Inhibitors for the free enzyme showed no effect on the entrapped enzyme.

INTRODUCTION

Liposomes have been used as a carrier for enzyme replacement therapy (1-11) due to its weakly antigenic properties and protective effect against enzymes to induce immune response.

Hypophosphatasia is a hereditary bone disease which is characterized by deficiency or abnormality of serum and bone alkaline phosphatase. This disease results in severe bone defects due to lack of calcification of bone matrix and of cartilage (12).

Treatments have been carried out by oral intake of high dosage of phosphate (12) and by enzyme replacement therapy of alkaline phosphatase rich plasma (13). At present, there is no effective treatment for this disease.

The calcification in epiphyseal cartilage and embryonic bone may be carried out by the hydrolysis of pyrophosphate by the alkaline phosphatase forming inorganic phosphate. This enzymatic hydrolysis is taken place in the extracellular membrane-bound matrix vesicles. The inorganic phosphate therefore deposites in these vesicles promoting bone mineralization (14). Recent study has shown that the matrix vesicles are essential for extracellular mineralization (15). In the

hypophosphatasia patients, it is suggested that the vesicles do not form (12).

It seems likely that enzyme replacement therapy using alkaline phosphatase-containing liposomes can be effective for this disease by supplying alkaline phosphatase as well as vesicles. In the present study, we report the properties of human placental alkaline phosphatase entrapped in multilamellar liposomes. The information obtained from this study will be useful in the future for the treatment of hypophosphatasia.

MATERIALS AND METHODS

Human placental alkaline phosphatase, p-nitrophenyl phosphate, L-phenylalanine, L-cysteine, L-tryptophan, ethylenediaminetetraacetic acid (EDTA) and 2-mercapto-ethanol were purchased from Sigma Co., (U.S.A.). Phosphatidylcholine was purified from egg yolk by a two-step column technique i.e. an alumina column and silicic acid column as described previously (16). General chemicals were of analytical grade.

Preparation of Human Placental Alkaline Phosphatase-Containing Liposomes

Phosphatidylcholine was dissolved in chloroform in a 500 ml round-bottom flask. The solution was dried in a rotary evaporator under reduced pressure at 37°C to form a thin film on the flask. Human placental alkaline phosphatase dissolved in buffer solution (10 μg/ml) was added to give a lipid concentration of 10 mg/ml.

Multilamellar liposomes were formed by constant vortexing for 5 minutes on a vortex mixer (Thermolyne, Syborn, U.S.A.) and sonicating for 30 seconds in a bath sonicator (Bransonic 220, Smith kline Co., U.S.A.). The liposome dispersion was hydrated at 37°C for 2 hours. The preparation was centrifuged at 140,000 g for 30 minutes. The precipitate was washed with buffer solution for 3 times.

Assay of Alkaline Phosphatase

The activity of alkaline phosphatase was measured by the hydrolysis of p-nitrophenol from p-nitrophenyl phosphate in carbonate buffer at pH 10.5 and 37°C for 10 minutes. The absorbance of the yellow product of p-nitrophenol was measured at 405 nm. 1 μmole of p-nitrophenol released by the enzyme per minute was defined as 1 unit.

Assay of the Entrapped Alkaline Phosphatase

Triton-X100 solution was used to lysis the liposomes. Equal volumes of enzyme-containing liposome dispersion and 3% triton-X100 solution were mixed well to obtain a clear solution for enzyme activity assay. No interference of triton-X100 was found with the assay.

Leakage of Alkaline Phosphatase from Liposomes

Alkaline Phosphatase-containing liposomes were suspended in the buffer solution and stored at 25°C.

The dispersions were centrifuged at 140,000 g for 1 hour at different time intervals. Precipitate was checked for the alkaline phosphatase activity remained in the liposomes.

Temperature Effect

To examine the optimum temperature for alkaline phosphatase activity, the entrapped and free enzyme were reacted in different temperature from 25 to 65°C. Due to the presence of triton-X100 (cloud point=64°C), turbid solution was produced after heating. 6.7% sodium dodecyl sulfate solution was used to lysis the liposomes. The activity of the enzyme was not interfered by the sodium dodecyl sulfate in the assay.

Thermal Stability

To determine the thermal stability, the entrapped and free enzyme were incubated in a water bath at the desired temperature from 20 to 75°C for 10 minutes. The treatment was terminated by immersing in an ice bath. The activity of the enzyme was examined at 37°C.

pH Effect

The reactions were carried out in the buffers of different pH for the entrapped and free enzyme at 37°C. The buffer systems used were sodium acetate buffer (pH 5.20), Tris-HCl buffers (pH 7.40 and 8.40) and carbonate buffers (pH 9.70, 10.70 and 11.25).

Reaction Kinetics

Purified human placental alkaline phosphatase (17) was used. Kinetic parameters for the entrapped enzyme, free enzyme and enzyme with empty liposomes were determined according to the Lineweaver-Burk equation

$$\frac{1}{V} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[S]}$$

where V is the initial reaction rate, [S] is the substrate concentration, V_m is the maximum reaction rate and K_m is the Michaelis constant.

Inhibition Effect

The inhibitors for alkaline phosphatase used were amino acids (L-phenylalanine, L-cysteine and L-tryptophan) and chelators (EDTA and 2-mercaptoethanol). The activity of the entrapped enzyme, free enzyme and enzyme with empty liposomes was measured after treated with the inhibitors at 37°C for 10 minutes.

RESULTS AND DISCUSSION

Leakage of Alkaline Phosphatase from Liposomes

Leakage of alkaline phosphatase from liposomes is shown in Figure 1 plotted as the concentration of the enzyme remained in the liposomes against time after preparation. The curve shows an abrupt decrease in the concentration of the enzyme in the liposomes in 1 hour after preparation and then the concentration remains constant after 7 days observation. The concentration

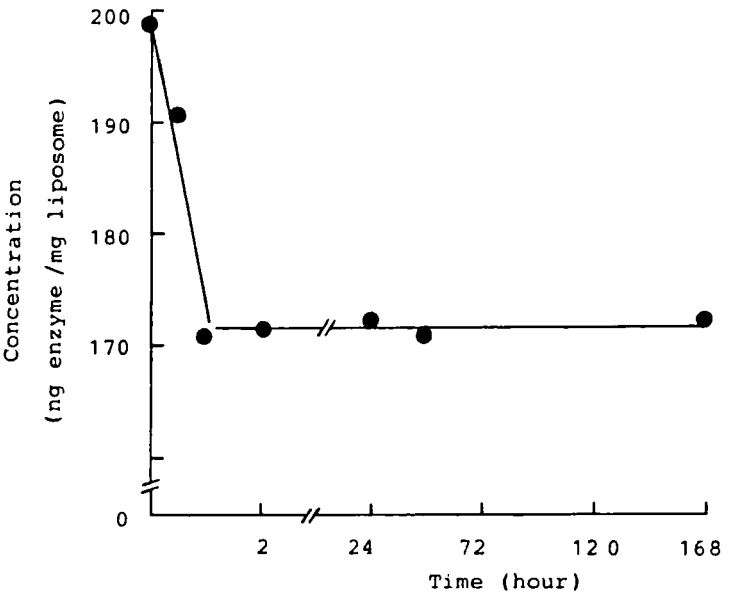

FIGURE 1. Leakage of human placental alkaline phosphatase from liposomes.

of the enzyme entrapped in the liposomes after leakage is about 80% of the initial entrapped concentration.

Small molecular weight substances entrapped in liposomes leak easily through diffusion (19). Human placental alkaline phosphatase is a macromolecule which molecular weight is about 70,000 - 200,000 (18). The diffusion of a large molecule such as human placental alkaline phosphatase is a considerable slow process. Also, it is difficult for this large size enzyme to pass through the multilamellar membrane of the liposomes. Therefore, the entrapped alkaline phosphatase would have little leakage from liposomes.

However, as indicated in Figure 1, the entrapped enzyme leaks easily from liposomes in the first hour after preparation. To explain this leakage behavior, one may take into account at the desorption of alkaline phosphatase from the surface of the liposomes. Human placental alkaline phosphatase is composed of hydrophilic and apolar amino acids in a ratio of 1.9 (20,21). The apolar amino acids of the enzyme molecules may be adsorbed on the surface of the liposomes by hydrophobic interaction (16,22) during the entrapping process. Therefore, upon standing for the leakage test, the adsorbed enzyme may be desorbed from the surface of the liposomes. Until equilibrium is attained, no more enzyme is desorbed from the surface showing a plateau value which represents the concentration of the entrapped enzyme in the liposomes.

Temperature Effect

The curves for the effect of reaction temperature for the entrapped and free alkaline phosphatase are shown in Figure 2. The optimum reaction temperature for the entrapped and free enzyme shows a similar peak at 46°C.

Thermal Stability

The results of thermal stability for the entrapped and free alkaline phosphatase are shown in Figure 3. The activity for the entrapped and free enzyme is

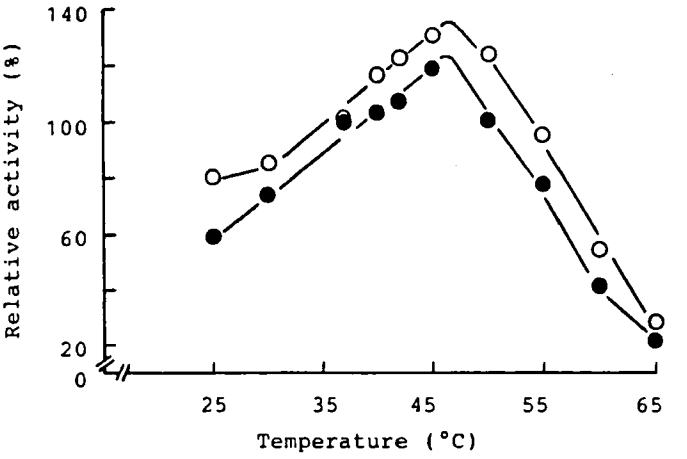

FIGURE 2. Optimum reaction temperature for human placental alkaline phosphatase. ○, enzyme entrapped in liposomes; ●, free enzyme.

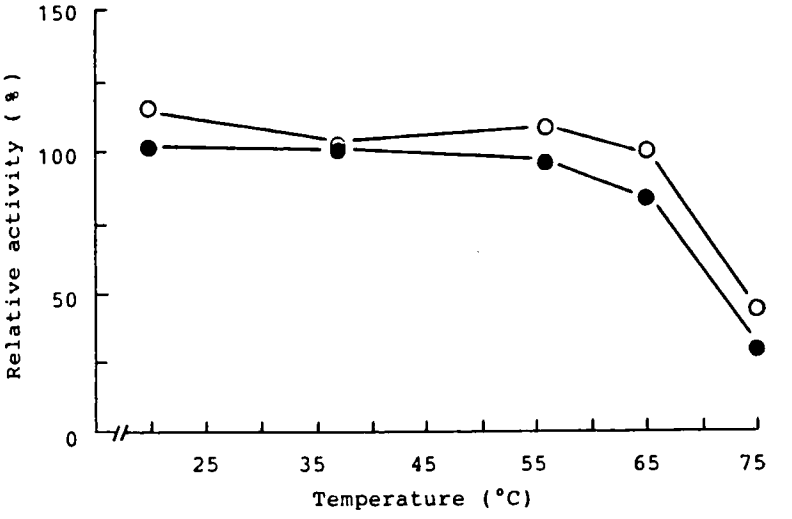

FIGURE 3. Thermal stability of human placental alkaline phosphatase. ○, enzyme entrapped in liposomes; ●, free enzyme.

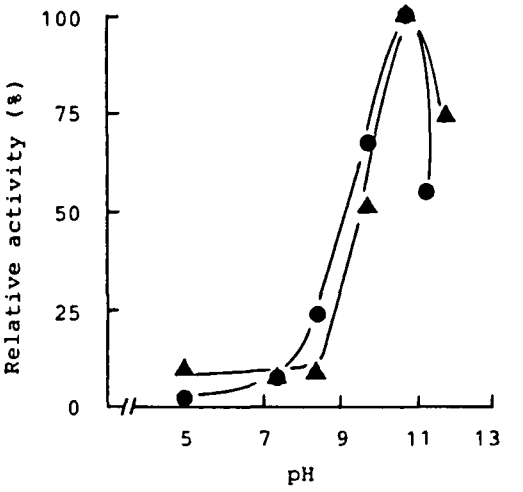

FIGURE 4. Optimum reaction pH for human placental alkaline phosphatase. ●, enzyme entrapped in liposomes; ▲, free enzyme.

similar in the temperature range studied i.e. the activity remains constant in the temperature from 22 to 55°C and decreases with increasing temperature from 55 to 75°C.

pH Effect

The curves for the effect of pH on the activity for the entrapped and free alkaline phosphatase are shown in Figure 4. The optimum pH value for the reaction for the entrapped enzyme is 10.7 which is in good agreement with that of the free enzyme.

Reaction Kinetics

The Lineweaver-Burk plots for the entrapped enzyme, free enzyme and enzyme with empty liposomes are given

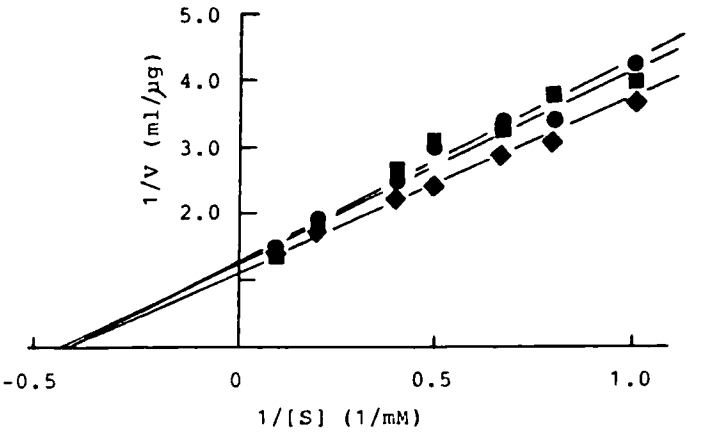

FIGURE 5. Lineweaver-Burk plots of human placental alkaline phosphatase. ◆, enzyme entrapped in liposomes; ●, enzyme with empty liposomes; ■, free enzyme.

in Figure 5. The V_m and K_m values obtained from the intercepts of the vertical axis and the horizontal axis respectively are shown in Table 1. Similar results for the reaction kinetics for the entrapped enzyme, free enzyme and enzyme with empty liposomes were obtained.

From the results for temperature effect, thermal stability, pH effect and reaction kinetics , it appears that the activity of the entrapped enzyme remains unchanged like the free enzyme and the presence of phosphatidylcholine exerts little effect on the activity of the enzyme.

Inhibition Effect

L-phenylalanine, L-tryptophan, L-cysteine, 2-mercaptoethanol and EDTA inhibit human placental

TABLE 1

Kinetic parameters for human placental alkaline phosphatase.

	K_m(mM)	V_m(μg/ml)
Entrapped Enzyme	1.85	0.83
Free Enzyme	2.34	0.82
Enzyme with Empty Liposomes	2.24	0.77

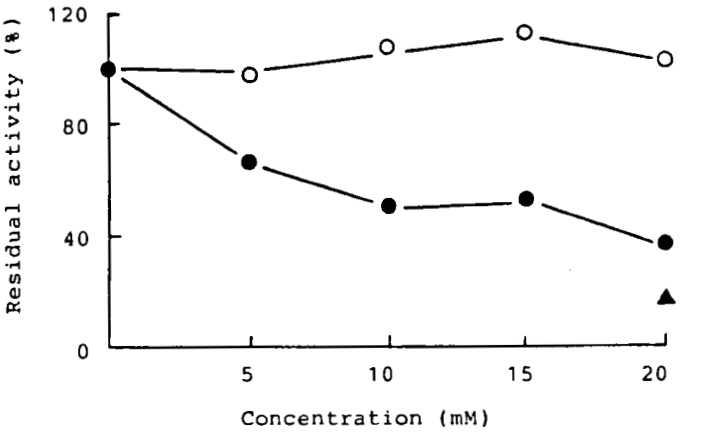

FIGURE 6. The effect of L-phenylalanine on human placental alkaline phosphatase. o, enzyme entrapped in liposomes; ▲, enzyme with empty liposomes; ●, free enzyme.

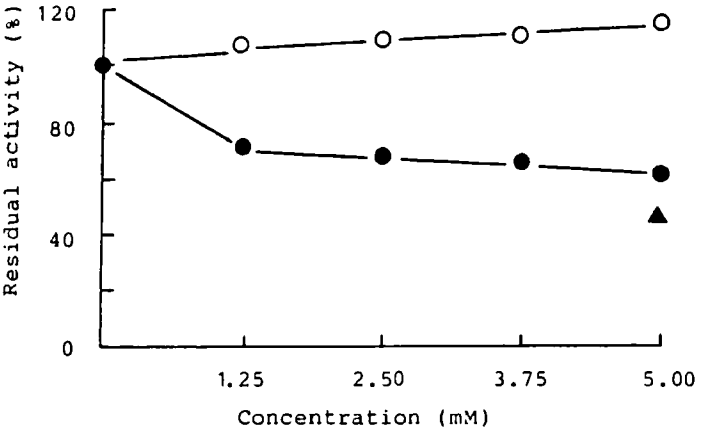

FIGURE 7. The effect of L-tryptophan on human placental alkaline phosphatase. o, enzyme entrapped in liposomes; ▲, enzyme with empty liposomes; ●, free enzyme.

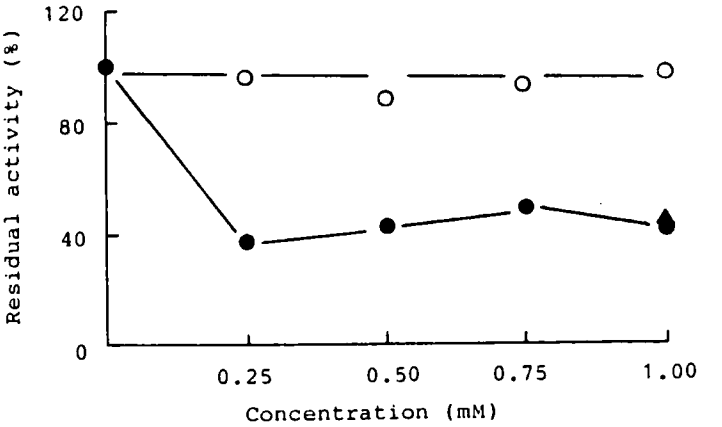

FIGURE 8. The effect of L-cysteine on human placental alkaline phosphatase. o, enzyme entrapped in liposomes; ▲, enzyme with empty liposomes; ●, free enzyme.

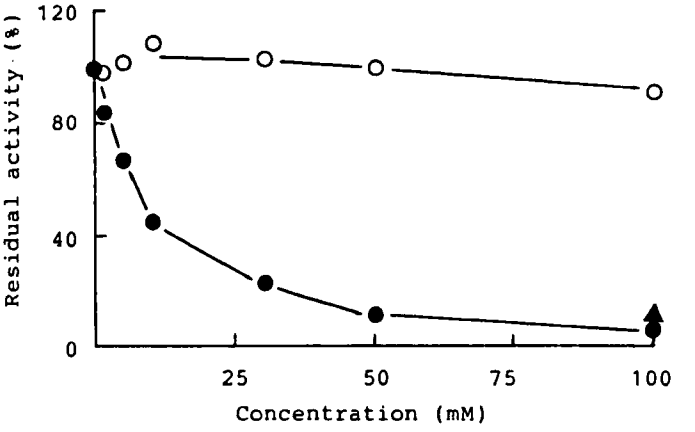

FIGURE 9. The effect of 2-mercaptoethanol on human placental alkaline phosphatase. O, enzyme entrapped in liposomes; ▲, enzyme with empty liposomes; ●, free enzyme.

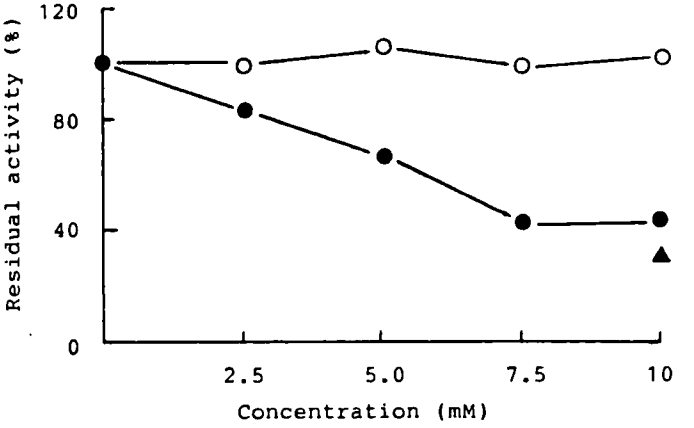

FIGURE 10. The effect of EDTA on human placental alkaline phosphatase. O, enzyme entrapped in liposomes; ▲, enzyme with empty liposomes; ●, free enzyme.

alkaline phosphatase 79%, 46%, 87%, 15% and 90% respectively at a concentration of 10^{-3}M (18). As shown in Figures 6, 7, 8, 9 and 10, it is clear that for the free enzyme and enzyme with empty liposomes, the activity decreases as the concentration of the inhibitors increases; whereas, for the entrapped enzyme, the activity is stable within the concentration range of inhibitors studied indicating that these inhibitors affect little on the activity of the entrapped enzyme. This may be due to the fact that the entrapped enzyme was protected by the liposome against inhibition.

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