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Heparin column analysis of serum type 5 tartrate-resistant acid phosphatase isoforms

Yoshihiko Igarashi[a,*], Minako Y. Lee[b], Shigeru Matsuzaki[a]

[a]*Dokkyo University School of Medicine, Department of Biochemistry, Mibu Tochigi 321-0293, Japan*
[b]*University of Washington School of Medicine, Department of Biological Structure, Seattle, WA 98195-7420, USA*

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Abstract

The objective of the present study was to develop a specific method for the separation of tartrate-resistant acid phosphatase (TRAP) derived exclusively from osteoclasts. Heparin column-bound TRAP in human serum was separated into three peaks of TRAP activity when eluted with a linear gradient of sodium chloride. The last peak corresponded to TRAP 5b which was first named according to its electrophoretic mobility [Clin. Chem. 24 (1978) 309] and was considered to be derived from osteoclasts [J. Bone Miner. Res. 13 (1998) 683]. The second peak was found to be TRAP 5a. The height of the last peak varied from age to age. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Heparin column; Enzymes; Tartrate-resistant acid phosphatase

1. Introduction

There are four isoenzymes of acid phosphatase (EC 3.1.3.2) in human serum, i.e., erythrocytic, lysosomal, prostatic, and macrophagic acid phosphatase [1]. The erythrocytic, platelet [2] and macrophagic forms are resistant to inhibition by dextrorotatory tartrate. Tartrate-resistant acid phosphatase (TRAP), one of the macrophagic acid phosphatases, is regarded as a marker for osteoclasts and is closely associated with bone resorption [3]. It is inhibited by fluoride ion [4,5], while the erythrocytic and platelet acid phosphatases are not.

Physiological functions of TRAP in the osteoclast are not fully understood [6–8]. TRAP-gene-knockout mice show only moderate osteopetrosis [9]. High blood levels of TRAP are associated with active bone remodeling [10,11]. For example, elevated serum TRAP levels have been reported in Paget's disease of the bone [13], hemodialysis [14], primary hyperparathyroidism [15], metastatic malignancies involving bone resorption [16] and multiple myeloma [17]. Very recently, transgenic mice overexpressing TRAP were shown to exhibit an increased rate of bone turnover developing osteoporosis [12]. These findings provide evidence that TRAP is involved in normal bone homeostasis in vivo.

The TRAP isoenzymes have long been analyzed using electrophoresis [18], CM-Sepharose column chromatography [19] or isoelectric focusing [20]. TRAP was named as type 5 acid phosphatase based on its fast electrophoretic mobility toward the cathode on acidic acrylamide gel [21]. The band 5 was further separated into bands 5a and 5b by

*Corresponding author. Tel.: +81-282-872-127; fax: +81-282-867-268.
E-mail address: bioiga@dokkyomed.ac.jp (Y. Igarashi).

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electrophoresis [19]. TRAP 5b was likely to be derived from osteoclasts and TRAP 5a from some other cells [22]. These analytical methods require a large amount of serum to detect TRAP activity. Thus, they could not be utilized for diagnosis of above-mentioned diseases and other bone metabolic diseases. TRAP 5b assays for diagnosis in human serum using two different methods have been published recently. Nakanishi et al. [23] developed a kinetic assay method for detecting band 5b TRAP activity using an inhibitor for band 5a. Halleen et al. [24] developed a novel assay method specific for 5b using a monoclonal antibody. However, their antibody did not distinguish 5b from 5a. The present study deals with a heparin column analysis of TRAP that has been improved to separate TRAP 5b from erythrocytic TRAP and TRAP 5a acid phosphatase.

2. Experimental

2.1. Samples

All the blood samples were collected without using heparin. Sera from cord blood were stored at −80°C. All the blood samples were obtained after informed consent. Sera obtained from children of 5 to 11 years of age (average 7.4 years), were collected in small volumes and stored at −80°C. Before applying to the column, these sera were pooled. Sera of healthy adult volunteers were individually analyzed immediately after sampling.

2.2. Column

Heparin columns, HiTrap Heparin (5 ml) were purchased from Amersham Pharmacia Biotech. A BioLogic HR system (Bio-Rad) was used to analyze human sera attaching to the heparin column at 4°C. The serum was dialyzed against 20 m*M* Tris–HCl, 0.1 *M* NaCl, pH 7.2. After filtration, 2–10 ml of serum was applied to a 5-ml heparin column with a flow-rate of 0.5 ml/min. The column was washed with 72 volumes of equilibrated buffer and eluted by a linear gradient of sodium chloride from 0.1 to 1.05 *M*. The eluted heparin column was regenerated following the manufacturer's instruction.

2.3. Enzyme assay

TRAP activity was analyzed by a colorimetric method [10]. The final substrate concentration of *p*-nitrophenyl phosphate in the assay medium was 100 m*M* with 40 m*M* sodium (+) tartrate and with or without 45 m*M* sodium fluoride in 200 m*M* citrate buffer, pH 5.5. A 100-μl volume of reaction mixture was incubated at 37°C for 1 h, and the reaction was stopped with 50 μl of 0.1 *M* NaOH. The absorbance of the mixture was measured at 415 nm vs. a reference wavelength at 492 nm using an MTP-32 microplate reader (Corona Electric, Japan). Controls; NaOH was added just before addition of the fractionated serum to reaction buffer and incubated as the samples.

2.4. Electrophoresis

Native (un-denatured) acid phosphatases were analyzed by electrophoresis on acid acrylamide disk gel [18]. A separating gel was 7.5% acrylamide [acrylamide–bisacrylamide 30:0.8 (g)/100 ml] and was polymerized with 0.14% ammonium persulfate and 0.5% (v/v) *N,N,N′,N′*-tetramethylethylenediamine (TEMED) in 375 m*M* potassium acetate buffer, pH 3.9. A 1-ml volume of the mixture was poured into a 70×5 mm I.D. glass tube. A stacking gel consisted of 2.5% acrylamide [acrylamide–bisacrylamide 10:2.5 (g)/100 ml] in 250 m*M* potassium acetate buffer, pH 4.5. The stacking gel was diluted with the same volume of distilled water and was polymerized by light with riboflavin (0.5 mg/100 ml) and 0.058% TEMED. It was piled 5 mm on the separating gel. A 100-μl volume of sample was mixed with the same volume of 50% glycerol containing 4.5% bovine serum albumin (BSA). All of this mixture was applied on the stacking gel. Electrophoresis was performed in 35 m*M* β-alanine, pH 4.0 at 4°C adjusting 5 mA per tube for 2 h.

Ampholine PAGplate (pH 3.5–9.5) were purchased from Amersham Pharmacia Biotech. The electrophoresis was performed 1500 V for 3.5 h following the manufacturer's description. Samples

were run along along side. The activity staining of TRAP was performed according to Lee et al. [25].

3. Results

An elution pattern of cord serum showed an unbound peak and three TRAP active peaks after separation with a linear gradient of sodium chloride (0.1 to 1.05 *M*) (Fig. 1a). The serum proteins that were measured using UV absorbance at 280 nm were separated into two UV peaks; one of them was unbound (95% or more) and the other (less than 5%) was eluted at the same peak as the first TRAP activity peak. The last peak of TRAP activity eluted was the highest among the TRAPs bound on the heparin. The tartrate and fluoride ion resistant acid phosphatase (TFRAP) activity were eluted at the unbound peak and at the first eluted peak, respectively (Fig. 1b). However, peaks of TRAP and TFRAP were not necessarily eluted at the same position. Not all the fractions of the first peak (peak I) were inhibited to the same extent by fluoride; later fractions in this peak were rather resistant to fluoride. Thus, the TFRAP of peak I eluted a little later than the TRAP. When a small amount of hemolyzed serum (15 μM on the basis of heme [26]) was applied, the elution pattern of TRAP and TFRAP activity became quite different as shown in Fig. 1c. Two large peaks of TFRAP appeared in the hemolyzed serum. One of them was the unbound peak and the other was peak I eluted from the heparin column. The second (peak II) and the third (peak III) peaks were not affected by hemolysis in the absence of fluoride ion. When TRAP was inhibited by fluoride, peaks II and III disappeared almost completely. Thus, it was shown that the erythrocytic TRAPs were eluted at the unbound and the peak I on heparin column chromatogram. The TRAP activity appeared only at the unbound peak when a dialyzed hemolysate was applied to the heparin column (data not shown); no peaks appeared after elution with NaCl. Re-chromatography of unbound fractions of this experiment showed essentially no TRAP peaks in the bound eluted fractions. Therefore, it is very unlikely that non-binding of some TRAPs was due to column overload; band 5 TRAP in 6 ml hemolysate serum did not overload the heparin column.

The typical elution patterns of TRAP from cord, child and adult serum are shown in Fig. 1d. We could not collect enough volume of serum from a single child to analyze the TRAP activity by a heparin column. It is reported that there is little sex difference in the TRAP activity of the children, less than 10 years of age [27,28]. Therefore, the sera from two boys and three girls (average age was 7.4 years) were pooled for the enzyme analysis. The chromatographic analysis showed peak III of the sera from children was 2.5 times higher than that of cord serum. In contrast, the elution pattern of an adult serum showed that the height of peak III was approximately one-third that of cord serum. When heparin columns were loaded with 2 to 8 ml of cord serum, the enzyme activities in the eluted fractions were proportional to the serum volume applied to the column. Therefore, the sample volume was not exceeding the capacity of the column we used.

To assess the stability of the TRAP activity, the dialyzed cord serum was stored at 4°C for 38 days and was analyzed by heparin columns on the fourth, 21st and 38th days. Each elution pattern was found to be similar (data not shown).

Each of the three peaks eluted with NaCl was concentrated using centrifugal filter devices. Aliquots of each fraction were applied to the acid polyacrylamide gel electrophoresis and TRAP was stained with pararosaniline as a coupling dye (Fig. 2). The results showed that peaks I and III corresponded to TRAP 5b and the peak II to TRAP 5a which were named by Lam et al. [19].

The TRAP activity of the concentrated peak III in cord serum was analyzed by the isoelectric focusing as described in Experimental (Fig. 3a, lane 1). The main band of TRAP activity was found close to the cathode and a minor band at center of Ampholine flat gel, pH range 3.5–9.5. When peak III of child and adult TRAPs was applied to Ampholine gel (Fig. 3a, lanes 2 and 3, respectively), the main band was found at the same position as that of the cord TRAP (alkaline position).

The pH optima for these three types of cord TRAPs were tested to examine their enzymatic

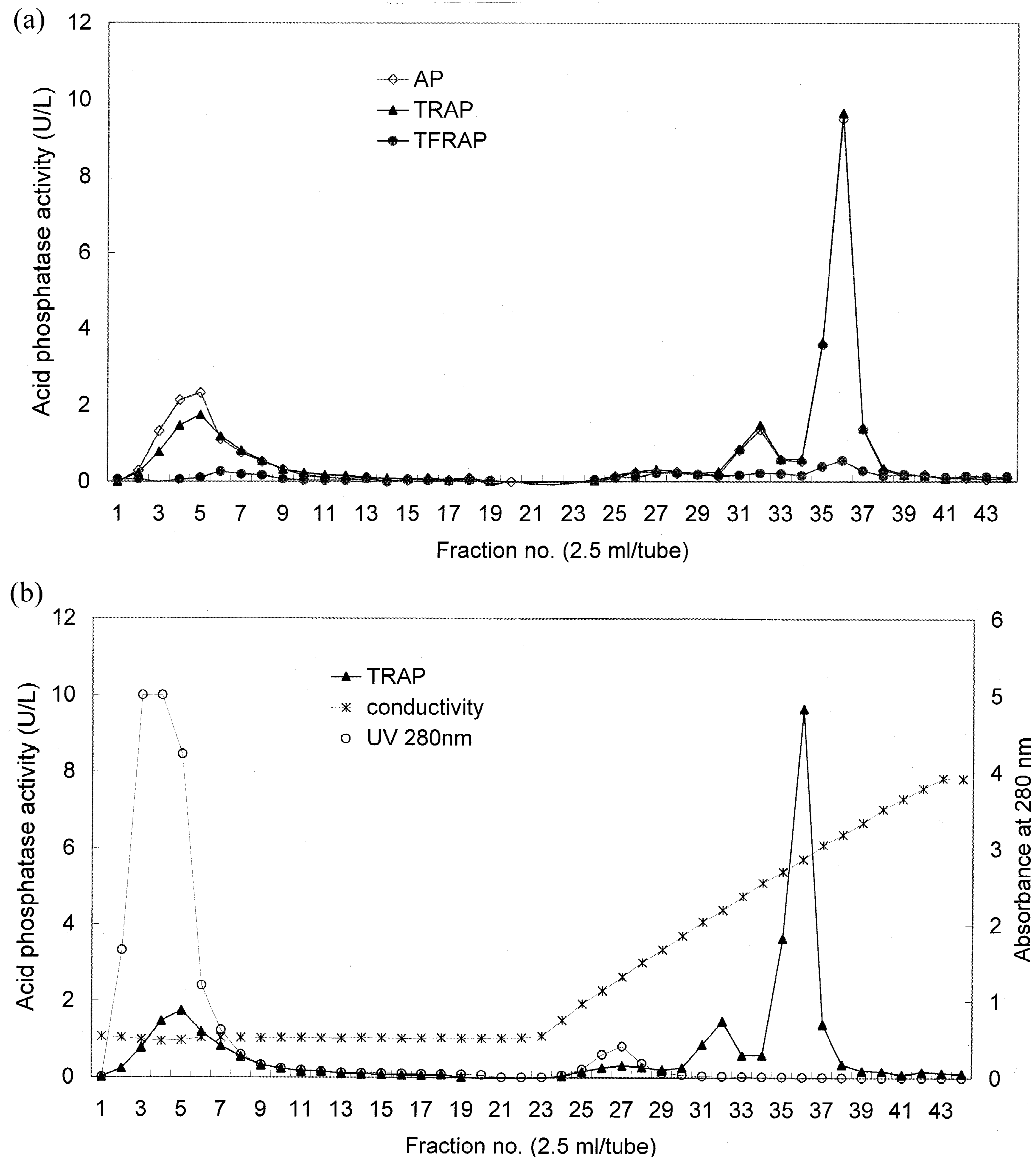
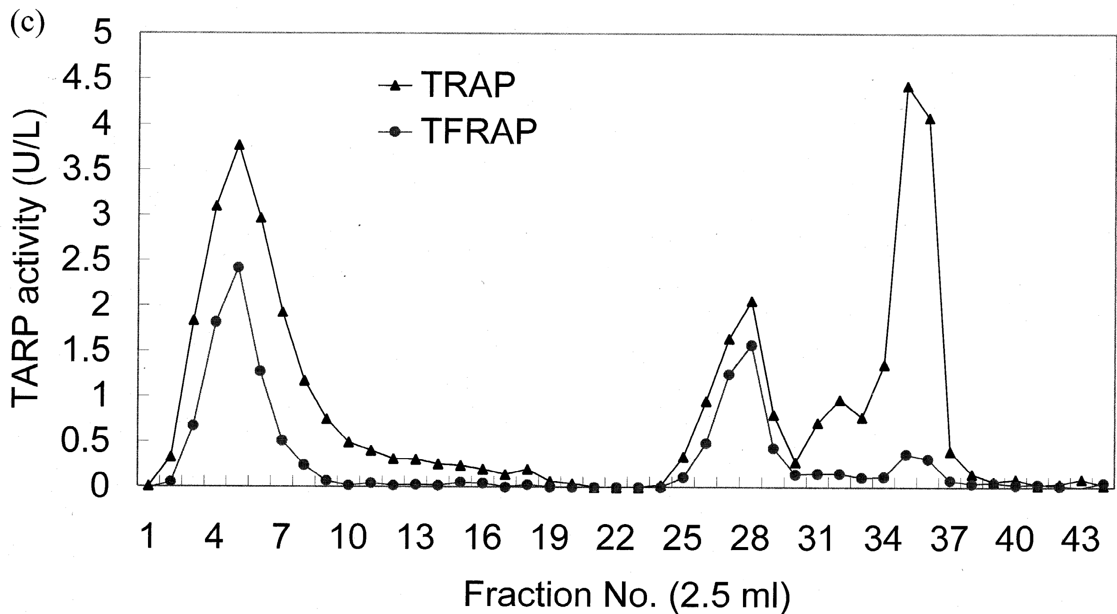


Fig. 1. Separation. (a) The chromatographic separation of acid phosphatase activity in the cord serum on the 5 ml of the heparin column. The cord serum was dialyzed against the equilibrium buffer; 20 m*M* Tris–HCl, 0.1 *M* NaCl, pH 7.2. A 6-ml volume of the serum was applied to the heparin column. Fractions (2.5 ml each) were collected with the equilibrium buffer (fractions 1–20), and the bound TRAPs were eluted by a NaCl linear gradient from 0.1 *M* to 1.05 *M* (star) and fractionated 2.5 ml each. Between fractions 20 and 21, the column was washed with 72 volumes of the equilibrium buffer. The protein was monitored using UV absorbance at 280 nm (open circle). The activity of eluted TRAPs was assayed as described in Experimental (filled triangle). (b) The activity of acid phosphatase was assayed without inhibitor in 0.1 *M* citrate buffer at pH 5.5 (open diamond). The activity of eluted TRAPs was assayed with (filled circle) and without (filled triangle) 25 m*M* of sodium fluoride. (c) The elution profile of the cord serum containing hemolysate. The activity of TRAPs was assayed with (filled circle) and without (filled triangle) 25 m*M* sodium fluoride. (d) The elution profiles on heparin column of the cord, child and adult serum. An 8-ml volume of each serum was applied on the heparin column. Unbound TRAPs were fractionated 5 ml each from fractions 1 to 10. After washing the column, TRAPs were fractionated 2.5 ml each from fractions 11 to 34. Numbers in parentheses show the age.



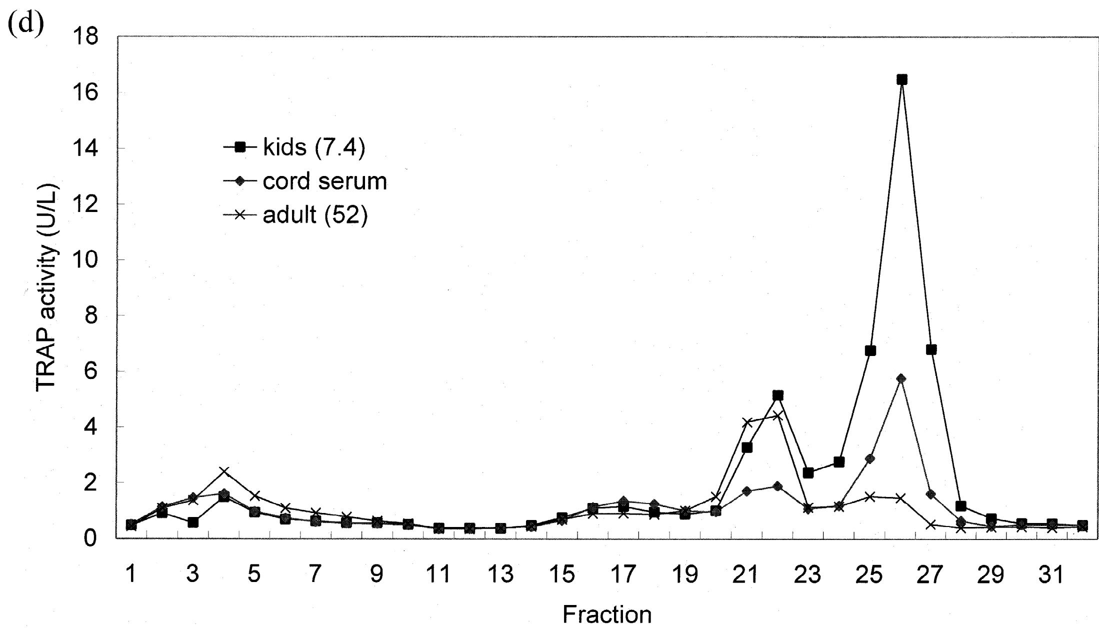


Fig. 1. (*continued*)

characteristics (Fig. 3b). The optimal pH of peak I and peak II was pH 5.5 and that of peak III, pH 6. There was a slight difference in pH optimum between peak II TRAP and peak III TRAP.

4. Discussion

For decades, total TRAP activity has been regarded as a bone resorption marker in the serum

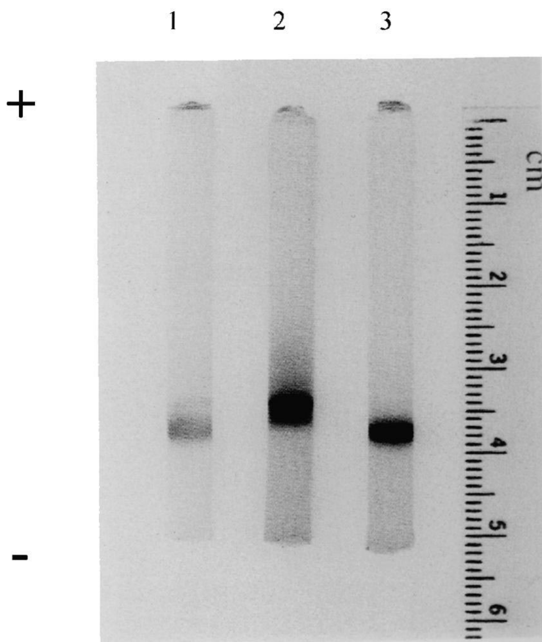


Fig. 2. Identification. Electrophoretic analyses of cord serum TRAP separated on the heparin column. Lane 1 corresponds to peak I of the heparin column chromatography. Lane 2 corresponds to peak II and lane 3 to peak III.

[28,29]. In serum, total TRAP activities are derived from bone, erythrocyte and platelet. Band 5a has never been identified in any other sources except hairy cells [30]. Halleen et al. purified band 5 TRAP from human bone that had the molecular mass of 32 000 and the optimum pH of 5.7 and contained no sialic acid [31]. It has been shown that band 5a TRAP is sialylated but 5b is not [22]. Thus, the band 5b TRAP must be derived from bone. The measurement of TRAP 5b instead of total TRAP in the serum would reflect the status of bone resorption more accurately. We found that young people whose bone remodeling is active had a high peak III, while elderly people have a much lower peak III. In Fig. 1d, serum from children demonstrated higher peak III than peak II. Thus, this heparin column can separate distinctly the TRAP 5b peak from the 5a. Peak II of TRAP was nearly constant for the children and the adults except for the cord serum. Total TRAP could serve as a bone resorption marker only in children [27,32]. Because TRAP 5b peak has very low activity in the adult serum, total TRAP activity would not reflect bone resorption. This is the reason why TRAP 5b should be analyzed correctly for evaluation of bone resorption. Nakanishi et al. [23] claimed that 5b activity could be determined in the presence of heparin at pH 6.6. They showed a good correlation between TRAP 5b and total TRAP (excluded TFRAP). However, they neglected unbound TRAP; total TRAP contained higher than 10 U/l activity compared with TRAP 5b. On the heparin column, unbound fractions contained three type of acid phosphatases, i.e., acid phosphatase, TRAP and TFRAP activity. The ratio of TFRAP to TRAP was very low (1:5) in this peak. The ratio of acid phosphatase to TRAP was near unity. It means that the unbound peak contains not only erythrocytic TRAP but also other TRAPs. As an inevitable consequence of the hemolysate, total TRAPs would be increased. The analysis done by our heparin column method was not affected by hemolysate as shown in Fig. 1c. Our results show that heparinized sera are not suitable for the estimation of TRAP activity because heparin binds TRAP and partially inhibits TRAP activity. TRAP 5b bound to the heparin column stronger than 5a at pH 7.2 and TRAP 5b activity was inhibited about 10% with heparin (25 U/ml final). In our assays p-nitrophenylphosphate (pNPP) was used as the substrate of TRAP at pH 5.5. Nakanishi et al. used 2,6-dichloro-4-acetylphenyl phosphate as the substrate and assayed at pH 6.6. The apparent discrepancy of our results and those of Nakanishi et al. may be partially explained on the basis of different substrate and different pH optima in assays.

Halleen et al. showed that monoclonal antibody O1A captured TRAP 5 (5a and 5b) [24]. The activity of TRAP 5b was near optimal at pH 6.1, but 5a that has the optimal pH of 5.0 was less than one fifth of its maximum activity at pH 6.1. Consequently their captured TRAP 5 assay detects only TRAP 5b at pH 6.1. In our experiments (Fig. 1d), TRAP 5a in the sera of elderly persons was usually higher than 5b; it was not so rare to find TRAP 5a to be more than three times as high as TRAP 5b. In such sera, the Mab O1A method may overestimate the actual TRAP 5b levels. Alternatively, anti-TRAP 5b antibody can be useful for the development of enzyme-linked immunosorbent assay (ELISA) for TRAP assay but any attempt to obtain a highly specific

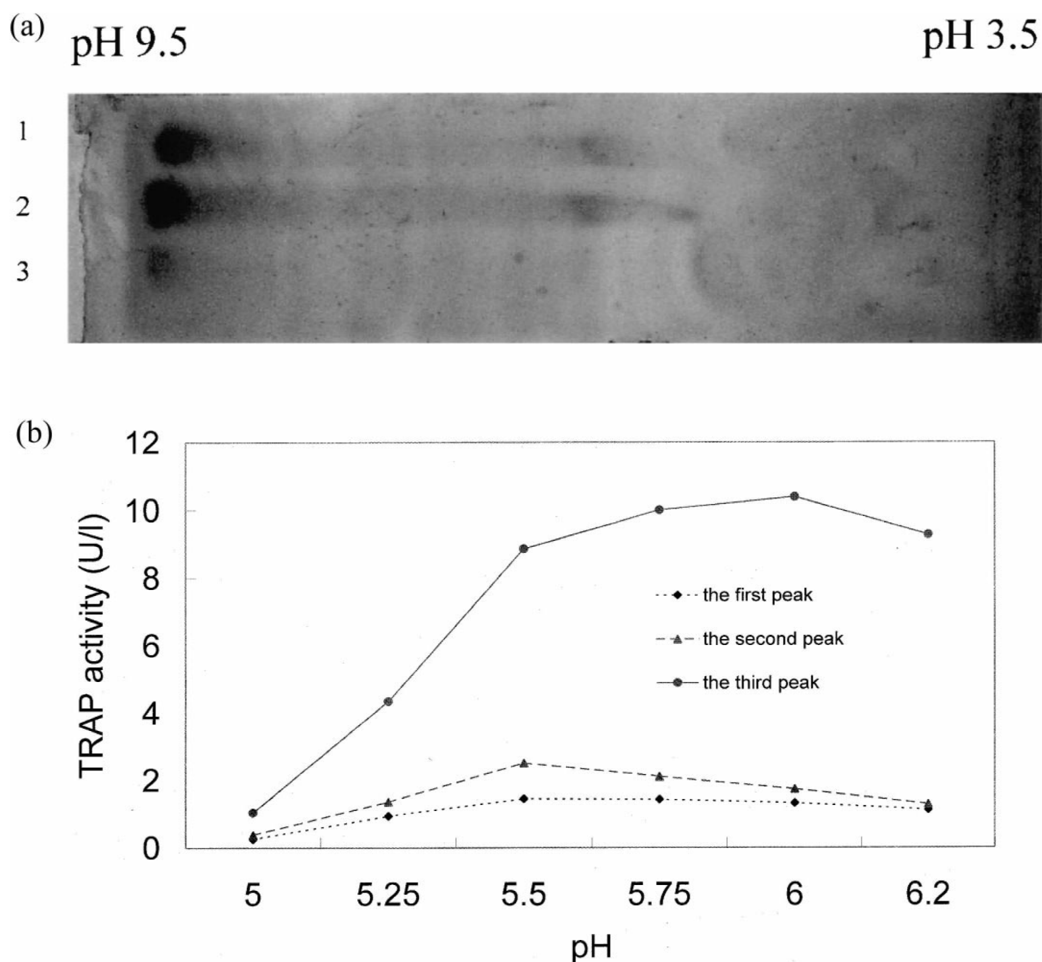


Fig. 3. Characterization. (a) Isoelectric focusing of the peak III of TRAPs. Lane 1 corresponds to cord sera. Lane 2 corresponds to child sera. Lane 3 corresponds to adult sera. Electrophoretic conditions are as described in Experimental. (b) pH effect on acid phosphatase activity. The enzymes in the cord serum separated on the heparin column were assayed at different pH values of 100 m*M* citrate buffer as mentioned in Experimental. The each top peak fraction separated was assayed. Peak I (filled diamond), peak II (filled triangle) and peak III (filled circle).

antibody has been without success to date [24,29,33]. Our method utilizing the peak III TRAP may be much better to estimate the bone turnover than other methods for the present. One cannot neglect the possibility, however, that some TRAP activity derived from alveolar macrophages is contaminated.

The TRAP gene is a single one in the human genome and only a single mRNA species is detected [34]. Alternative splicing or post-transcriptional modification would occur in TRAP RNA [35] or enzyme [36]. The origin of TRAP 5a is not known so far [33]. Peak I of the eluted fractions might be a part of TRAP 5b that was shown by the acid acrylamide electrophoresis. However, the peak position of macrophagic TRAP was not coincident with the erythrocytic TRAP that was somewhat shifted to higher salt concentrations. The first TRAP peak may consist of at least two different enzymes; one is fluoride sensitive and the other is resistant to this anion.

The results of our present study have shown that TRAPs 5b and 5a of acid phosphatase are distinctly

separated as peaks III and II, respectively, by pre-packed heparin column chromatography. This method would be useful for the purification of TRAPs 5a and 5b. There was a slight difference in pH optimum between the peak II TRAP (pH 5.5) and the peak III TRAP (pH 6) as shown in Fig. 3b. These pH optima for peak II and III TRAPs are similar to those of TRAP 5a and 5b acid phosphatase from human serum [19], respectively. Band 5 acid phosphatases have usually been separated using CM-Sepharose [19]. This method can be applicable to the estimation of bone resorption in child sera but not to adult sera, because CM-Sepharose bound the TRAP so strongly that the small amount of adult TRAP attached to the resin could not be eluted efficiently.

The isoelectric point (p*I*) of TRAP in the cord serum has been estimated to be 7 using Rotofor preparative iso-electric focusing [37]. In this report, the p*I* of the cord TRAP was an alkaline pH at close cathode for main band and a minor band neutral pH. The main bands of child and adult TRAPs were moved to the same position as the cord TRAP. The p*I* of adult bone TRAP was estimated approximately 9 by Ampholine gel [34]. The p*I* of cord TRAP was not different from that of the adult TRAP. Sialic acid molecules attached on the 5a polypeptide decrease the p*I* of 5a and/or shift a different three-dimensional structure from 5b [22]. These changes of sialic acid induce a slightly different binding property to the heparin column as peaks II and III in Fig. 1a.

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