Highly Sensitive Solid-Phase Immunoenzymometric Assay for Placental and Placental-Like Alkaline Phosphatases with a Monoclonal Antibody and Monodisperse Polymer Particles

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We have used a mouse monoclonal antibody (H7) to placental alkaline phosphatase (PLAP, EC 3.1.3.1) in developing an immunoenzymometric assay for PLAP and PLAP-like enzymes. The antibody is bound to sheep anti-mouse IgG (Ab2) covalently coupled to tosylated shell-and-core light (1.07 g/cm3) monodisperse polymer particles. Adding the H7-Ab2-polymer particle suspension to a PLAP-containing sample gives maximal binding of the antigen within 10 min. PLAP and PLAP-like enzymes remain active and bound to the solid-phase throughout all assay manipulations, and can thus be saved for future testing. In testing the enzymes for inhibition by L-Phe, L-Phe-Gly-Gly, l-Leu, and l-homoarginine, the effect of all the inhibitors is fully reversible. The assay is highly versatile, and its sensitivity (routinely 0.05 µg/L) can be increased 1000-fold by adjusting the sample volume and incubation time (sample volume is irrelevant between 50 µL and 5 mL). We have measured the basal activities of PLAP in men and women and, by using enzyme inhibitors, have characterized it as corresponding to the PLAP-like phenotypes described in normal human tests.

Additional Keyphrases: isoenzymes · enzyme inhibition assay · phenotypes · reference interval · cancer

Human placental alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, PLAP) is a polymorphic enzyme, expressed in high amounts by the syncytiotrophoblast after the 12th week of pregnancy (1, 2). Trace activities of PLAP have also been demonstrated in normal cervix (3, 4) and lung (5).

PLAP-like enzymes are expressed in trace amounts in normal human testis (5, 6) and thymus (5), and are present in high concentrations in tumor tissue as well as in the serum and fluids of patients with different malignancies, especially seminoma of the testis (7–9) and ovarian tumors (10–12).

PLAP and PLAP-like enzymes can be distinguished by use of carefully cross-absorbed polyclonal antibodies (13), or monoclonal antibodies (14), and by their differential susceptibility to inhibition by hydrophobic amino acids and peptides (15, 16). Some investigators have suggested that PLAP and PLAP-like enzymes may result from the expression of two different structural genes (13, 14, 17).

Here we report our development of an immunoenzymometric assay (IEMA) in which the solid-phase reagent is a murine monoclonal antibody (H7) that reacts with an epitope common to the allelic variants of PLAP and the different phenotypes of PLAP-like enzymes. The solid-phase matrix of the assay is monodisperse polymer particles. The assay is fast and simple, is much more sensitive than other currently available methods, and allows quantification of PLAP and PLAP-like enzymes in biological fluids. By using this assay in conjunction with enzyme inhibitors, we have been able to measure and characterize previously undetectable basal concentrations of PLAP-like antigen in sera of healthy individuals.

Materials and Methods

Samples

Serum samples were obtained from healthy blood donors attending the State Serum Institute, Copenhagen, Denmark, and from women in their 15th to 16th week of pregnancy, who were undergoing routine screening for fetal neural tube defects at the State Serum Institute.

Reagents

Antibodies. The H7 monoclonal antibody was produced as described (14) by fusing spleen cells of BALB/c mice (immunized with purified FS phenotype of PLAP) with X63-Ag8 P856 myeloma cells (15). The H7 monoclonal antibody is of IgGk κ subclass, and reacts with the common phenotypes of PLAP as well as with all the phenotypes of testicular PLAP-like enzymes (14). Sheep anti-mouse IgG were produced and purified as previously described (20).

Hydropellic monodisperse particles. We used monodisperse polymer particles (MPP) of the shell-and-core type prepared by copolymerization of methylicacrylate, hydroxyethyl methacrylate, and ethylene glycol dimethacrylate in the presence of particles of methylisobenzene, 2.9-µm in diameter, according to Ugelstad et al. (21). The diameter of the final particles was 3.3 µm, the density 1.07 g/cm3, and the surface area 1.7 m2/g. The particles are commercially available as Dynospheres® XP 4101 (Dyno Industrier A/S, Oslo, Norway).

Solid-phase reagent. Sheep anti-mouse IgG (Ab2) was bound to p-toluensulfonyl chloride-activated (tosylated) MPP as previously described (22). To bind the H7 monoclonal antibody to the MPP-Ab2, we incubated overnight, with end-over-end rotation, the H7 monoclonal antibody/particle mixture, including trace amounts (20 000 cpm) of 125I-labeled H7 monoclonal antibody, to monitor the effectiveness of the binding procedure. The solid-phase reagent thus prepared was extensively washed with pH 7.5 assay buffer. The buffer contained, per liter, 50 mmol of Tris, 0.15 mol of NaCl, 0.1 g of bovine serum albumin, 0.1 mL of Tween 20 (polyoxyethylene (20) sorbitan monolaureate; ICI Americas, Inc.), and 0.1 g of thimerosal. The reagent was then diluted so as to contain 0.2 µg of H7 monoclonal antibody per 100 µL of particle suspension. Upon centrifugation, the particles

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4 Nonstandard abbreviations: PLAP, placental alkaline phosphatase; MPP, monodisperse polymer particles; Ab, antibody.
form a firm pellet, approximately 1 μL in volume, which is
easily dispersed upon addition of wash buffer or substrate.

**Purified PLAP.** A placenta containing the FF phenotype
of PLAP was obtained shortly after delivery, homogenized, and
purified by a combination of methods as reported
elsewhere (23). The homogeneity of this purified PLAP was
determined by sodium dodecyl sulfate/polyacrylamide gel
electrophoresis under reducing conditions and by cross-
immunoelectrophoresis. Its specific activity was 1200 KU/g
under the assay conditions reported here. To prepare stan-
dards, we added increasing amounts of PLAP (0.2 to 10
μg/L) to pooled human sera containing no detectable PLAP-
lke antigen as determined by radioimmunoassay (23). One
d hundred milliliters of standard at each concentration was
prepared, aliquoted, and stored at -70 °C for future use.

**Procedures**

**Catalytic assays.** We measured alkaline phosphatase ac-
tivity as recommended by the Scandinavian Society for
Clinical Chemistry and Clinical Physiology (24), using a
substrate solution of 1 mol/L diethanolamine buffer, pH 9.8,
containing 0.5 mmol of MgCl2 and 10 mmol of p-nitrophenyl
phosphate (Sigma Chemical Co., St. Louis, MO) per liter.
For the inhibition studies, we added individual inhibitors to
this basic buffered substrate solution at the following con-
centrations: L-homoarginine (5 mmol/L), L-Phe (5 mmol/L),
L-Phe-Gly-Gly (0.5 mmol/L), and L-Leu (1 mmol/L).

**Assay procedure.** Incubate samples (50 μL, or as specified)
in polystyrene disposable tubes with 100 μL of MPP-Ab2-H7
substrate for 30 min. Next, using an Oxford pipetor, add 2
μL of assay buffer, and centrifuge for 10 min at 1500 × g.
Decant the supernatant fluid by inversion, and wash the
pellet with 2 mL of assay buffer. Add 0.5 mL of buffered
substrate solution to each tube, vortex-mix, then incubate in
a water-bath at 37 °C for 3 h. To stop the enzymic reaction,
add 0.5 mL of 40 mmol/L L-Phe in diethanolamine buffer
to each tube. Vortex-mix again, centrifuge for 10 min at 1500
× g, then transfer 200 μL of the supernates to a 96-well
microtiter plate and record the absorbance at 405 nm (we
used a Titertek Multiskan spectrophotometer from Flow
Labsatories, McLean, VA). Concurrently, process stan-
dards containing known amounts of PLAP (0.2 to 10 μg/L)
in the same way. Check all tubes periodically during the
incubation with substrate; if an assay tube develops color
faster than the 10 μg/L standard tube (500 pg of bound
PLAP), stop that reaction earlier than for the standard
(curve, i.e., at 30, 60, 90, or 120 min). Read the results from
the standard curve, then multiply by the appropriate time
factor.

**Results**

**Assay conditions.** In the present immunoassay for PLAP,
the enzymic activity of the antigen is used as the detection
step, after specific binding to a solid-phase monoclonal
antibody, i.e., MPP-Ab2-H7. As Figure 1 shows, the reaction
rate is proportional to the amounts of bound PLAP.
The quantity of monoclonal antibody per assay tube (0.2 μg)
suffices to bind as much as 10 ng of PLAP under the
specified assay conditions, i.e., 20-fold the amounts shown
in Figure 1. Because of the unusual heat stability of PLAP,
the incubation time can be greatly prolonged without damage to
the catalytic activity of the enzyme. Thus by changing
incubation time the sensitivity of the assay can be adjusted
as needed.

The Michaelis–Menten constants (Km) of free PLAP and
testis PLAP-like enzyme, as well as the Km of the enzymes
bound to the MPP-Ab2-H7 antibody, are identical (0.30–0.34
mmol/L), thus validating the use of this H7 monoclonal
antibody IEMA for quantifying PLAP-like enzyme by using
known concentrations of PLAP as standard. Furthermore, if
the specific activity of PLAP is known, PLAP and PLAP-
lke enzyme can be quantified without use of a standard
curve.

The advantage of working with uniform-size polymer
particles as solid-phase matrix can be fully realized from
the next two experiments. Figure 2 shows a comparison of the
effectiveness of binding of PLAP by the H7 monoclonal
antibody bound to monodisperse polymer particles (Figure
2A) or bound to a microtiter well (Figure 2B). Uptake of
PLAP by the MPP-Ab2-H7 solid-phase reagent is complete
within 10 min of reaction, while nearly 24 h are needed for
the complete uptake of PLAP by the H7 antibody when
bound to microtiter wells. This difference is most probably
attributable to the very effective interaction between antigen
and antibody provided by the homogeneous and stable
suspension of these low-density polymer particles, because the
amount of antibody and total exposed area are identical
in both assays.

Another important advantage of the polymer particles
system with respect to microtiter plate systems is exemplif-
ied in Figure 3. When microtiter wells are used, sample
volume is usually restricted to 200 μL. When the MPP
suspension is used, sample size can be varied from 50 μL up
to 5 mL, and uptake of the antigen is linearly related to
volume. Thus, by increasing sample size alone, the sensitivity
of this IEMA can be increased 100-fold.

**Inhibition studies.** The H7 monoclonal antibody used in
this assay shows very high affinity for PLAP, approximately
1.3 × 1011 L/mmol (18). The antigen can only be eluted from
H7 immunosorbent columns by highly denaturing condi-
tions such as use of 5 mmol/L phosphate buffer (pH 12) or 4.5
mol/L KSCN (25). Under the present assay conditions (1
mol/L diethanolamine buffer, pH 9.8) there is no appreciable
loss of bound antigen. After sequential exposure to dieth-
anolamine buffer (pH 9.8) and assay buffer (pH 7.5), and
even after continuous exposure to pH 9.8 for 48 h, 98 to
100% of the enzyme remains bound, as shown by tests of
total bound [125I]labeled PLAP (not shown) and total bound
alkaline phosphatase activity (see below).

The experiment depicted in Table 1 was designed to study
the reversibility of enzyme inhibition as well as the stability

![Fig. 1. Linear response with increasing amounts of bound PLAP and
prolonged incubation times](https://example.com/fig1.png)
of the enzyme upon alternate exposure to substrate and "washing" solutions. Five sets of tubes (labeled 1–5, triplicate assays) containing the same amount of MPP-Ab2-H7-bound PLAP, 5 ng, were exposed to substrate solutions for 30 min, to measure total activity of PLAP (cycle 1). After washing as described above in Materials and Methods, substrate (tube 1) or substrate plus inhibitors (tubes 2–5) was added to the tubes and incubated for 30 min to measure activity remaining (and percent inhibition) in the presence of L-homogarginine, L-Phe, L-Phe-Gly-Gly, and L-Leu (cycle 2). After washing, substrate was once again added to all tubes to measure the total activity that could be accounted for upon "washing away" the inhibitor (cycle 3). Thereafter, each substrate–inhibitor solution was tested on each set of tubes with alternating washing and total-activity determinations (cycle 4–9). The set of tubes labeled 6 was not exposed to buffered substrate solutions until the last cycle. The activity of this "virgin" set was identical to the activities of the sets subjected to the eight previous cycles, indicating the complete stability and analytical recovery of the bound PLAP activity. These experiments indicate that (a) the inhibition of PLAP by the different amino acids and peptides is reversible, because full enzymic activity is accounted for after "washing away" the inhibitor, and (b) similar inhibition results are obtained when identical parallel samples are subjected to different inhibitor solutions, or when the same single sample is treated with different sequential washings and inhibitor solutions. In all cases, the inhibition profiles of the bound enzyme were identical to those patterns previously obtained with the enzymes in solution (17). The commonly used means of stopping the alkaline phosphatase enzymic reaction (with p-nitrophenyl phosphate as substrate), addition of 1 mol/L NaOH, causes a considerable increase in pH, which will denature the enzyme and cause its dissociation from the antibody. The effect of the enzyme inhibitors is fully reversible (Table 1), so they can be successfully used to stop the enzymic reaction, without denaturing the enzyme. Thus by the addition of L-Phe to the reaction mixture (20 mmol/L final concentration) and immediate centrifugation (i.e., by physically removing the enzyme) the enzymic reaction of PLAP and PLAP-like enzymes is effectively stopped and full activity remains when the inhibitor is "washed away." Guided by this principle, we have used the same standard-curve tubes for more than 20 cycles with no appreciable loss of enzymic activity.

Analytical variables. This assay gives highly satisfactory results in terms of precision and reproducibility. Analysis of two different serum pools containing different amounts of PLAP gave the following within-assay variation (n = 19 determinations each): 5.9 (SD 0.20) μg/L (CV 3.4%) and 1.2 (SD 0.10) μg/L (CV 8.3%). The between-assay variation calculated on the same samples on 10 consecutive days was 5.8 (SD 0.4) μg/L (CV 6.9%) and 1.2 (SD 0.15) μg/L (CV 12.5%).

Concentrations of PLAP or PLAP-like enzymes in serum. The present assay offers unique possibilities to measure and characterize extremely low concentrations of PLAP-like enzyme such as those present in a normal, healthy population. Using a 1-mL sample volume, we measured PLAP-like antigen concentrations in healthy nonpregnant individuals. The distribution of these values (Figure 4) is in agreement with the result reported previously for 126 normal persons, as obtained by using an enzyme immunosassay (26) with a sensitivity of 0.4 μg/L, where 70% of the population had values for the antigen that were below the level of detection. The upper limit of normality, defined as the 100 percentile, nevertheless seems to be extended from their value of 1.85
Table 1. Sequential Inhibition and Recovery of Enzymic Activity

<table>
<thead>
<tr>
<th>Set</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tr>
<td>2</td>
<td>100.5</td>
<td>(P) 26.7</td>
<td>97.5</td>
<td>(PGG) 23.5</td>
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<td>(L) 93.1</td>
<td>101.8</td>
<td>(H) 94.5</td>
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<tr>
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<td>(L) 91.4</td>
<td>102.2</td>
<td>(H) 98.9</td>
<td>101.3</td>
<td>(P) 26.9</td>
<td>102.2</td>
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<tr>
<td>4</td>
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<td>(L) 84.8</td>
<td>98.3</td>
<td>(H) 93.9</td>
<td>100</td>
<td>(P) 26.1</td>
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<td>(PGG) 22.7</td>
<td>98.6</td>
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<tr>
<td>5</td>
<td>99.8</td>
<td>(H) 89.2</td>
<td>99.7</td>
<td>(P) 27.7</td>
<td>101.5</td>
<td>(PGG) 23.2</td>
<td>96.1</td>
<td>(L) 91.1</td>
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*Each of three tubes per set contained 5 ng of PLAP bound to MPP-Ab2-H7, and was assayed for total alkaline phosphatase activity in cycles 1, 3, 5, 7, and 9. In cycles 2, 4, 6, and 8, each set was assayed for total activity (T) or activity remaining in the presence of L-Phe (P), L-Phe-Gly-Gly (PGG), L-Leu (L), or L-homoarginine (H). The activity of each set was compared with the activity of set 1 in each cycle. All tubes were washed between cycles as described in Materials and Methods.

$^a$Mean (n = 3) percentage of total alkaline phosphatase activity.

**Discussion**

The enzyme-antigen immunoassay principle, in which the enzymic activity of PLAP is measured at the detection step, has been described previously (27, 28). The principle has been used to screen for positive hybridoma cultures secreting anti-PLAP monoclonal antibodies. An enzyme immunoassay involving the H317 monoclonal antibody (29) has been used to measure PLAP concentrations in serum throughout pregnancy. Five monoclonal antibodies (F11, D10, C2, H7, and B10) to PLAP-allelic variants have previously been developed and characterized (14). The antibodies react with epitopes mapped on two different antigenic domains of the molecule, and they detect structural differences between PLAP-allelic variants and normal (and tumor-derived) PLAP-like enzymes. One of these antibodies (H7) reacts with an epitope common to all forms of PLAP.

Table 2. Inhibition of MPP-Ab2-H7-Bound PLAP and PLAP-Like Enzymes by Some Hydrophobic Amino Acids and Peptides

<table>
<thead>
<tr>
<th>Source of enzymes bound by MPP-Ab2-H7</th>
<th>n</th>
<th>% inhibition, mean (and SD), by</th>
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<tr>
<td></td>
<td></td>
<td>L-Homoarg</td>
</tr>
<tr>
<td>PLAP (FF phenotype)</td>
<td>3</td>
<td>10.1 (1.0)</td>
</tr>
<tr>
<td>PLAP-like (testis)</td>
<td>3</td>
<td>21.5 (5.4)</td>
</tr>
<tr>
<td>Normal serum</td>
<td>30</td>
<td>16.5 (3.5)</td>
</tr>
<tr>
<td>Pregnancy serum</td>
<td>10</td>
<td>n.d.</td>
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and PLAP-like antigens. We have chosen to use this H7 antibody in the present assay, so as to be able to quantify PLAP and PLAP-like enzymes simultaneously. The choice of this high-affinity antibody and of a new solid-phase matrix (monodisperse polymer particles) has proven rewarding and opens a new range of possibilities for PLAP determinations.

The H7 monoclonal antibody has been bound to the particles by using a link of sheep anti-mouse polyclonal antibody with specificity for the Fc region of the mouse immunoglobulin. This sheep anti-mouse spacer arm places the H7 antibody in a very favorable orientation. In our experience, direct coupling of the antibody of interest to solid-phase particles results in a considerable loss of binding capacity. The monodisperse polymer particles we used here have a density approximating that of normal human serum. Therefore continuous mixing or end-over-end rotation is not necessary, even when large volumes of PLAP-containing samples are being incubated, because the particles will remain in a homogeneous suspension throughout the incubation (up to 24 h). This homogeneous suspension of the particles in the sample fluid provides very effective surface contact of the antibody with the antigen population. Effectiveness of binding is greatly increased as compared with the more commonly used microwell wells as solid-phase matrix, where the antigen has to diffuse toward the antigens coating the wells. Binding of the antigen to the MPF-Ab2-H7 reagent is complete within 10 min; in contrast, 24 h is required for complete binding by the H7 antibody bound to microwell particles. Thus assay time is considerably shortened.

The H7 monoclonal antibody shows very high affinity for PLAP and PLAP-like enzymes. After binding is accomplished, the association is completely stable throughout all assay manipulations. In practice this implies that precious sample material (i.e., tumor enzymes from biological fluids) can be assayed and "stored" bound to the particles. The particles can subsequently be split and assayed for the enzyme susceptibility to different amino acids (as in the present paper) or can be probed for structural differences by using monoclonal antibodies labeled with horseradish peroxidase or in assays equivalent to the "sandwich" enzyme immunoassay and immunoradiometric assay described elsewhere (14, 18).

Previously, with the polyclonal sandwich enzyme immunoassay (26), the upper normal limits of basal PLAP serum concentrations could be measured in a reference population, but 70% of the population had PLAP concentrations that were undetectable by that assay (0.4 μg/L sensitivity limit). With the present assay we could easily measure the whole distribution of normal values in a healthy reference population and, in turn, were able to characterize the antigen as corresponding to a PLAP-like enzyme by use of enzyme inhibitors. We don't know which tissue is contributing this normal basal level of PLAP-like enzyme. PLAP-like antigens have been described in normal human testis, thymus, and endometrium—any of these tissues could be responsible. A recently described immunoassay involving the H317 monoclonal antibody (29) has not detected any measurable PLAP-like enzymes in normal human testis and in normal serum, probably because of the more restricted specificity of the H317 antibody, which recognizes neither the FF-allelic variant of PLAP nor the testicular PLAP-like enzymes (McLaughlin and Johnson, personal communication, 1984). Thus, in this respect, the H317 antibody resembles the previously described F1 and C2 monoclonal antibodies (14). The present assay based on the H7 monoclonal antibody is valid for the quantification of both PLAP and PLAP-like enzymes. This specificity is essential if one is to follow values for PLAP and PLAP-like enzymes as a marker of malignant disease. Our preliminary results in the follow-up of patients with different ovarian tumors indicate that both PLAP and PLAP-like enzymes might be expressed in certain tumor patients, and even that both types of enzymes can be expressed by the same tumor, either concomitantly or sequentially, as a result of treatment.

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References


