[Chem. Pharm. Bull. 30(6)2105—2109(1982)]

Differential Determination of the Heat-stable Alkaline Phosphatase Activity in Serum[1)]

Kazuyuki Hirano,[a] Yuichi Iizumi,[a] Mamoru Sugiura,*,[a] Jun Miyazaki,[b] Kazumasa Miki,[b] Shiro Iino,[b] Hiroshi Suzuki,[b] Toshitsugu Oda,[b] and Masako Morikawa[c]

Gifu College of Pharmacy,[a] 6-1, Mitahora-higashi 5 Chome, Gifu 502, Japan, First Department of Internal Medicine, Faculty of Medicine, University of Tokyo,[b] 3-1, Hongo 7 Chome, Bunkyo-ku, Tokyo 113, Japan, and Department of Pharmacology, Tokyo College of Pharmacy,[c] 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

(Received November 16, 1981)

A procedure for the determination of the heat-stable alkaline phosphatase activity in serum was developed by using antibody-conjugated paper disks and inhibition by sodium thiocyanate. The placental alkaline phosphatase adsorbed on a paper disk conjugated with antibody was completely inhibited by 3 M sodium thiocyanate, but the activity of intestinal alkaline phosphatase adsorbed on the paper disk was unaffected by 3 M sodium thiocyanate. The results obtained by the proposed method showed a good correlation with those obtained by the heat-inactivation method ($r=0.991$). These results strongly suggest that the proposed method can be utilized as a routine clinical test for the determination of serum placental-type alkaline phosphatase, *i.e.*, placental, Regan, Nagao and Kasahara isoenzymes, from patients with cancer.

Keywords——determination of alkaline phosphatase activity; heat-stable alkaline phosphatase; antibody-conjugated paper disk; sodium thiocyanate; cancer

Introduction

It is well known that tumor tissues frequently contain placental-type alkaline phosphatase isoenzymes including Regan, Nagao and Kasahara isoenzymes.[2-4)] These alkaline phosphatases are the heat-stable enzymes. The determination of the heat-stable alkaline phosphatase activity in serum is sometimes performed as an aid to the diagnosis of cancer. Methods based on heat stability, electrophoretic mobility, radioimmunoassay, immunochemical assay and inhibition measurements[5-11)] have been described for the determination of placental alkaline phosphatase in sera of patients with cancer and pathological pregnancies. However, the heat-inactivation method requires careful control of conditions, such as the incubation temperature, the thickness of the tube and the pH of the sample, if reproducible results are to be obtained. The immunological relationship between the intestinal and placental isoenzymes is now controversial. The authors and other investigators observed cross-reaction between the two isoenzymes.[8,9,12-15)] The inhibitory method using L-phenylalanine, L-homoarginine and L-levamisole may be affected by inactivation of the enzyme.[10,11,16,17)] Thus it is difficult to determined the activity of the heat-stable enzyme accurately by the above methods. We have discovered that the placental isoenzyme is completely inhibited by 3 M sodium thiocyanate, while the intestinal isoenzyme is very resistant to it.[18)] We therefore used anti-placental alkaline phosphatase antibody-conjugated paper disks and activity inhibition by sodium thiocyanate to develop a method for the determination of the heat-stable alkaline phosphatase activity and applied it to measure the activity in sera from cancer patients and cases of pregnancy.

Materials and Methods

Materials——Filter paper (No. 51A) for the preparation of the paper disks was purchased from Toyo Roshi Co., Ltd. Sodium thiocyanate was obtained from Nakarai Chemicals, Ltd. Other reagents used were

of analytical reagent grade.

Preparation of Antibody——Antibodies were obtained in rabbits immunized with purified human alkaline phosphatases in complete Freund's adjuvant according to the previous paper.[19] Antibodies were purified according to the method of Fahey.[20]

Preparation of Antibody-conjugated Paper Disk——Antibodies to placental, intestinal and liver alkaline phosphatases were conjugated with paper disks according to the method of Ceska and Lundkvist.[21] Filter papers with a diamter of 6 mm were punched out and soaked with distilled water. They were then mixed with 80 ml of CNBr solution (2.7 g of CNBr in 80 ml of distilled water). The pH was brought to 10.5 with 1 N NaOH for 30 min. The liquid was then aspirated off and the disks were washed with 100 ml of 5 mM $NaHCO_3$. This washing procedure was repeated 12 times. These disks were then washed twice with 100 ml of distilled water. Ten mg of antibody was mixed with 2 g of the activated paper disks with stirring at 4°C for 3 h. The disks were then washed with 200 ml of 0.1 M $NaHCO_3$. The whole washing procedure was performed at room temperature. The remaining reactive groups were blocked with 40 ml of 50 mM ethanolamine in 0.1 M $NaHCO_3$ for 3 h at room temperature. The disks were again washed twice with 100 ml of 0.5 M $NaHCO_3$ and then with 0.1 M acetate buffer (pH 4.0). This latter washing procedure was repeated once more with an incubation period of 30 min. The disks were then washed twice with 100 ml of incubation buffer (see below). They were stored at 4°C in a small volume of incubation buffer.

Incubation Buffer——Fifty mM Tris–HCl buffer (pH 8.0) containing 0.9% NaCl, 0.3% bovine serum albumin, 0.05% sodium azide and 0.6% Triton X-405.

Assay Procedure——Proposed Method: One piece of paper disk conjugated with antibody was used per tube. To each tube was added 100 μl of incubation buffer and 50 μl of serum or standard enzyme solution. The tubes were covered with a sheet of parafilm and the whole rack was placed on a standard laboratory horizontal shaker and incubated overnight at 4°C. The solution in the tube was aspirated and the paper disk was then washed three times with 2 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 0.9% NaCl. All assays were performed in duplicate. To one tube was added 100 μl of 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM $MgCl_2$. To the other tube was added 100 μl of 3 M sodium thiocyanate in 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM $MgCl_2$. These tubes were incubated at 37°C for 30 min. Then the activity of alkaline phosphatase on the paper disks was determined by the method of Kind and King.[22]

Heat-inactivation Method: 0.2 ml of serum was heated for 10 min at 65°C in a thin-walled (5 × 0.5 cm) glass tube which had been preincubated at 65°C. The samples was cooled in an ice bath and centrifuged at 12000 × *g*. Fifty μl of the supernatant was taken for the measurement of the heat-stable alkaline phosphatase activity by the method of Kind and King.

Results and Discussion

Partial Cross-reaction of Placental and Intestinal Alkaline Phosphatases

As shown in Fig. 1, placental and intestinal alkaline phosphatases were precipitated by anti-placental alkaline phosphatase antibody, but liver alkaline phosphatase did not react with

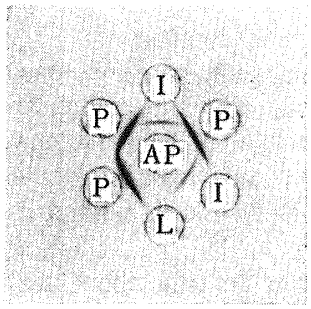

Fig. 1. Immunological Relationship of Human Alkaline Phosphatases

AP, anti-placental alkaline phosphatase antibody; P, purified placental alkaline phosphatase (10 μg, 83 King-Armstrong units); I, purified intestinal alkaline phosphatase (10μg, 96 King-Armstrong units); L, purified liver alkaline phosphatase (10 μg, 137 King-Armstrong units). The gel plate was stained for protein with Amido Black 10B.

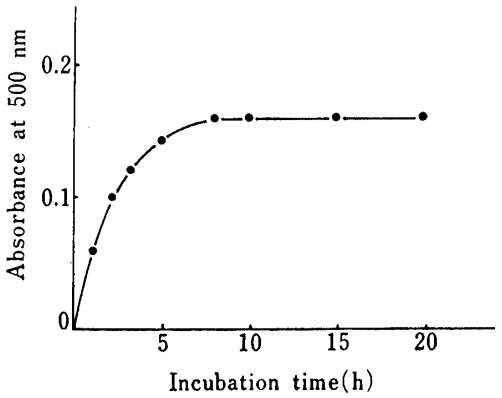

Fig. 2. Effect of Incubation Time on the Proposed Method

Twenty King-Armstrong units of the purified placental alkaline phosphatase was added to each tube. After incubation of the mixture at 4 °C for the indicated time, the activity was determined as described in the text.

that antibody. Ouchterlony double diffusion demonstrated a partial immunological cross-reaction of the placental and intestinal isoenzymes. This result suggests that placental and intestinal alkaline phosphatases share antigenic determinants.[11-14] On the paper disks conjugated with anti-placental alkaline phosphatase antibody, placental and intestinal alkaline phosphatases were adsorbed, but liver alkaline phosphatase was not adsorbed at all. Thus it is impossible to discriminate placental and intestinal isoenzymes by this immunological technique. A monospecific antiserum can be prepared by the absorption of anti-placental alkaline phosphatase antiserum with intestinal alkaline phosphatase.[15] However, monospecific antibody could not be prepared in large quantities by the above absorption method (data not shown).

Incubation Time of the Proposed Method

The incubation time required for the immunoreaction was examined. As can be seen in Fig. 2, plateau values in the immunoreaction were reached at over 8 h. Therefore, overnight incubation, *e.g.* 18 h, was selected as the standard condition. However, it is necessary for the proposed method to shorten the incubation time required for the immunoreaction.

Inhibitory Effect of Sodium Thiocyanate on Alkaline Phosphatase Activity

The effect of sodium thiocyanate on alkaline phosphatase adsorbed on the paper disk was investigated. As can be seen in Fig. 3, the liver and placental alkaline phosphatases adsorbed on the paper disks carrying antibodies to each isoenzyme were completely inhibited by treatment with 3 M sodium thiocyanate at 37 °C for 30 min, while the intestinal alkaline phosphatase activity remained at more than 90% of the original level. As compared with the native enzymes, the enzymes adsorbed on the paper disks were stable to 3 M sodium thiocyanate.[18] This result suggests that the enzymes were stabilized by immobilization on the paper disks. It is usually considered that hydrophilic sites of the placental and liver isoenzymes are denatured by 3 M sodium thiocyanate.

Kinetic Parameters of the Enzyme adsorbed on the Paper Disk

The K_m and V_{max} values of the enzymes adsorbed on the paper disks were compared with those of the native enzymes for disodium phenylphosphate as a substrate. The kinetic parameters are summarized in Table I. The K_m values of the enzymes on the paper disks increased approximately 2-fold as compared with those of the native enzymes, and the V_{max} values were decreased. These results suggest that substantial conformational changes and denaturation of the enzymes are not caused by the immobilization, although the affinity of the substrate for adsorbed enzymes is generally decreased more or less.[23-25]

Calibration Curve and Time Course of Alkaline Phosphatase Activity by the Proposed Method

The added enzyme and paper disk were incubated according to the proposed method, and the enzyme activity of the supernatant was determined. The amount of enzyme adsorbed on the paper disk was calculated from the residual activity in the supernatant. The amount of enzyme adsorbed on the paper disk was 60±6.5% (n=10) (mean±S.D.) of the added enzyme. We consider that the activity of the enzyme adsorbed on the paper disk decreases because the free enzyme combines with antibody on the paper disk . The adsorbed enzyme exhibited 74± 3.4% of the activity of the corresponding amount of free enzyme. Thus, under the above conditions, the calibration curve for the purified placental enzyme was prepared. The calibration curve was linear up to 20 King–Armstrong units. The time course of alkaline phosphatase activity determined by the proposed method was linear during a reaction period of 90 min.

Reproducibility and Recovery of the Proposed Method

The within-day and day-to-day reproducibility were investigated with sera from pregnancy cases. The results were 2.2±0.9% (n=20) and 3.7±1.0% (n=8) (mean±S.D.), respectively. The recovery of the proposed method was 97±3.1% (n=5) (mean±S.D.).

Correlation between the Proposed Method and the Heat-inactivation Method

The placental alkaline phosphatase activity was determined by the proposed method and the heat-inactivation method using sera from cancer patients and cases of pregnancy. As shown in Fig. 4, the correlation between the values obtained by these two methods is given by the linear regression equation $Y=1.10\,X+0.21$, in which the correlation coefficient (r) is 0.991 ($n=28$). Thus the activity obtained by the proposed method is very well correlated with that found by the heat-inactivation method.

In the separation of alkaline phosphatase isoenzymes by the electrophoretic technique, there is a problem in interpretation arising from diffusion or overlapping of activity on the electrophoretic support medium. In the separation of these isoenzymes by the immunological technique, there is the problem that the placental and intestinal isoenzymes share partially cross-reactive antigenic determinants. Warnock and Reisman[26] reported that some hepatoma alkaline phosphatase isoenzymes displayed a greater degree of heat stability. Therefore, the heat-inactivation method cannot precisely evaluate the heat-stable placental alkaline phosphatase. By the use of the paper disk conjugated with anti-placental alkaline phosphatase anti-

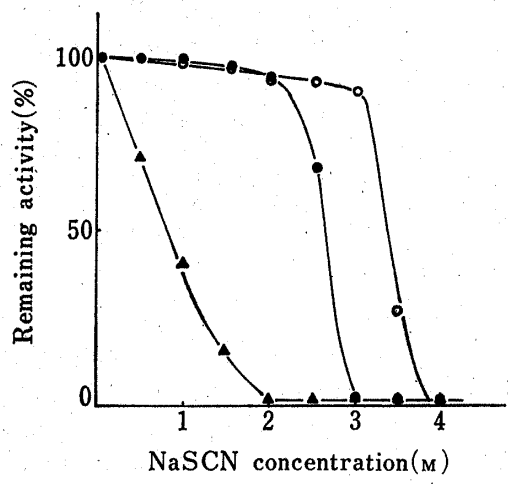

Fig. 3. Effect of Sodium Thiocyanate on Alkaline Phosphatases adsorbed on Paper Disks

Twenty King–Armstrong units of purified alkaline phosphatase was added to the assay system. The adsorbed enzymes on the paper disk were incubated with various concentration of sodium thiocyanate in 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM $MgCl_2$ at 37 °C for 30 min. —●—, human placental alkaline phosphatase; —○—, human intestinal alkaline phosphatase; —▲—, human liver alkaline phosphatase.

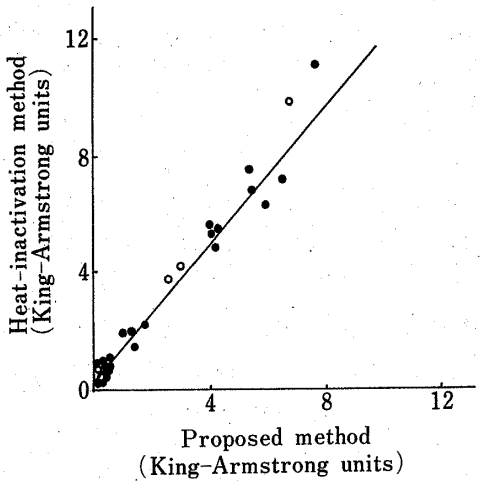

Fig. 4. Correlation of Human Placental Alkaline Phosphatase Activity determined by the Proposed Method and by the Heat-inactivation Method

Placental alkaline phosphatase activity in sera from cases of pregnacy (●) or cancer patients (○) was determined by the proposed method and by the heat-inactivation method as described in the text.
$Y=1.10X+0.21$
$r=0.991$
$n=28$

TABLE I. Kinetic Parameters of the Native Enzymes and the Enzymes adsorbed on Paper Disks

	Native enzyme		Adsorbed enzyme	
	K_m (mM)	V_{max} (nmol·mg^{-1}.min^{-1})	K_m (mM)	V_{max} (mmol·mg^{-1}.min^{-1})
Placenta	1.6	0.55	2.8	0.44
Intestine	1.5	0.64	2.8	0.51
Liver	1.5	0.91	2.3	0.78

body, however, the placental and intestinal isoenzymes can be separated from serum, and the placental isoenzyme can be determined precisely by sodium thiocyanate treatment. The addition of 3 M sodium thiocyanate to the assay system did not interfere with the enzyme reaction or color development. The placental-type alkaline phosphatase isoenzyme activity in serum from some patients with cancer may be determined specifically and precisely by the proposed method, though there may be some loss of the adsorbed enzyme activity from the paper disk. It should be possible to microanalyze the heat-stable alkaline phosphatase in sera from cancer patients by the proposed method using a fluorescent substrate. The proposed method should have wide application for the specific determination of various enzyme activities, such as kallikrein, γ-glutamyltransferase, *etc.*, in body fluids.

References and Notes

1) This paper forms Part 189 of "Studies on Enzymes" by M. Sugiura.
2) W.H. Fishman, N.R. Inglis, L.L. Stolbach, and M.J. Krant, *Cancer Res.*, **28**, 150 (1968).
3) T. Nakayama, M. Yoshida, and M. Kimura, *Clin. Chim. Acta*, **30**, 546 (1970).
4) K. Higashino, M. Hashinotsume, K.Y. Kang, Y. Takahashi, and Y. Yamamura, *Clin. Chim. Acta*, **40**, 67 (1972).
5) C.L. Anstiss, S. Green, and W.H. Fishman, *Clin. Chim. Acta*, **33**, 279 (1971).
6) N.R. Inglis, D.T. Guzek, S. Kirley, S. Green, and W.H. Fishman, *Clin. Chim. Acta*, **33**, 287 (1971).
7) B. Jacoby and K.D. Bagshawe, *Cancer Res.*, **32**, 2413 (1972).
8) M. Usategui-Gomez, F.M. Yeager, and A. Fernandez de Castro, *Cancer. Res.*, **33**, 1574 (1973).
9) F.G. Lehmann, *Clin. Chim. Acta*, **65**, 271 (1975).
10) P.M. Von Bayer, F. Galb, and E. Knoth, *J. Clin. Chem. Clin. Biochem.* **17**, 605 (1979).
11) H. Van Belle, M.E. De Broe, and R.J. Wieme, *Clin. Chim. Acta*, **23**, 454 (1977).
12) M. Sugiura, K. Hirano, Y. Iiizumi, J. Miyazaki, K. Miki, S. Iino, H. Suzuki, and T. Oda, *Chem. Pharm. Bull.*, **30**, 1387 (1982).
13) L. Fishman, N.R. Inglis, and W.H. Fishman, *Clin. Chim. Acta*, **38**, 75 (1975).
14) F.G. Lehmann, *Clin. Chim. Acta*, **65**, 257 (1975).
15) F.G. Lehmann, *Biochim. Biophys. Acta*, **616**, 41 (1980).
16) W.H. Fishmann, S. Green, and N.I. Inglis, *Nature* (London), **198**, 685 (1963).
17) Chi-Wen and W.H. Fishman, *J. Biol. Chem.*, **247**, 3082 (1972).
18) J. Miyazaki, S. Iino, K. Miki, T. Oda, and H Suzuki, *Igaku no Ayumi*, **116**, 23 (1981).
19) S. Iino, K. Aba, T. Oda, H. Suzuki, and M. Sugiura, *Clin. Chim. Acta*, **42**, 161 (1972).
20) J.D. Fahey, "Methods in Immunology and Immunochemistry," Vol. 1, Academic Press, New York and London, 1967, p. 323.
21) M. Ceska and U. Lundkvist, *Immunochemistry*, **9**, 1021 (1972).
22) P.R.N. Kind and E.J. King, *J. Clin. Pathol.*, **7**, 322 (1954).
23) J.H. Woychik and M.V. Wondolowski, *Biochim. Biophys. Acta*, **289**, 347 (1972).
24) K. Sukegawa and S. Takahashi, *Eiyo to Syokuryo*, **28**, 33 (1975).
25) J.H. Woychik and M.V. Wondolowski, *J. Milk Food Technol.*, **36**, 31 (1973).
26) M.L. Warnock and R. Reisman, *Clin. Chim. Acta*, **24**, 5 (1969).