and to butanol extraction[22,23]. Unfortunately, the application of these methods to the kidney did not yield good results. In the former experiments[24], a significant solubilization has been achieved through the simultaneous treatment of the microsomal suspension with deoxycholate and trypsin. Instead, we have at present introduced the use of lipase to avoid possible alteration of the structure of the enzyme during tryptic digestion; the employment of lipase ensures a very efficient extraction also. The procedure is rapid, easy, reproducible and can be carried out with small quantities of material[25].

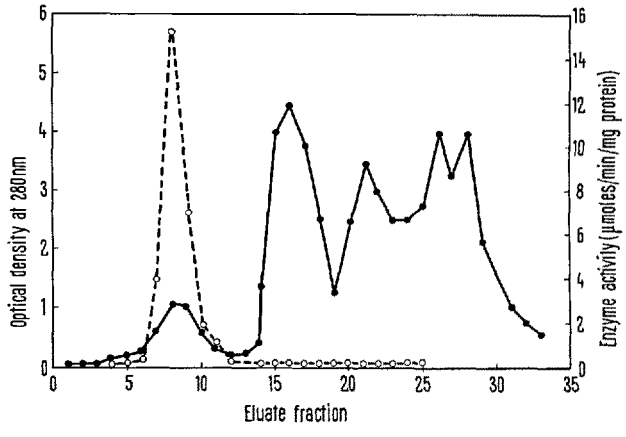


Fig. 1. Fractionation on DEAE-Sephadex A-25 of alkaline phosphatase from a microsomal extract containing 30 mg of proteins. The extract was prepared from kidney microsomes by the procedure described in the text and absorbed to a column of DEAE-Sephadex (20 × 2 cm). Elution was achieved with a linear gradient formed from 100 ml of 0.05 *M* *Tris*/HCl buffer (pH 8.1) in the mixing chamber and the same buffer + 1 *M* NaCl in the reservoir flask. The eluate fractions (1.8 ml) were assayed for O.D. at 280 nm (solid line curve) and for alkaline phosphatase activity (broken line curve).

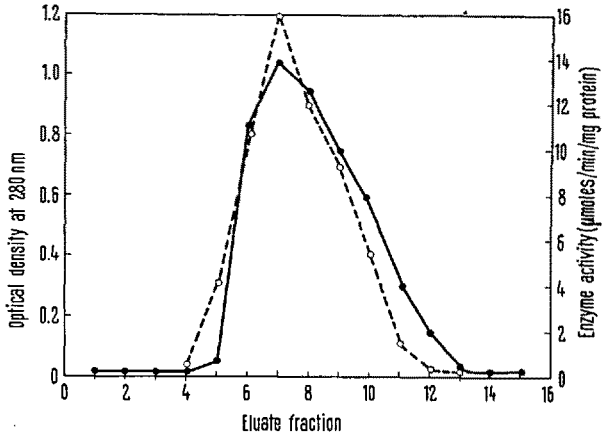


Fig. 2. Refractionation on DEAE-Sephadex A-25 of the alkaline phosphatase obtained from the enzyme peak in Figure 1. Same column and system as in Figure 1.

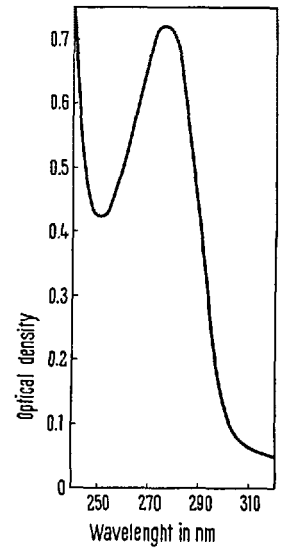


Fig. 3. Absorption spectrum of purified alkaline phosphatase protein.

*Riassunto.* Viene descitto un nuovo metodo per la purificazione della fosfatasi alcalina da rene di ratto. La metodica comprende 4 tappe: (1) isolamento della frazione microsomiale; (2) solubilizzazione dell'enzima dalle strutture microsomiali per mezzo del desossicolato e della lipasi; (3) frazionamento cromatografico su DEAE-Sephadex A-25 ed eluzione con gradiente di concentrazione; (4) ricromatografia delle frazioni attive sullo stesso scambiatore anionico. All'elettroforesi su gel d'amido l'enzima ottenuto si comporta come una proteina omogenea.

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