Sensitive Fluorometry of Heat-Stable Alkaline Phosphatase (Regan Enzyme) Activity in Serum from Smokers and Nonsmokers

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We developed a simple, sensitive enzymatic assay involving the fluorogenic substrate naphthol AS-MX phosphate ([3-hydroxy-2-naphthoic acid 2,4-dimethylanilide) phosphate] to measure heat-stable alkaline phosphatase (EC 3.1.3.1), the Regan isoenzyme, in human serum. The day-to-day CV was 5.7% for a serum activity of 0.080 arbitrary units/L. Measurable amounts of enzyme were detected in most normal individuals. The mean for 51 nonsmokers was 0.068 (SD 0.037) arb. units/L; for 25 smokers it was 0.440 (SD 0.360) arb. units/L. Activity of this isoenzyme in smokers was as much as 10-fold the upper normal limit for nonsmokers. Activation of this tumor marker by smoking has not received attention hitherto. We conclude that a truly normal range can only be established among nonsmokers. The isoenzymes in smokers, nonsmokers, and pregnant women were similar in their heat stability, immunologic cross-reactivity, and inhibition by l-phenylalanine.

Additional Keyphrases: enzyme activity - reference interval - cancer

The Regan enzyme, a heat-stable isoenzyme of alkaline phosphatase (HSAP; EC 3.1.3.1) was first reported in 1968 by Fishman et al. (1). This isoenzyme has been found in tumor tissue and in the serum of patients with various neoplasms. It has many characteristics in common with the placental alkaline phosphatase of pregnancy, such as heat stability, inhibition by l-phenylalanine, immunologic cross-reactivity, and electrophoretic mobility (1-3).

It has not only been found in neoplasms but also constitutes a small fraction of the total alkaline phosphatase activity in the serum of normal individuals (4, 5).

Previous methods for determination of HSAP in cancer patients have involved such commonly used substrates as p-nitrophenyl phosphate (6), beta-glycerophosphate (7), indoxyl phosphate (8), beta-naphthyl phosphate (9), and phenyl phosphate (10). These methods, however, do not consistently detect the presence of HSAP activity in the serum of normal individuals, and they discriminate poorly between normal and abnormal results.

Ideally, assuming that oncologic gene repression normally is incomplete, an assay for a tumor marker should be sensitive enough to demonstrate detectable concentrations in serum of non-tumor subjects.

Previously, the most sensitive methods for determining HSAP have been immunological (4, 11-14) procedures. Here, we describe an enzymatic method for measuring HSAP with high sensitivity and precision, in which we use the fluorogenic substrate "naphthol AS-MX-phosphate." This substrate has been used in the past for staining alkaline phosphatase isoenzymes after electrophoresis and for measuring its total activity in serum (15, 16).

The principle of the present assay is as follows. After the serum is heat-inactivated, the residual alkaline phosphatase is allowed to react with naphthol AS-MX phosphate at pH 10.2 at 37 °C for 15 min. The reaction is stopped by adding acetone. Precipitated protein is removed by centrifugation, and the liberated naphthol AS-MX in the decanted supernate is measured fluorometrically.

One arbitrary unit of enzyme activity is defined as that activity by which 1 μmol of naphthol AS-MX per minute is liberated by the enzyme in 1 L of serum from naphthol AS-MX phosphate (5 mmol/L), at pH 10.2 and 37 °C.

Materials and Methods

Reagents. Naphthol AS-MX (3-hydroxy-2-naphthoic acid 2,4-dimethylanilide), naphthol AS-MX phosphate, and N,N-dimethylformamide were purchased from Sigma Chemical Co., St. Louis, MO 63178. Diethanolamine and acetone were reagent grade. Rabbit antisem to placental alkaline phosphatase was kindly provided by Dr. W. H. Fishman, La Jolla Cancer Research Foundation, La Jolla, CA.

Apparatus. Fluorescence was measured with a Turner Fluorometer (GK Turner Associates, Palo Alto, CA 94303); λε = 405 nm and λм = 535 nm. Corning filters 47B and 2A-12 were used.

Blood donors. Serum was sampled from 76 healthy individuals (51 nonsmokers and 25 smokers), from a pregnant woman, and from several patients with malignant disease.

Controls. Sera with high and low HSAP activity were respectively pooled, frozen in 1-mL aliquots, and subsequently treated the same as patients' samples. Stored at −20 °C, these were stable for longer than one year.

Stock standard. Naphthol AS-MX, 0.75 mmol/L, was prepared by dissolving 21.9 mg of naphthol AS-MX in 100 mL of NN-dimethylformamide. Stored in a brown bottle and protected from the light, this solution is stable for four months at 4 °C.

Standard curve. Working standards were prepared by mixing 5, 10, 15, and 20 μL of the stock standard with 1-mL aliquots of heat-inactivated pooled serum. This provides standards with concentrations corresponding to 3.75, 7.50, 15.0, and 30.0 μmol/L.

Assay buffer. Diethanolamine, 5 mol/L, was adjusted to pH 11 with hydrochloric acid. Stored at 4 °C, it is stable for one year. A 10-fold dilution of this buffer is used in preparing the substrate. The final reaction mixture, 250 μL substrate plus 250 μL of serum, will have a pH of 10.2 because of the buffering capacity of the serum.

Substrate. Naphthol AS-MX phosphate solution, 10 μmol/L, was prepared by adding 45.1 mg (or an equivalent weight corrected for water content) to 10 mL of diethanolamine buffer (stock buffer 10-fold diluted). It should be freshly prepared daily. Alternatively, if kept frozen in aliquots and protected from light, it is stable for three months at −20 °C.

Procedures

Heat inactivation of samples. We heat 1-mL aliquots of
serum, controls, and patients' samples in 12 × 75 mm glass tubes for 7 min at 65 °C, in a water bath. Plastic tubes are unsuitable because of poor heat conduction. When the contents of the tubes have cooled to room temperature, the serum is ready to be assayed. Occasionally, the serum clots during heating, but this can be avoided by allowing the blood to clot for more than 30 min before collecting the serum.

Assay procedure. Pre-warm 13 × 75 mm glass tubes—a blank and a tube for each standard, control, and patient's sample—in a 37 °C heating block or water bath for 2–5 min. Add 250 μL of substrate solution to each tube, then 250 μL of standards, controls, and patients' samples to the corresponding tubes except blank tubes. Vortex-mix, incubate for 15 min, and then stop the enzyme reaction by adding 3 mL of acetone to all tubes, including the blank tubes. Then add 250 μL of standards, controls, and patients' samples to the corresponding blank tubes and vortex-mix. Centrifuge samples and blanks at 2000 × g, decant the supernate into a fresh set of 13 × 100 mm test tubes, and measure the fluorescence of the samples vs. blanks within 30 min.

Neutralization of HSAP with rabbit anti-human placental alkaline phosphatase antibody. After incubating heat-inactivated normal human serum with increasing concentrations of antibody for 4 h at room temperature, we measured the residual alkaline phosphatase activity in the incubation mixture, as corrected for the alkaline phosphatase activity contributed by the anti serum. The antibody did not cross react with other alkaline phosphatase isoenzymes.

Heat-inactivation time curves. Serum samples from a healthy nonsmoking subject, a pregnant woman, a patient with a metastatic gastric carcinoma, and a patient with a highly increased total alkaline phosphatase activity (1530 U/L) were heated in 12 × 75 mm glass tubes at 65 °C. Aliquots from each tube were removed from the heating bath every minute for 15 min. Residual alkaline phosphatase activity was then determined as described.

Inhibition with L-phenylalanine. We measured the inhibition of HSAP by L-phenylalanine according to the procedure of Fishman and Ghosh (17) as modified (18).

Results

Figure 1 shows a typical standard curve for the enzyme assay, which demonstrates linearity over the full range of the curve. Linearity of the assay with duration of incubation is shown in Figure 2. Spontaneous hydrolysis of the substrate in the blank tubes was negligible at pH 10.2. Fluorescence did not increase in the blank tubes, even after 160 min. Because the background varies considerably, a blank tube is required for each sample. Omission of a blank tube decreases sensitivity.

The reliability of this assay is based on the assumption that heat denaturation at 65 °C rapidly inactivates all other serum alkaline phosphatase isoenzymes, leaving HSAP virtually intact. Figure 3 shows the heat-inactivation curves for serum from a tumor patient with above-normal HSAP activity, from a pregnant woman, from a normal healthy subject, and from a patient with an abnormally high total alkaline phosphatase activity secondary to metastasis to the bones. After an initial precipitous decline, the residual activity decreases slowly by about 2% per minute. From the Figure one can conclude that heating for 7 min at 65 °C suffices, even for samples of high initial total alkaline phosphatase activity.

We tested assay performance with the two serum pools. The results are shown in Table 1. The coefficients of variation are satisfactory for both the high and low control.

A reference interval, established from data on 76 apparently healthy individuals (mostly laboratory personnel), is shown in Table 2. However, when we divided this population into smokers and nonsmokers, significantly different ranges were obtained for the two groups (see Table 2 and Figure 4). Most smokers have a several-fold increase of HSAP activity over that for the normal nonsmoking population. In fact, as discussed below, the increases of HSAP in smokers was often of the same magnitude as that seen in our cancer patients. To show the relationship between the placental enzyme, the HSAP from a normal population, and that from tumor patients, we compared heat stability and the degree of inhibition of enzyme activity by L-phenylalanine (17, 18) and by rabbit anti-human placental alkaline phosphatase antibody with those of the placental enzyme. As shown in Figure 3 and Table 3, these properties are similar.

### Table 1. Between-Assay Precision of HSAP Assay

<table>
<thead>
<tr>
<th></th>
<th>High control</th>
<th>Low control</th>
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<tbody>
<tr>
<td>Mean, arb. units/L</td>
<td>1.48</td>
<td>0.0801</td>
</tr>
<tr>
<td>SD, arb. units/L</td>
<td>0.064</td>
<td>0.0048</td>
</tr>
<tr>
<td>CV, %</td>
<td>4.0</td>
<td>5.7</td>
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</tbody>
</table>

*n = 31, each different days.

### Table 2. Distribution of HSAP Activity in an Apparently Healthy Population

<table>
<thead>
<tr>
<th>HSAP activity, arb. units/L</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Range (±2 SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers</td>
<td>51</td>
<td>0.068</td>
<td>0.037</td>
<td>0–0.144</td>
</tr>
<tr>
<td>Smokers</td>
<td>25</td>
<td>0.44</td>
<td>0.360</td>
<td>0–1.17</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>0.196</td>
<td>0.282</td>
<td>0–0.77</td>
</tr>
</tbody>
</table>

### Table 3. Comparison of L-Phenylalanine and Antibody Inhibition of HSAP from Various Sources

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Placenta</th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Phenylalanine, 20 mmol/L</td>
<td>80</td>
<td>77 (8)*</td>
<td>79 (5)*</td>
</tr>
<tr>
<td>Rabbit anti-human placental</td>
<td>45</td>
<td>43</td>
<td>50</td>
</tr>
<tr>
<td>alkaline phosphatase antibody</td>
<td></td>
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*Mean (and SD) for three subjects each.
Fig. 2. Effect of duration of incubation on HSAP activity with the substrate naphthol AS-MX phosphate in a sample from a healthy nonsmoking individual.

Fig. 3. Heat-inactivation curves for alkaline phosphatases from (a) term pregnancy serum, (b) a patient with a metastatic gastric carcinoma, (c) a normal healthy nonsmoking individual, (d) same as c, but ordinate expanded × 10, and (e) patient with a total serum alkaline phosphatase activity of 1530 U/L secondary to breast carcinoma metastatic to bones.

Discussion

The kinetic procedure we used for measuring HSAP is sensitive enough to serve as a serum tumor-marker assay. It is simple enough to be performed by any laboratory, with readily available equipment and reagents. We combined three factors to achieve this: (a) heat inactivation of all other serum alkaline phosphatases, (b) use of a substrate producing a fluorescent reaction product, and (c) a five- to 10-fold decrease in the fluorometric background reading by use of acetone as a protein precipitant. Our assay mixture contains a large amount of serum, 250 μL per 500 μL total volume. It was necessary to eliminate protein before fluorometric reading, and we found acetone to be the most satisfactory protein precipitant for this purpose. The reaction product is soluble, protein is completely precipitated, the supernatant fluid is crystal clear, and background fluorescence is very low.

Precision is excellent and day-to-day variability is negligible. Heating serum for 7 min at 65 °C inactivates all of the alkaline phosphatase isoenzymes other than the placentallike isoenzymes. The method is 80–100 times more sensitive than kinetic methods in which phenyl phosphate is used to measure HSAP (5, 19).

Although we did not systematically search for specific interfering substances, we encountered none in our assays of more than 500 serum samples, including specimens from normal subjects and from patients with various neoplastic and non-neoplastic diseases. Lipemia does not interfere.

With this method we can reproducibly measure small quantities of HSAP in most healthy individuals. The fraction we most often found composed <0.3% of the total alkaline phosphatase in serum.

An unexpected finding that, to our knowledge, has not received previous attention was the frequently and often prominently increased activity of HSAP in the serum of apparently healthy smokers. This phenomenon does not appear to be sex-related. Patients not uncommonly deny a smoking history and may admit to smoking only after more questioning; moreover, smoking history obtained from chart review is frequently misleading. In previous reports no clearcut normal ranges emerged (4, 5, 12–14, 19). This is not surprising in light of Figure 4 and Table 2, which indicate that a true "normal" range can be ascribed only in nonsmoking subjects. From our data on smokers, we suggest that the reported incidence of an increase of this tumor marker in serum in the presence of various tumors needs to be redefined and the clinical usefulness of the marker reconsidered. We are currently re-evaluating HSAP activity in nonsmoking patients with tumors. Our preliminary re-
sults show that in 80–90% of such patients with above-
normal HSAP activity, the values are within the range
found in smokers.

The presumed phenomenon of gene activation for this
tumor marker by smoking is interesting. This is not restrict-
ed to this antigen but, as is well known, also occurs in
relation to carcinobromic antigen (20–22). However, our
preliminary data show that increases in the placental-like
alkaline phosphatase are much more dramatic, with a four-
to 10-fold increase for the pack-per-day cigarette smoker,
than for carcinobromic antigen.

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