Improved method for measuring tartrate-resistant acid phosphatase activity in serum

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We describe an improved method for the kinetic measurement of tartrate-resistant acid phosphatase (TrACP; EC 3.1.3.2) activity in serum. Of the TrACP derived from erythrocytes, platelets, and macrophages (osteoclasts and others), that from the first two sources is also resistant to fluoride, whereas skeletal TrACP is sensitive to fluoride. Thus, osteoclast-derived TrACP can be measured specifically by exploiting its sensitivity to fluoride. We measured the activity of tartrate-resistant and fluoride-sensitive acid phosphatase (TrFsACP) by using 2,6-dichloro-4-acetylphenyl phosphate as substrate at pH 6.2. The activity of TrFsACP in serum was increased by adding hexadimethrine bromide (Polybrene) to the reaction mixture. This method was not influenced by hemolysis with hemoglobin concentrations as great as 0.9 g/L. The mean ± SD values of TrFsACP activity by this method were 20.4 ± 2.8 and 16.4 ± 2.3 U/L for young (ages 20–29 years) men (n = 34) and women (n = 50), respectively. The highest mean TrFsACP activity was found among children younger than 15 years, followed by that in elderly subjects (older than 60).

Sensitive and specific serum markers for bone resorption and formation are essential for clinical assessment for bone metabolism. Serum band 5 tartrate-resistant acid phosphatase (TrACP; EC 3.1.3.2)[4] is ordinarily expressed in certain differentiated cells of the mononuclear phagocyte system, notably osteoclasts and alveolar macrophages [1], and pathologically in Gaucher's cells [2] and in the hairy cells of leukemia reticuloendotheliosis [3]. Serum band 5 TrACP is one of the most abundant enzymes in osteoclasts [4] and is regarded as a marker for them [5, 6]. During the process of bone resorption, osteoclasts secret a large quantity of TrACP into the resorption lacunas [6]. In hairy cell leukemia, however, serum band 5 TrACP activity remains within the reference interval for otherwise healthy persons, in spite of extremely high enzyme activity in the leukemic cells [7]. As a result, the serum activity of band 5 TrACP constitutes a potential index for the rate of bone resorption [8, 9].

Several different types of assay systems have been reported for measuring TrACP activity or TrACP mass in serum, but most are not adaptable to automated analyzers [4, 8–10]. Kinetic methods described so far [11, 12] also have several disadvantages, e.g., interference by bilirubin or hemoglobin [13]. Further, TrACP released from platelets and erythrocytes during blood collection and clotting constitute an important source of error in determinations of band 5 TrACP that use tartrate as inhibitor [14]. Protein phosphatase-inhibiting effects of polyanionic substances like heparin are neutralized with hexadimethrine bromide (Polybrene) [15]. In the presence of tartrate, fluoride shows inhibiting effects on skeletal TrACP activity [16] but not on erythrocytic TrACP activity [17].

Here, we describe a method for assaying band 5 TrACP activity as tartrate-resistant fluoride-sensitive acid phosphatase (TrFsACP), based on the sensitivity of this band to tartrate and fluoride, by using a continuous-monitoring method with 2,6-dichloro-4-acetylphenyl phosphate (DCAPP) substrate [18] and including Polybrene in the reaction mixture.

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[4] Nonstandard abbreviations: TrACP, tartrate-resistant acid phosphatase; TrFsACP, tartrate-resistant fluoride-sensitive acid phosphatase; TrFrACP, tartrate-resistant fluoride-resistant acid phosphatase; DCAPP, 2,6-dichloro-4-acetylphenyl phosphate; MPRM, mono-poly resolving medium; and PNPP, *p*-nitrophenyl phosphate.

Materials and Methods

Chemicals. DCAPP was obtained from Nitto Boseki Co. (Koriyama, Japan). Polybrene was obtained from Aldrich Chemical Co.. Sodium L-(+)-tartrate, *p*-nitrophenyl phosphate (PNPP), and heparin were obtained from Sigma Chemical Co. Mono-Poly resolving medium (MPRM; Dainippon Pharmaceutical Co.) consisted of 80 g/L Ficoll 400, 56.5 g/L sodium diatrizoate, and 113 g/L meglumine

diatrizoate; its relative density was 1.114 and osmolarity was 458 ± 10 mOsmol/L. All other analytical-grade chemicals were purchased from Wako Pure Chemical Co.

Reaction solutions. Buffer solution I consisted of 150 mmol of 2-(-morpholino)ethanesulfonic acid, 80 mmol of Poly-brene, 60 mmol of sodium L-(+)-tartrate, and 5 g of bovine albumin per liter (pH 6.2). Buffer solution II consisted of buffer solution I plus sodium fluoride (45 mmol/L). The substrate solution was 45 mmol of DCAPP and 50 mmol of Tris per liter (pH 4.0).

Enzymes. The enzymes used in this study were prepared from bone, erythrocytes, platelets, and leukocytes. Briefly, for the bone source, a bovine tibia was cleaned of soft tissue, sawed into small cubes, and washed with potassium chloride (0.1 mol/L) to remove marrow and blood. The bone pieces were ground to powder in a mill and then homogenized with a Polytron homogenizer for 15 s in 1.5 volumes of a solution of, per liter, 1 g of Triton X-100, 0.3 mol of potassium chloride, 0.5 mmol of phenylmethylsulfonyl fluoride, 2.5 mmol of benzamidine, and 50 mmol of aminocaproic acid. The extract was collected by centrifugation at 10 000*g* for 20 min at 4 °C and stored at −80 °C until use.

Platelets, erythrocytes, and leukocytes were prepared as follows to obtain the various forms of enzymes used. Briefly, 5 mL of heparinized blood from an apparently healthy subject was layered on top of 3 mL of MPRM in a 10-mL plastic tube and centrifuged at 200*g* for 20 min at room temperature. Platelet-rich plasma, the middle leukocyte band (buffy coat), and erythrocyte sediment were separated and washed twice with isotonic saline by centrifugation at 400*g* for 5 min for each washing.

The numbers of platelets, erythrocytes, and leukocytes were adjusted to counts of 25×10^4, 30×10^4, and 3000, respectively, per microliter with use of a Sysmex Micro cell counter (Model NE-7000; TOA Medical Electronics Co.). To 1-mL separate suspensions of platelets, erythrocytes, and leukocytes 5 μL of Triton X-100 was added; the suspensions were then vortex-mixed for 1 min and centrifuged at 1000*g* for 10 min at 4 °C. The supernatants thus recovered were stored at −80 °C until use.

Serum samples. Blood samples collected from 300 apparently healthy Japanese subjects [150 males (ages 5–69 years) and 150 females (ages 5–69 years)] by clean venipuncture were allowed to clot at room temperature for 2–4 h and centrifuged at 1000*g* for 10 min at room temperature. Serum thus separated was transferred into 1.5-mL tubes and stored at −80 °C until used (within 6 months).

Assay procedure. TrACP activity was measured with use of a centrifugal analyzer (Cobas Fara; Hoffmann-La Roche). Briefly, 150 μL of buffer solution I was added to 15 μL of sample and the mixture was incubated at 37°C for 5 min.

The enzyme–substrate reaction was initiated by adding 60 μL of substrate solution. The change in absorbance at 340 nm was monitored at 20-s intervals for 5 min. The millimolar absorptivity for the hydrolysis product (2,6-dichloro-4-acetylphenol) is $21.49 \text{ L} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ at 340 nm [18]. One IUB unit (1 U) of TrACP activity is defined as 1 μmol of DCAPP hydrolyzed per minute at 37 °C in the presence of 40 mmol of sodium L-(+)-tartrate per liter at pH 6.2. Tartrate-resistant fluoride-resistant acid phosphatase (TrFrACP) activity was assayed by using buffer solution II instead of buffer solution I. TrFsACP activity was estimated by subtracting TrFrACP activity from TrACP activity.

Statistics. All results were expressed as mean ± SD. Statistical significance was evaluated by using a Student's *t*-test for unpaired data.

Results

Inhibitory effect of fluoride on TrACP. We examined the inhibitory effect of fluoride on the TrACP activity of bone, platelet, erythrocyte, and leukocyte extracts at a fixed concentration of tartrate (60 mmol/L in the buffer solution) and various concentrations of fluoride (0 to 75 mmol/L; Fig. 1). The TrACP activity of serum and bone extract decreased by 63% and 88%, respectively, at the fluoride concentration of 45 mmol/L, but no further decrease was observed at greater fluoride concentrations. In contrast, the TrACP activity of platelets, leukocytes, and erythrocytes was decreased 6%, 14%, and 7%, respectively, at the fluoride concentration of 45 mmol/L.

Effect of Polybrene. We examined the TrFsACP activity of serum at various concentrations of Polybrene in buffer solution (0 to 100 mmol/L). The TrFsACP activity in
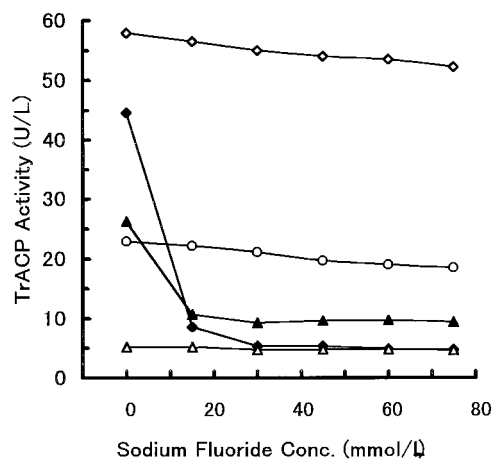

Fig. 1. Effect of sodium fluoride on TrACP activity in platelet extract (◇); leukocyte extract (○); erythrocyte extract (△); bone extract (◆); and serum (▲).

Buffer solution consisted of, per liter, 150 mmol of 2-(-morpholino)ethanesulfonic acid, 80 mmol of Polybrene, 60 mmol of sodium L-(+)-tartrate, and 5 g of bovine albumin (pH 6.2).
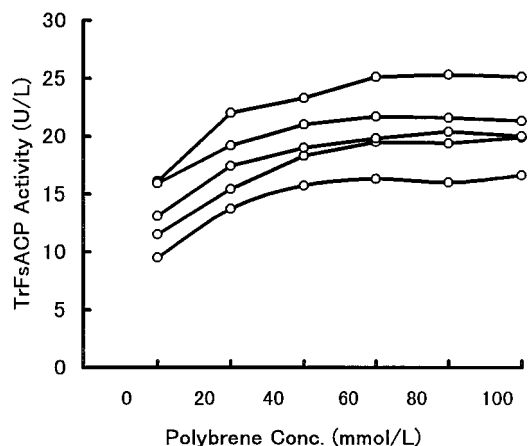
Fig. 2. Effect of Polybrene on TrFsACP activity on five apparently healthy individuals' serum (combined with different Polybrene concentrations in buffer solution).

healthy individuals' serum (n = 5) was greatest when Polybrene was present at 80 mmol/L (Fig. 2). Accordingly, we used a Polybrene concentration of 80 mmol/L in this study.

To evaluate the effect of heparin (polyanionic phosphatase inhibitor), we performed the assay by adding various concentrations of heparin (0 to 100 000 IU/L) to the serum and measuring the TrFsACP activity with and without Polybrene in the buffer solution(s). Heparin, in the absence of Polybrene, inhibited TrFsACP activity; however, this effect was reversed with the addition of Polybrene to the buffer solution. Addition of Polybrene not only restored the TrFsACP activity to its original baseline value but also increased it to above baseline, indicating that Polybrene also neutralized other phosphatase inhibitors besides heparin (Fig. 3).

We also compared the effect of including Polybrene in buffer solution on the activity of TrFsACP in serum samples collected from 62 Japanese men and 63 Japanese women (all at ages 20–39 years). The respective mean ± SD

TrFsACP values in the buffer solution without Polybrene were 14.9 ± 3.0 and 12.2 ± 2.7 U/L. Adding Polybrene increased the TrFsACP values to 20.7 ± 2.9 and 16.6 ± 2.6 U/L, respectively, i.e., by 41.8% ± 18.9% in the men and by 38.5% ± 19.9% in the women.

To further characterize TrFsACP, we examined the effect of Polybrene on the kinetics of TrFsACP activity in serum. Lineweaver–Burk plots of the results showed a linear relationship of enzyme activity with substrate concentration only in the presence of Polybrene in the buffer solution (Fig. 4). The K_m value of the enzyme activity for DCAPP in the presence of Polybrene was 4.1 mmol/L.

Effect of hemolysis. We also evaluated the effect of hemolysis on TrFsACP activity by assaying heparinized blood samples with hemoglobin concentration ranging from 0.3 to 0.9 g/L. The blood samples were hemolyzed by passage through a small-gauge needle. The TrFrACP activity in the hemolyzed sample increased with the increase in hemoglobin concentration. TrFsACP activity, however, was not affected by hemolysis up to a hemoglobin concentration of 0.9 g/L (Table 1).

Effect of storage. In view of the labile nature of TrFsACP activity, we examined the effect of storage temperature (room temperature vs −80 °C) on the stability of TrFsACP activity in serum samples collected from five healthy individuals. TrFsACP activity in these samples did not change within 12 h at room temperature and as long as 12 months at −80 °C. We also allowed aliquots of three of the blood samples to clot for different intervals (1, 2, 4, 6, and 12 h) at room temperature. TrFrACP activity in serum increased with clotting time to a plateau after 4 h of clotting; TrFsACP activity in serum was independent of clotting time.

Assay imprecision. Within- and between-run imprecision was evaluated with two different serum samples assayed 20 times each. Aliquots of the serum samples were stored
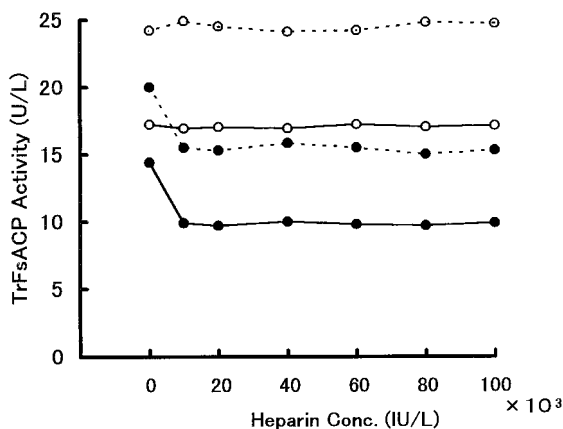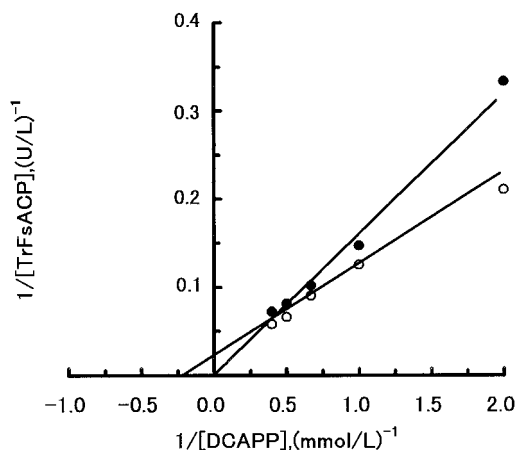

Fig. 3. Effect of heparin on two apparently healthy individuals' serum TrFsACP activity with Polybrene (○) and without Polybrene (●) in buffer solution.

Fig. 4. Lineweaver–Burk plots of TrFsACP activity of serum sample with Polybrene (○) or without Polybrene (●) in buffer solution.

Table 1. Effect of hemolyzation on serum TrFsACP and TrFrACP activity (U/L).

Sample	Hemoglobin conc., g/L	Not hemolyzed		Hemolyzed	
		TrFsACP	TrFrACP	TrFsACP	TrFrACP
1	0.3	17.6	12.0	17.9	15.0
2	0.4	21.3	11.1	21.5	15.8
3	0.5	29.5	11.9	29.4	18.0
4	0.5	18.1	10.8	18.3	16.9
5	0.9	15.3	8.7	15.1	20.9

at $-80\,°C$ until use. The means (and within-run CVs) of TrFsACP activity run were 16.9 U/L (3.8%) and 25.3 U/L (2.7%), respectively. The means (and between-run CVs) of TrFsACP activity of were 16.9 U/L (4.6%) and 24.4 U/L (5.7%), respectively. The upper limit of linearity of the present TrFsACP assay, determined by measuring bone extract, was 90 U/L.

Method comparisons. We compared the PNPP method (TrACP) previously reported by Lau et al. [8] (x) with the present method for quantifying TrACP and TrFsACP activity (y) in 50 samples (Fig. 5). Agreement between the PNPP method and the present method for TrACP was $y = 1.33x + 4.64$ ($r = 0.89$); agreement with the TrFsACP assay was $y = 0.84x + 7.31$ ($r = 0.77$).

Assays of samples from apparently healthy subjects. The TrFsACP activity concentrations in apparently healthy Japanese men and women of ages 20–69 years ranged from 20.4 to 23.6 U/L and from 16.4 to 24.0 U/L, respectively. Both sexes showed increasing TrFsACP activity with age (Table 2), the concentrations being significantly higher in the men older than 50 years and in women older than 40 years in comparison with the younger subjects (20–29 years). However, the values were highest in children of <15 years.
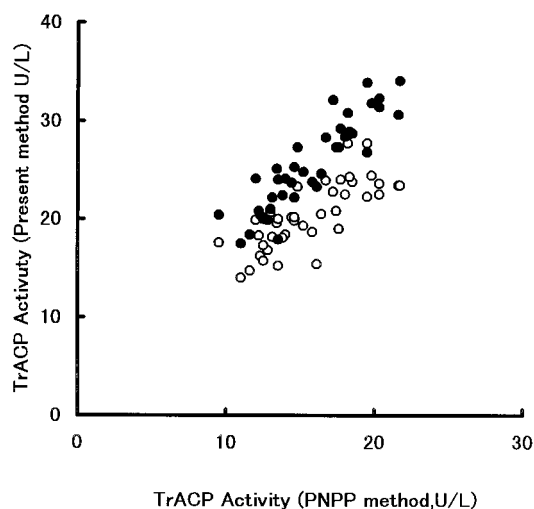

Fig. 5. Correlation between PNPP method and present method: TrACP (●),TrFsACP (○).

Table 2. Serum TrFsACP activity in healthy subjects.

	Mean ± SD activity, U/L (n)	
Age range, years	Males	Females
5–9	40.6 ± 6.4 (17)[a]	39.2 ± 9.9 (7)[b]
10–14	47.9 ± 11.3 (21)[a]	37.1 ± 13.5 (10)[b]
15–19	27.4 ± 7.4 (12)	18.8 ± 5.0 (8)
20–29	20.4 ± 2.8 (34)	16.4 ± 2.3 (50)
30–39	20.4 ± 2.9 (30)	16.4 ± 2.3 (16)
40–49	20.8 ± 2.6 (11)	18.8 ± 3.2 (22)[b]
50–59	22.3 ± 3.7 (12)	22.9 ± 3.6 (19)[b]
60–69	23.6 ± 2.7 (13)[a]	24.0 ± 2.6 (18)[b]

[a] $P < 0.001$ vs men 20–29 years old.
[b] $P < 0.001$ vs women 20–29 years old.

Discussion

Four true isoenzymes of ACP, namely, lysosomal, prostatic, macrophagic, and erythrocytic, have been identified on the basis of their origin and genetic encoding. ACP isoenzymes originating from lysosome and prostate have a high molecular mass (100 kDa) and are inhibited by tartrate and fluoride. In contrast, ACP isoenzymes from macrophages (37 kDa), erythrocytes (17 kDa), and osteoclasts (30–37 kDa) are resistant to tartrate treatment [20]. Further, ACP derived from erythrocytes resists both fluoride and tartrate treatment [17].

Keeping in view these properties of ACP isoenzymes from the various specific sources, we attempted to improve the available assay method to better measure the osteoclastic and other macrophagic TrACP (TrFsACP) activity in serum. This improved method excludes TrACP originating from erythrocytes and platelets, at least, and probably also from other cellular sources. Further, it obviates the preincubation of serum samples at 37 °C for 1 h to inactivate erythrocyte-derived TrACP. This method also avoids the need to perform the test for TrACP relatively quickly after collection of the blood sample. However, this method may also measure other macrophagic TrACP activity—which we did not investigate in this study.

TrACP activity was ~5–10% higher in serum than in the corresponding heparinized plasma, suggesting some release of TrACP during blood clotting [8]. Including heparin in the reaction mixture lowers the ACP activity. Heparin at acidic pH is known to precipitate the globulin causing turbidity in the incubation mixture and may coprecipitate TrACP [14]. We observed consistently lower TrFsACP activity in serum with various added concentrations of heparin. This decrease, however, was reversed by including Polybrene in the reaction mixture; indeed, adding Polybrene consistently increased the ACP activity above the baseline value for the absence of heparin. The Lineweaver–Burk plots showed a linear relationship of activity with substrate concentration in the presence of Polybrene. Heparin is a linear anionic polyelectrolyte with negatively charged sulfate and carboxyl groups. Proteins with a high content of basic amino acids have some

polycationic character that may inhibit the activity of TrFsACP. Naturally occurring heparin *[21]* and possibly other TrACP inhibitors in serum samples can thus be neutralized with the use of Polybrene.

The prostatic ACP activity in serum kept at room temperature decreased, being totally inactive after 72 h. The decrease was due to the increase in pH that resulted from the loss of carbon dioxide in the serum. Serum ACP activity is well preserved at room temperature, however, when the serum is buffered to pH 6.2–6.6 with disodium hydrogen citrate *[22]*. TrACP activity in serum samples did not change after 24 h at room temperature *[8]*. In the present study, TrFsACP activity in serum was stable for at least 12 h at room temperature and for 1 year at −80 °C.

Serum TrFsACP activity in healthy Japanese measured with this improved method was higher than the TrACP value reported by Scarnecchia et al. *[23]*, presumably because of the different substrate and pH of the reaction buffer and the inclusion of Polybrene in our reaction mixture. The higher TrFsACP values observed in children younger than 15 years agreed with these reported by Schiele et al. *[24]*. Also, the increased TrFsACP activity we found among women over 40 suggests an increased bone turnover in older women as their gonadal function diminishes.

In conclusion, the present method was highly sensitive for measuring TrFsACP, which is specific for osteoclasts and other macrophages. It therefore appears to be very useful in the clinical assessment of bone metabolism. Further, the present method is adaptable to automation.

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