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HIGHLY SENSITIVE ASSAY FOR ALKALINE AND ACID PHOSPHATASE ACTIVITIES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A highly sensitive and specific assay for alkaline and acid phosphatases in biological materials, such as plasma and saliva, has been established. Phenol, formed enzymatically from the substrate phenylphosphate, was determined by high-performance liquid chromatography with electrochemical detection. The retention time of phenol was 7 min and no other peaks were observed. The method is rapid and sensitive with a detection limit for phenol of as little as 5 pmol. Thus, as little as 0.5 μl of rat plasma or 10 μl of human saliva is required for both alkaline and acid phosphatase assays. The assay is accurate and reproducible. Using this assay, alkaline and acid phosphatase activities in saliva were found to be 1.12 ± 0.12 nmol/min/ml and 9.79 ± 1.23 nmol/min/ml, respectively. This new assay method should be applicable to extremely small biological samples.

INTRODUCTION

Both alkaline phosphatase (EC 3.1.3.1, orthophosphoric monoester phosphohydrolase) and acid phosphatase (EC 3.1.3.2) are organ-specific enzymes that catalyse the hydrolysis at their pH optima. These enzymes in serum are accepted as a diagnostic tool for many diseases [1–8] and are usually assayed by colorimetric and fluorimetric methods [9–18]. Methods generally used for assaying phosphatase monitor the absorbance of *p*-nitrophenol or phenol liberated enzymatically from the substrate [11, 12, 18].

Electrochemical detection (ED) is highly sensitive and it discriminates selectively electrochemically active materials. A sensitive and selective electrochem-

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ical detector is widely used for high-performance liquid chromatography (HPLC) of phenolic compounds, such as catecholamines and phenolic steroids in biological materials [19, 20]. The product, *p*-nitrophenol or phenol, is electrochemically active and can be measured by ED with high sensitivity.

In this experiment, phenylphosphate was used as a substrate for the determination of alkaline and acid phosphatase activities in human saliva. We developed a highly sensitive HPLC–ED method.

EXPERIMENTAL

Materials

Disodium phenylphosphate and disodium *p*-nitrophenyl phosphate were obtained from Daiichi (Tokyo, Japan), and L-*p*-bromotetramisole oxalate was from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals used were of analytical grade. Disodium phenylphosphate was purified by recrystallization before use to remove any contaminating phenol.

Chromatographic conditions

The HPLC system consisted of a Waters Model 6000A solvent-delivery system (Waters, Milford, MA, U.S.A.), an LC amperometric detector E-502 (Irika, Kyoto, Japan), an autosampler AS-48 (Toyo Soda, Tokyo, Japan) and a Develosil ODS-7 analytical column (150×4.6 mm I.D.). The mobile phase was a mixture of 10 m*M* acetate buffer (pH 4.0)–methanol (70:30, v/v). The flow-rate was 1.5 ml/min. The electrode potential was set at 1.2 V against an Ag/AgCl reference electrode for the detection of phenol.

Samples

Whole saliva and duct saliva from parotid and sublingual glands were collected from fifteen healthy men aged between 18 and 24 years. Prior to taking food, ca. 1.5 ml of unstimulated saliva were drooled into a polypropylene tube. Duct saliva was collected by a cotton roll method. The saliva sample was then centrifuged at 1600 *g* for 10 min and the supernatant removed for enzyme analysis.

Enzyme assays

The assay for the enzymatic activity of alkaline phosphatase was performed as follows. The standard incubation mixture consisted of the following components in a total volume of 200 μl (final concentrations in parentheses): 10 μl of 0.1 *M* disodium phenylphosphate (5 m*M*), 100 μl of 0.1 *M* carbonate buffer, pH 10.2 (50 m*M*), saliva and distilled water. For the blank incubation, the same procedure was carried out without addition of saliva. The reaction was started by addition of saliva and was carried out at 37 °C for 30 min. The reaction was terminated by adding 50 μl of 25% trichloroacetic acid. After a few minutes in an ice-bath, the mixture was centrifuged at 1600 *g* for 5 min. An aliquot (20 μl) of the resulting supernatant was analysed by HPLC. For the assay of acid phosphatase, the same procedure as for alkaline phosphatase was used except that 100 μl of 0.1 *M* citrate buffer (pH 4.8) were used instead of carbonate buffer in the incubation mixture.

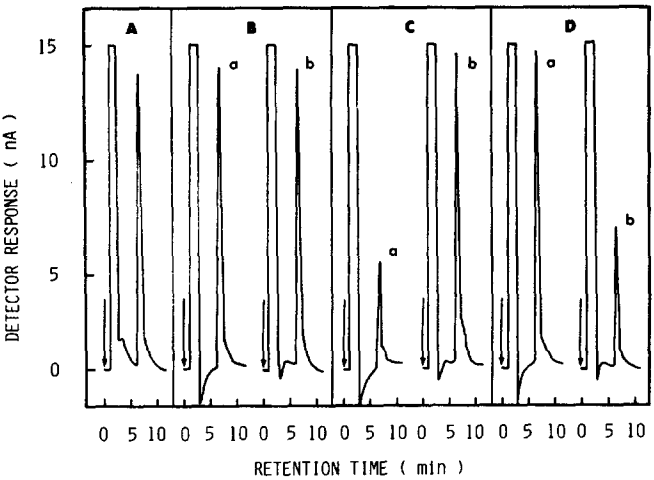


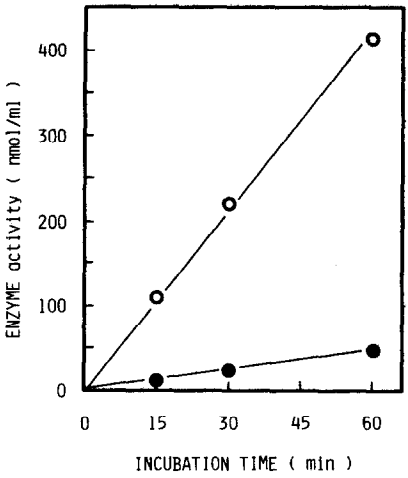
Fig. 1. HPLC elution patterns of alkaline and acid phosphatase incubation mixtures with human saliva as an enzyme source. The conditions are described in Experimental. (A) A Standard of 3.5 nmol of phenol in incubation mixture. (B) Experimental incubation was carried out with (a) 80 μl of saliva at pH 10.2 and (b) 10 μl of saliva at pH 4.8. Activities of alkaline and acid phosphatase, calculated from the charts, were 1.4 and 11.7 nmol/min/ml saliva, respectively. (C) In the presence of 0.1 m*M* L-*p*-bromotetramisole, experimental incubation was carried out with (a) 80 μl of saliva at pH 10.2 and (b) 10 μl of saliva at pH 4.8. Activities of alkaline and acid phosphatase in the presence of L-*p*-bromotetramisole were 0.4 and 12.1 nmol/min/ml saliva, respectively. (D) In the presence of 2.5 m*M* L-tartrate, experimental incubation was carried out with (a) 80 μl of saliva at pH 10.2 and (b) 10 μl of saliva at pH 4.8. Activities of alkaline and acid phosphatase in the presence of L-tartrate were 1.5 and 5.3 nmol/min/ml saliva, respectively.

With this procedure, the coefficients of variation with replicates of the same saliva sample for alkaline and acid phosphatase activity were 3.2 and 1.4%, respectively, and the precision was satisfactory.

RESULTS AND DISCUSSION

Phenol and *p*-nitrophenol were measured with very high sensitivity by HPLC–ED. The calibration curves of the peak height of the current intensity versus the injected amounts of phenol or *p*-nitrophenol were linear from 5 to 200 pmol. Detection sensitivity was higher for phenol than *p*-nitrophenol under the conditions used. Therefore, phenylphosphate was used as a substrate for both alkaline and acid phosphatase assays.

The chromatographic patterns of the alkaline and acid phosphatase reactions with human whole saliva as an enzyme sample are shown in Fig. 1. Fig. 1A shows the chromatogram of 3.5 nmol of phenol in the reaction mixture: the substrate, phenylphosphate, was not observed on the chromatogram. The experimental incubation (Fig. 1B) with 0.1 *M* carbonate buffer (pH 10.2) (a) or 0.1 *M* citrate buffer (pH 4.8) (b) formed phenol. In the presence of 0.1 m*M* L-*p*-bromotetramisole (Fig. 1C), alkaline phosphatase activity (a) decreased to ca. 30%, whereas acid phosphatase activity (b) was not affected. In contrast, 2.5 m*M* L-tartrate



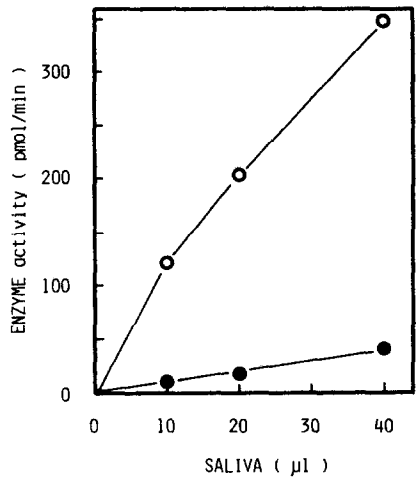


Fig. 2. Relationship between the reaction time and the rate of phenol formation using human saliva as an enzyme source at 37°C. Alkaline (●) and acid phosphatase activities (○) are expressed as nmol/ml of saliva.

Fig. 3. Alkaline and acid phosphatase activities in human saliva as a function of enzyme concentration. Both alkaline (●) and acid phosphatase activities (○) are expressed as pmol/min.

(D) strongly inhibited the activity of acid phosphatase (b), without affecting the alkaline phosphatase activity (a).

The rate of phenol formation using human whole saliva as an enzyme sample proceeded linearly up to 60 min (Fig. 2). Alkaline and acid phosphatase activities in human whole saliva as a function of enzyme concentration are shown in Fig. 3. The reaction rate was linear with up to 40 μl of saliva as an enzyme source.

The effect of pH on the hydrolysis of phenylphosphate by rat plasma and human whole saliva was measured in the pH range 2.6–11.6, using universal buffer (Fig. 4). There were two optimal pH values for the hydrolysis of phenylphosphate by serum and saliva. The activities of hydrolysis reached a maximum at pH 4 and 10, corresponding to acid and alkaline phosphatase activity, respectively.

Both enzyme activities were measured at temperatures between 4 and 56°C, under standard conditions of pH and substrate concentration (Fig. 5). Both alkaline and acid phosphatase activities increased up to 37°C. At 56°C, thermal inactivation was observed for alkaline phosphatase, but the acid phosphatase activity was further increased.

Using the described procedure, we measured the activities of both alkaline and acid phosphatase in human whole, parotid and sublingual saliva. The averages of the activities in fifteen whole saliva samples were 1.12 nmol/min/ml for alkaline phosphatase and 9.79 nmol/min/ml for acid phosphatase (Table I). The acid phosphatase activity in parotid saliva was significantly higher than that in sublingual saliva.

This assay is highly sensitive. The limit of sensitivity was ca. 5 pmol of phenol, which permits the assay of alkaline and acid phosphatase in only 0.5 μl of plasma or 10 μl of human saliva. The enzyme concentration detectable in human saliva

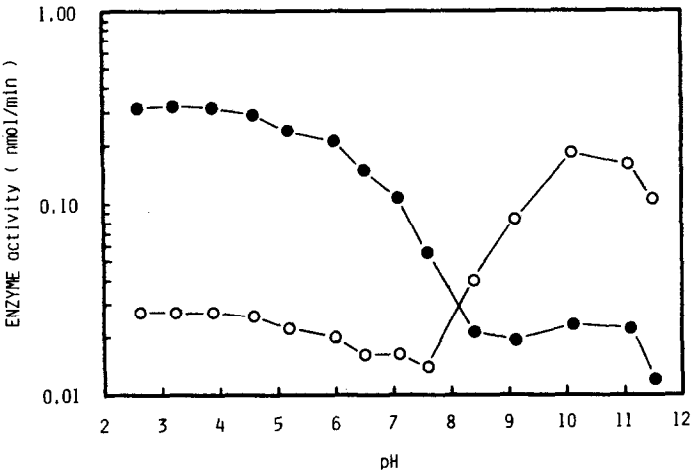


Fig. 4. Effects of pH on hydrolysis of phenylphosphate. The sample of 0.5 μl of rat plasma (○) or 40 μl of human saliva (●) was incubated with universal buffer (pH 2.6-11.6) under the standard conditions. The rate of phenol formation from phenylphosphate is expressed as nmol/min.

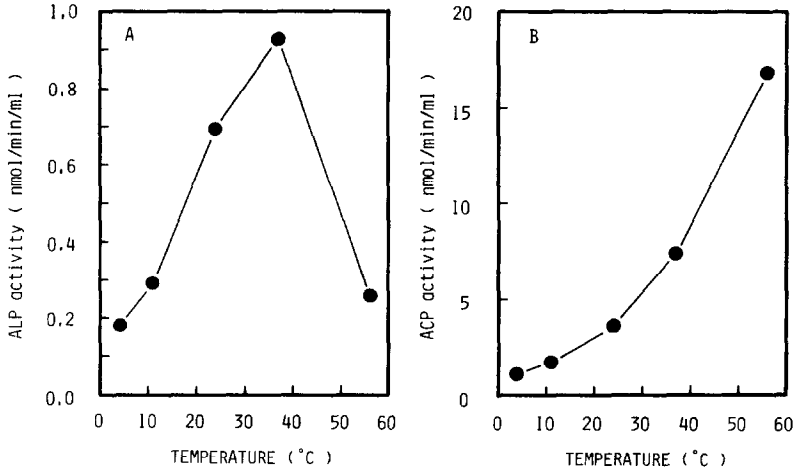


Fig. 5. Effects of temperature on enzyme activity. Alkaline (A) and acid (B) phosphatase activities were measured at various temperatures under the standard conditions.

is markedly lower than that of a colorimetric method using phenolphthalein monophosphate as a substrate. In the colorimetric method, 200 μl of human saliva were used for determination of alkaline phosphatase activity [21]. Also, our method is simple and rapid and the results are available within 8 min after incubation and protein precipitation. The assay is also accurate and reproducible: the reaction product, phenol, was detected with no interfering substances on the chromatogram.

The physiological significance of these phosphatases in saliva was not obvious. Nevertheless, alkaline phosphatase was confined to myoepithelial cells of the salivary gland, which have been shown to participate actively in the expulsion of saliva [22, 23]. It has been suggested that a marked reduction in the activity of

TABLE I

ALKALINE AND ACID PHOSPHATASE ACTIVITIES IN HUMAN SALIVA

Enzyme activity was measured under the standard conditions. Samples of parotid and sublingual saliva were collected from fifteen healthy men by a cotton roll method.

Sample	Enzyme activity (mean ± S.D.) (nmol/min/ml)	
	Alkaline phosphatase	Acid phosphatase
Whole saliva	1.12 ± 0.46	9.79 ± 4.59
Parotid saliva	1.22 ± 0.77	15.39 ± 3.55
Sublingual saliva	0.77 ± 0.60	8.67 ± 3.76

alkaline phosphatase may result in the functional impairment of these cells, leading to a decrease in the flow of saliva. This new assay method may be suitable for the determination of the physiological function of these phosphatases in saliva or the salivary gland, since it requires as little as 10 μl of human saliva for both alkaline and acid phosphatase assays.

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REFERENCES

1 H.D. Kay, Br. J. Exp. Pathol., 10 (1929) 253.
2 H.Q. Woodard, G.H. Twombly and B.L. Coley, J. Clin. Invest., 15 (1936) 193.
3 A.B. Gutman, T.L. Tyson and E.B. Gutman, Arch. Int. Med., 57 (1936) 379.
4 W.H. Fishman, N.I. Inglis and M.J. Krant, Clin. Chim. Acta, 12 (1965) 298.
5 M.M. Kaplan and L. Rogers, Lancet, ii (1969) 1029.
6 A.C. Aisenberg, M.M. Kaplan, S.V. Rider and J.M. Goldman, Cancer, 26 (1970) 318.
7 M.D. Reynolds, H.M. Lemon and W.W. Byrnes, Cancer Res., 16 (1956) 943.
8 L.R. Tuchman, G. Goldstein and M. Clyman, Am. J. Med., 27 (1962) 159.
9 C.H. Coleman, Clin. Chim. Acta, 13 (1966) 401.
10 J.H. Wilkinson and A.V. Vodden, Clin. Chem., 12 (1966) 701.
11 O.A. Bessey, O.H. Lowry and M.J. Block, J. Biol. Chem., 164 (1946) 321.
12 P.R.N. King and E.J. King, J. Clin. Pathol., 7 (1954) 322.
13 D.W. Moss, Clin. Chim. Acta, 5 (1960) 283.
14 D.B. Land and E. Jockin, Anal. Biochem., 16 (1966) 481.
15 G.G. Guilbault, S.H. Sandar, R. Glazer and J. Hayner, Anal. Lett., 1 (1968) 333.
16 B. Reiz and G.G. Guilbaut, Clin. Chem., 21 (1975) 1791.
17 A. Vaughan, G.G. Guilbault and D. Hackney, Anal. Chem., 43 (1971) 721.
18 E.B. Gutman and A.B. Gutman, J. Biol. Chem., 136 (1940) 201.
19 T. Kurahashi, H. Nishino, S. Parvez, H. Parvez, H. Kojima and T. Nagatsu, in H. Parvez, M. Malsot, S. Parvez and T. Nagatsu (Editors), Progress in HPLC, Vol. II: Electrochemical Detection in Medicine and Chemistry, VNU Science Press, Utrecht, 1986, pp. 3-36.
20 M. Basrat-Malsot, H. Parvez, S. Parvez and T. Nagatsu, in H. Parvez, M. Malsot, S. Parvez and T. Nagatsu (Editors), Progress in HPLC, Vol. II: Electrochemical Detection in Medicine and Chemistry, VNU Science Press, Utrecht, 1986, pp. 261-292.
21 K.G. Boyer and J.T. France, Int. J. Fertil., 21 (1976) 43.
22 B.I. Bogart, J. Histochem. Cytochem., 16 (1968) 572.
23 I.M. Chaudhry, D.W. Gandor and S.J. Gerson, Arch. Oral Biol., 26 (1981) 399.