Development of a Kinetic Assay for Band 5b Tartrate-resistant Acid Phosphatase Activity in Serum

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Background: Band 5 tartrate-resistant acid phosphatase (TrACP; EC 3.1.3.2) consists of two isoenzymes, bands 5a and 5b, of which band 5b TrACP is considered to be derived from bone. However, no kinetic method for the specific measurement of band 5b TrACP in serum is available. Our aim was to develop a kinetic assay method for the specific measurement of band 5b TrACP in serum.

Methods: Band 5b TrACP was measured kinetically in serum as tartrate-resistant fluoride-sensitive heparin-resistant ACP with 2,6-dichloro-4-acetylphenyl phosphate as substrate at pH 6.6.

Results: Heparin inhibited band 5a TrACP but had no effect on band 5b TrACP in serum or in bone extract. The presence of EDTA or ascorbic acid had no effect, but dithiothreitol inhibited enzyme activity. The within-run (n = 20) and between-run (n = 20) CVs of band 5b TrACP activity were 3.3–5.8% and 5.0–7.3%, respectively. The mean ± SD values of band 5b TrACP activity in males (n = 25) and females (n = 57) 20–29 years of age by this method were 8.0 ± 2.2 U/L and 6.4 ± 1.8 U/L, respectively. The band 5b TrACP value was significantly higher in females >50 years of age compared with the younger subjects (20–29 years). The highest band 5b TrACP values were among children younger than 15 years.

Conclusions: This kinetic assay is a simple and specific method for the measurement of band 5b TrACP in serum samples and is useful in the evaluation of bone turnover activity.

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Serum band 5 tartrate-resistant acid phosphatase (TrACP; EC 3.1.3.2) usually is expressed in certain differentiated cells of the mononuclear phagocyte system, notably osteoclasts and alveolar macrophages (1), and pathologically in Gaucher 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 (5, 6). Thus, the serum concentrations of band 5 TrACP constitute a potential index for the bone resorption rate (7, 8).

On the basis of the catalytic and ionic properties and carbohydrate content, two isoenzymes of band 5 TrACP, bands 5a and 5b, have been identified (9). Band 5a TrACP is sialylated, whereas band 5b lacks sialic acid. It has been shown that the removal of sialic acid from band 5a by sialidase changes band 5a to 5b (10). Increased band 5b TrACP is liberated from osteoclasts during normal physiologic bone growth among healthy children and during metastasis of bone (11). The presence of band 5b TrACP in Gaucher serum in spite of the presence of band 5a TrACP in the spleen has been reported (10). Thus, the measurement of band 5b TrACP appears to be a specific marker for osteoclastic activity.

However, no kinetic method for the specific measurement of band 5b TrACP in serum has been available. Here we report a simple kinetic method for the measurement of this osteoclastic activity marker (band 5b TrACP) by modifying the method we described previously for the...
measurement of tartrate-resistant fluoride-sensitive acid phosphatase (TrFsACP) (12).

**Materials and Methods**

**CHEMICALS**

2,6-Dichloro-4-acylphenyl phosphate (DCAPP) was obtained from Nitto Boseki Co. Ltd. Sodium t-(+)-tartrate and heparin were obtained from Sigma, and polybrene was obtained from Aldrich. Carboxymethyl-agarose (CM-Sepharose) was obtained from Pharmacia Fine Chemicals. All other analytical grade chemicals were purchased from Wako Pure Chemical Co.

**REACTION SOLUTION**

The reaction solutions we reported previously (12) were used with some modification. Briefly, buffer solution I consisted of 150 mmol/L 2-(-hydroxy-3-morpholino)propanesulfonic acid, 60 mmol/L sodium t-(+)-tartrate, 23 kIU/L heparin, and 5 g/L bovine albumin (pH 6.6). Buffer solution II consisted of buffer solution I plus sodium fluoride (45 mmol/L). The substrate solution consisted of 45 mmol/L DCAPP and 50 mmol/L Tris (pH 4.0).

**SERUM SAMPLES**

Blood samples collected from 516 apparently healthy Japanese subjects, ages 5–79 years (210 males and 306 females), 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 use (within 6 months).

**BONE EXTRACT**

Bone extract samples were prepared as described previously (12). In brief, bovine tibia after the removal of soft tissue was cut into small cubes. The marrow and blood were removed, and the bone was ground to powder and homogenized in a solution containing Triton X-100, potassium chloride, phenylmethylsulfonyl fluoride, benzamidine, and 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.

**ASSAY PROCEDURE**

TrACP activity was measured using a centrifugal automatic 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 the addition of 60 μL of substrate solution. The change in absorbance at 340 nm was monitored at 20-s intervals for 5 min. The millimolar absorptivity of the hydrolysis product (2,6-dichloro-4-acylphenol) is 21.49 L·mmol⁻¹·cm⁻¹ at 340 nm. 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/L sodium t-(+)-tartrate and 23 kIU/L heparin at pH 6.6. Tartrate-resistant fluoride-resistant acid phosphatase activity was assayed using buffer solution II instead of buffer solution I. Band 5b TrACP activity was estimated by subtracting tartrate-resistant fluoride-resistant acid phosphatase activity from TrACP activity.

**FRACTIONATION ON CM-SEPHAROSE**

For biochemical characterization, serum collected from children was pooled and chromatographed on CM-Sepharose. The CM-Sepharose was packed into glass tubing [60 × 0.9 cm (i.d.)] and washed with 10 mmol/L acetate buffer, pH 5.0. The acidity of the serum sample was adjusted to pH 5.0 by the addition of acetic acid, and the coagulated proteins were removed by centrifugation at 20,000 g for 15 min. The supernatant fraction was passed through the column at 0.5 mL/min. The enzyme, which was retained on the column, was then eluted with a linear concentration gradient of sodium chloride: 0–1 mol/L in 299 mL of elution buffer (10 mmol/L acetate buffer, pH 5.0). Fractionated serum was subjected to TrFsACP measurement with added heparin or polybrene in reaction buffers I and II.

**ELECTROPHORESIS**

Acid phosphatase isoenzymes were analyzed by electrophoresis on acidic polyacrylamide gel. After the electrophoresis was completed, the gel columns were washed with 0.1 mol/L acetate buffer (pH 5.2) and stained with α-naphthyl phosphate-Fast Garnet GBC as described previously (9).

**pH optimum**

The optimum pH values for band 5a and 5b TrACP were determined using 150 mmol/L 2-(-morpholino)ethanesulfonic acid, pH 5.0–6.4, and 150 mmol/L 2-(-hydroxy-3-morpholino)propanesulfonic acid, pH 6.6–7.2, buffers in buffer solution I with added polybrene or heparin.

**STATISTICS**

All results were expressed as means ± SD. Statistical significance was evaluated by the Student t-test for unpaired data.

**Results**

**SEPARATION OF TRACP BANDS 5A AND 5B BY CATION-EXCHANGE CHROMATOGRAPHY**

Chromatography of sera from children showed at least four TrFsACP activity peaks (I, II, III, and IV) when polybrene was added to the reaction buffer (Fig. 1). However, only the fourth peak appeared when heparin was added to the reaction buffer. The activity of the fourth peak was the same with either polybrene or heparin added. Electrophoresis of peaks III and IV on acidic polyacrylamide gels (9) yielded band 5a and 5b TrACP, respectively (Fig. 2). These fractions were used to study the effect of heparin and pH on band 5a and 5b TrACP.
The effect of heparin on band 5a and 5b TrACP, bone extract, and serum is shown in Fig. 3. These fractions were collected after cation-exchange chromatography as mentioned above. Band 5a TrACP was significantly inhibited by heparin \( (P < 0.001) \), whereas band 5b TrACP was not. However, a slight inhibition in bone extract and serum by heparin was observed.

The optimal reaction pH for band 5a TrACP (peak III) was \( \approx 5.8 \), whereas band 5b TrACP (peak IV) showed maximum activity at pH 6.6. The addition of heparin in the reaction mixture selectively reduced band 5a TrACP activity to 0 at pH 6.6 (Fig. 4).

In view of the labile nature of band 5b TrACP activity, we examined the effect of storage temperature (room temperature vs \( -20^\circ C \)) on the stability of band 5b TrACP activity in serum samples collected from five healthy individuals. Band 5b TrACP activity in these samples did...
not change within 12 h at room temperature or after 12 months at −80 °C.

EFFECTS OF VARIOUS EFFECTORS ON BAND 5b TRACP
The effects of potential activators and inhibitors of band 5b TRACP are summarized in Table 1. The presence of EDTA or ascorbic acid had no effect, but dithiothreitol inhibited enzyme activity.

ASSAY IMPRECISION
The within- and between-run imprecision was evaluated with two different serum samples assayed 20 times each. Aliquots of the serum samples were stored at −80 °C until use. The within-run CVs of band 5b TRACP activity were 5.8% (at 11.3 U/L) and 3.3% (at 29.9 U/L), respectively. The between-run CVs were 7.3% (at 11.3 U/L) and 5.0% (at 29.9 U/L), respectively. The upper limit of linearity of the present band 5b TRACP assay, determined by measuring serum, was 110.0 U/L. The $K_m$ value of band 5b TRACP activity was 4.7 mmol/L when DCAPP was used as the substrate.

METHOD COMPARISONS
We compared the present method for band 5b TRACP (with heparin, pH 6.6) with the previously reported method for TrFsACP (with polybrene, pH 6.2), using randomly selected apparently healthy children (n = 22) and adults (n = 28). The correlation coefficient ($r$) between band 5b TRACP ($x$) and TrFsACP ($y$; with polybrene) was 0.97 [Deming regression equation: $y = (0.92 \pm 0.03)x + (10.0 \pm 0.61); n = 50; S_{y|x} = 2.26$. For children, the correlation was $r = 0.92 [y = (0.92 \pm 0.08)x + (10.0 \pm 2.26); n = 22; S_{y|x} = 2.65$. For adults, the correlation was $r = 0.78 [y = (1.16 \pm 0.18)x + (8.03 \pm 1.54); n = 28; S_{y|x} = 1.86; Fig. 5].

ASSAYS OF SAMPLES FROM APPARENTLY HEALTHY SUBJECTS
Band 5b TRACP activity in apparently healthy Japanese males and females, ages 20–39 years, ranged from 3.6 to 12.4 U/L and 2.8 to 10.0 U/L, respectively. Both sexes showed an increase in band 5b TRACP activity with age (Table 2). Band 5b TRACP activity was significantly higher in males >60 years and females >50 years compared with the younger subjects (20–29 years). Band 5b TRACP activity in females was slightly lower than in males at ages 20–39 years. However, the activity was highest in children <15 years of age.

**Discussion**
We previously reported an improved method for the measurement of TrFsACP, based on its fluoride-sensitive property, using DCAPP as substrate. The improved method, however, measured TrFsACP originating from osteoclasts and other macrophages and excluded TRACP originating from erythrocytes and platelets (12). To detect band 5b TRACP specifically, we introduced heparin into the reaction mixture instead of polybrene. In our previous report, we showed a marked reduction in TrFsACP activity when heparin was added at the concentration of 100 kIU/L, but the activity did not decrease further with increases in the heparin concentration. The unaffected

### Table 1. Effect of some compounds on activity of band 5b TRACP.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration, mmol/L</th>
<th>Band 5b TRACP activity, % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>1</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>103</td>
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<tr>
<td>EDTA</td>
<td>1</td>
<td>95</td>
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<tr>
<td></td>
<td>5</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>93</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>57</td>
</tr>
</tbody>
</table>

**Table 2. Serum band 5b TRACP activity in healthy subjects.**

<table>
<thead>
<tr>
<th>Age range, years</th>
<th>Males Mean ± SD activity, U/L (n)</th>
<th>Females Mean ± SD activity, U/L (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–9</td>
<td>23.3 ± 6.3 (11)$^a$</td>
<td>27.6 ± 7.5 (12)$^b$</td>
</tr>
<tr>
<td>10–14</td>
<td>24.9 ± 8.2 (13)$^a$</td>
<td>23.6 ± 11.9 (6)$^c$</td>
</tr>
<tr>
<td>15–19</td>
<td>16.6 ± 3.4 (6)$^a$</td>
<td>9.8 ± 4.6 (10)$^d$</td>
</tr>
<tr>
<td>20–29</td>
<td>8.0 ± 2.2 (25)</td>
<td>6.4 ± 1.8 (57)</td>
</tr>
<tr>
<td>30–39</td>
<td>8.2 ± 2.2 (47)</td>
<td>6.2 ± 1.8 (30)</td>
</tr>
<tr>
<td>40–49</td>
<td>8.3 ± 1.9 (41)</td>
<td>6.7 ± 2.4 (43)</td>
</tr>
<tr>
<td>50–59</td>
<td>8.6 ± 1.8 (24)</td>
<td>9.6 ± 2.7 (70)$^b$</td>
</tr>
<tr>
<td>60–69</td>
<td>8.8 ± 2.7 (43)</td>
<td>10.2 ± 2.5 (71)$^b$</td>
</tr>
<tr>
<td>70–79</td>
<td>10.4 ± 3.0 (15)$^a$</td>
<td>10.4 ± 3.3 (22)$^a$</td>
</tr>
</tbody>
</table>

$a$ P <0.001 vs men 20–29 years.  
$b$ P <0.001 vs women 20–29 years.  
$c$ P <0.05 vs women 20–29 years.  
$d$ P <0.01 vs men 20–29 years.
TrFsACP appeared to be band 5b TrACP, and the affected TrFsACP appeared to be band 5a and other unknown TrFsACP isoenzymes, as shown by CM-Sepharose chromatography performed separately with added heparin or polybrene. Our present finding showed a small inhibitory effect of heparin on bone extract as well as indicating that bone extract also contains a small amount of non-band 5b TrACP.

Modder (13) observed a maximal inhibitory effect of heparin at the concentration of 50 kIU/L, but this was accompanied by maximal turbidity in the reaction mixture. This turbidity presumably occurred because of the globulin-precipitating effect of heparin. However, no such turbidity was observed in our reaction mixture. This appears to be related to the low pH (4.9) of the reaction mixture used by Modder compared with ours (pH 6.6). At pH 6.6, we found a good correlation between band 5b TrACP (with heparin) and TrFsACP (with polybrene). The intercept of 10.0 observed in the added polybrene method appeared to include band 5a and other unknown TrACP isoenzymes.

Fukushima et al. (14), however, have suggested that intracellular TrACP is highly diffusible at a pH below 6.0 in an inactive form. When secreted to the extracellular acidic compartment (resorption lacunae), TrACP is transformed into an active form and infiltrates cartilage and bone matrices. Under light and electron microscopes, the TrACP at pH 6.5, using a lead-salt method, was found to be localized in cartilage and bone matrices, osteoclasts, and chondroclasts. The most favorable pH was 6.5. At pH 6.6, a peak of band 5b TrACP was observed in both the heparin- and polybrene-added reaction mixtures, whereas the band 5a TrACP activity was near 0 when heparin was added. The optimum pH of band 5a TrACP was between 5.6 and 5.8. Lam et al. (15) also reported a relatively lower optimal pH (5.0) for band 5a TrACP than for band 5b (5.5–6.0) in acidic polyacrylamide gel electrophoresis. These findings show that the optimal pH for band 5b is relatively higher than that for band 5a.

Previously, Allen et al. (16) and Cheung et al. (17) showed no inhibitory effect of EDTA on TrACP extracted from human bone and cord blood. On the other hand, dithiothreitol at low concentrations has been found to enhance enzyme activity but to inhibit activity at higher concentrations and prolonged exposure (17). In our study, we also observed no effect of EDTA on band 5b TrACP but a massive inhibitory effect of dithiothreitol even at low concentrations. The explanation for this discrepancy is not available.

Using this improved method, we also measured serum band 5b TrACP activity in apparently healthy Japanese. Band 5b TrACP activity in females increased and reached a plateau at >40 years of age; activity in males increased more slowly than in females and reached the same values at >70 years of age. The higher band 5b TrACP values observed children younger than 15 years was in agreement with TrFsACP values we reported previously (12) but at a relatively low activity. The low values obtained by this method appear to be attributable to the inhibition of band 5a and other unknown TrACP isoenzymes by heparin.

In conclusion, the improved method we reported previously (12) can be used for the specific measurement of band 5b TrACP with some modification (added heparin instead of polybrene at slightly higher pH). This method is simple and may be useful in the evaluation of bone turnover.

References