Heterogeneous Enzyme Immunoassay with Electrochemical Detection: Competitive and “Sandwich”-Type Immunoassays

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In these competitive and “sandwich”-type heterogeneous enzyme immunoassays, based on liquid chromatography with electrochemical detection, rabbit immunoglobulin G is used as a model compound. Alkaline phosphatase (EC 3.1.3.1), the labeling enzyme, catalyzes conversion of phenyl phosphate to phenol. After separation on an octyldecylsilane column, the enzyme-generated phenol is detected in a thin-layer cell at a carbon-paste working electrode. The detection limit for phenol is 5.0 nmol/L. The electrode response varies linearly with concentration over a range of three orders of magnitude. For the sandwich-type assay procedure the detection limit is 10 ng/mL; the linearity ranges over four orders of magnitude. The detection limit of the competitive immunoassay is 5 μg/mL. The dynamic range spans two orders of magnitude.

Additional Keyphrases: rabbit IgG · alkaline phosphatase · chromatography, liquid · carbon-paste electrode

During the last decade, much effort has been expended on the investigation and development of enzyme immunoassays (1, 2). Heterogeneous enzyme assay methods, developed for determining compounds of both large and small molecular mass, rely on measurement of an enzyme-generated product for quantifying the compound of interest (3, 4). Absorbance spectroscopy, being easily done, is the technique most commonly used to measure the enzyme product, despite its relative insensitivity as compared with modern fluorescence, electrochemical and radioimmunoassay techniques. The relatively poor detection limit with absorbance spectroscopy is one cause of its lack of sensitivity (5, 6).

Electrochemical detection can be successfully coupled to the homogeneous enzyme immunoassay (7). Here we demonstrate the extension of electrochemistry to the more general heterogeneous immunoassay. We describe two such immunoassays, a “sandwich”-type and a competitive-type assay, based on liquid chromatography with electrochemical detection of the enzyme-generated product.

Rabbit immunoglobulin G was chosen as a model compound and alkaline phosphatase (EC 3.1.3.1) as the enzyme label. Alkaline phosphatase catalyzes the conversion of phenyl phosphate to phenol.

In the competitive assay, solid-phase and solution-phase rabbit IgG compete for solution-phase IgG-specific antibody labeled with alkaline phosphatase. The amount of enzyme label bound to the solid-phase IgG is determined by incubation with the enzyme substrate. The sandwich-type assay involves the incubation of solution-phase IgG with antibody-coated cuvettes. The amount of IgG bound to the solid-phase antibody is determined by a second incubation with enzyme-labeled specific antibody, followed by reaction with the enzyme substrate solution. The phenol produced by the enzyme label in either assay format is quantitated by oxidative hydrodynamic amperometry in a thin-layer cell after passing through a 5-cm octyldecylsilane (C18) column.

Materials and Methods

Apparatus

For liquid chromatography with hydrodynamic amperometric detection we used a prototype electrochemical immunoassay system (Immunoanalytical Systems, Inc., West Lafayette, IN 47906). The working electrode was a paraffin-oil-based carbon paste, the reference electrode was a silver/silver chloride (Ag/AgCl) with KCl, 3 mol/L, and the auxiliary electrode was a stainless-steel block that composed one-half of the thin-layer cell. We slurry-packed a 5 cm × 2 mm precolumn with 10-μm irregularly shaped RSIL material (Alltech Associates, Deerfield, IL 60015) and used it to separate phenol from the components of the assay buffer. A 12 cm × 4 mm Knauer column, dry-packed with 37- to 44-μm pellicular C18 packaging material (Alltech Associates), was placed between the pump and the injection valve to saturate the mobile phase. In all hydrodynamic amperometric analyses we used as mobile phase a phosphate buffer (0.1 mol/L, pH 7.0) containing 0 to 40 mL of methanol per liter, a flow rate of 1.0 to 1.6 mL/min, and an applied potential of +870 mV vs Ag/AgCl. We also used a 20-μL injection loop.

Materials

Reagents. Polystyrene cuvettes (12 × 5 mm) were purchased from Gilford Instruments, Cleveland, OH 44135; polyoxyethylene (20) sorbitan monolaurate (Tween 20) was from Fisher Scientific, Cincinnati, OH 45242. Rabbit IgG, goat antibody to rabbit IgG, and a conjugate of alkaline phosphatase with the goat antibody were purchased from Sigma Chemical Co., St. Louis, MO 63138. Phenyl phosphatase was from Calbiochem-Behring Corp., La Jolla, CA 92037.

Buffers. The buffers we used were phosphate-buffered isotonic saline with Tween 20 (PBS Tween): per liter, 30 mmol of KH2PO4, 14.3 mmol of NaCl, and 0.5 mL of Tween 20 adjusted to pH 7.4 with NaOH (5.0 mol/L); carbonate buffer: per liter, 35 mmol of NaHCO3 and 15 mmol of Na2CO3 (pH 9.6); and potassium phosphate buffer: per liter, 45 mmol of KH2PO4 and 55 mmol of K2HPO4 (pH 7.0).

Enzyme substrate. The substrate solution contained 1.0 mmol of phenyl phosphate and 1.5 mmol of MgCl2 · 6 H2O per liter of carbonate buffer.

Rabbit IgG standard solutions. A 10 mg/L stock solution of IgG was prepared in PBS Tween. Standard IgG solutions were prepared by diluting the stock solution with PBS Tween.

Rabbit IgG coating solution. From a 70 mg/L stock solution of IgG in sodium carbonate buffer we prepared 0.1, 1.0, and 10 mg/L coating solutions by diluting the stock solution with the carbonate buffer.

Goat antibody coating solution. Goat antiserum to rabbit IgG, received lyophilized in individual vials, was reconstruc-

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tuted with 2.0 mL of carbonate buffer. We used antibody dilutions of 100-, 1000-, and 10 000-fold in the carbonate buffer.

Goat antibody–alkaline phosphatase conjugate dilutions. We used the antibody–enzyme conjugate as received, and diluted 250-, 1000-, and 10 000-fold with PBS Tween.

Immunoassay Procedures

Sandwich immunoassay. Polystyrene cuvettes were coated with the goat antibody by passive adsorption from 500 μL of carbonate buffer. The adsorption method of antibody immobilization was chosen over covalent coupling for this preliminary study because it eliminates a time-consuming centrifugation step. After coating, we washed the cuvettes three times with PBS Tween, allowing the PBS Tween to remain in the cuvettes for 10 min during each wash. We added 400 μL of the rabbit IgG standards to the cuvettes, incubated at room temperature, then washed the cuvettes three times with PBS Tween, allowing the wash solution to remain in the cuvettes for 5 min during each wash. We added 375 μL of the enzyme-labeled goat antibody solution to each cuvette, again incubated at room temperature, and washed the cuvettes consecutively with PBS Tween (twice) and carbonate buffer (twice). We added 300 μL of the enzyme substrate solution to the cuvettes, incubated at room temperature, then stopped the substrate reaction by adding 25 μL of 5.5 mol/L NaOH. Immediately before chromatographic injection, we added 25 μL of 5.5 mol/L HCl to the sample to return its pH to 9.6. This step reduced a large, slowly decaying spike of capacitive current that appeared when we injected the more alkaline sample. We injected 20 μL of the substrate reaction solution into the chromatograph. The peak heights obtained for the oxidation of phenol were used to construct a standard curve.

Competitive immunoassay. Polystyrene cuvettes were coated with rabbit IgG by passive adsorption from 500 μL of carbonate buffer. We washed the coated cuvettes three times with PBS Tween, allowing the solution to stand in the cuvettes for 10 min during each wash. We added 375 μL of the rabbit IgG standards and 25 μL of the labeled antibody to the cuvettes and incubated at room temperature. After incubation the contents of the cuvettes were aspirated and the cuvettes washed consecutively with PBS Tween (twice) and carbonate buffer (twice). We then added 300 μL of the enzyme substrate solution to each cuvette and incubated at room temperature for a given interval before stopping the enzyme reaction by adding 25 μL of 5.5 mol/L NaOH. Again, just before chromatographic injection of a 20-μL sample, we added 25 μL of 5.5 mol/L HCl to each sample. From the peak heights for the oxidation of phenol we constructed a standard curve.

Results and Discussion

Liquid Chromatography with Electrochemical Detection

Heterogeneous enzyme immunoassays have been developed by use of various procedures and enzyme labels (8). For electrochemical detection, it is advantageous to use an enzyme for which the substrate is electroactive but the product is electroinactive. Alkaline phosphatase, used extensively in enzyme immunoassays, catalyzes the conversion of phenyl phosphate to phenol by hydrolysis of the ester linkage to yield phenol and phosphoric acid.

Cyclic voltammetry showed that phenyl phosphate in carbonate buffer was electroactive in the potential range −200 mV to +1000 mV vs Ag/AgCl. Phenol, however, was electroactive in this potential range, giving rise to an irreversible oxidation wave with a peak current (iₚ) at +670 mV. To improve the detection limit, we used hydrodynamic amperometry with liquid chromatography for measuring the enzyme-catalyzed generation of phenol. Although the components of the substrate solution are electroinactive, its injection gave rise to a capacitive current spike. The faradic signal for the oxidation of phenol in the 1 to 100 nmol/L range would be completely overwhelmed by this capacitive spike. Therefore, phenol had to be separated from the assay buffer before we could measure low concentrations of phenol. For this we used a 5-cm precolumn packed with 10-μm C₁₈ material. Methanol was added to the phosphate buffer to adjust the retention time for phenol to about 2.5 min. A representative chromatogram for phenol under these conditions is shown in Figure 1. Because maximum peak current response occurred at potentials of +850 mV or more, we used a potential of +870 mV in all analyses. Detection of phenol with this system had a linear dynamic range from 9.0 nmol/L to 9.6 μmol/L (slope = 0.57 nA/mol, y-intercept = 0.30 nA, r = 0.999) and a detection limit of 5.0 nmol/L.

Sandwich-Type Enzyme Immunoassay

Figure 2 shows typical sandwich-type assay chromatograms for rabbit IgG standards in PBS Tween. The peak current is proportional to the amount of phenol produced, which in turn is directly proportional to the amount of rabbit IgG in the standard solution. Optimization of the sandwich immunoassay required evaluating two antigen/antibody incubation intervals, the antibody coating dilution, and the antibody–enzyme conjugate dilution. We evaluated the antibody coating dilutions (100-, 1000-, and 10 000-fold) and the antibody–enzyme conjugate dilutions (250-, 1000-, and 10 000-fold) in a checkerboard fashion; that is, we performed the assay for each antibody coating concentration with all three antibody–enzyme conjugate dilutions. Whereas the antibody coating dilution had little effect on the assay results for rabbit IgG standards between 250 to 0 μg/L, the antibody–enzyme conjugate dilution had a pronounced effect on assay sensitivity, with a 250-fold dilution resulting in the best assay. However, this dilution would require too much conjugate, so we compromised, incorporating a 1000-fold dilution. We arbitrarily chose a 1000-fold antibody coating dilution.
2. We evaluated the time required for the two antigen/antibody reactions to reach equilibrium by using a 10 \( \mu \)g/L rabbit IgG standard and varying the incubation time for each reaction, one at a time. The solid-phase antibody/antigen reaction required 3 h to reach equilibrium, but the antigen/labeled antibody reaction had not reached equilibrium after 7 h. As a compromise, we used antigen/antibody incubation intervals of 3 h each.

Figure 3 shows results for the sandwich-type assay, carried out under the above conditions and allowing 20 min for reaction with substrate. Concentrations of rabbit IgG ranged from 250 to 0 \( \mu \)g/L, with a detection limit of 0.1 \( \mu \)g/L. The CV for the peak current for 10 repetitions of the 10 \( \mu \)g/L standard was 3.8%. Using a 60-min substrate reaction and the above assay conditions lowered the detection limit to 50 ng/L (Table 1), which could be further improved to 10 ng/L by increasing the antigen/antibody incubation interval to 10 h, each with a 60-min substrate reaction interval (data not shown). Table 1 also shows results of a 30-min antigen/antibody incubation interval in conjunction with a 60-min substrate reaction interval. In this shortened assay the detection limit was 100 ng/L.

In the sandwich-type assay the amount of labeled antibody bound to the cuvettes is directly proportional to the concentration of rabbit IgG in the standard solution. Both under optimum and compromise assay conditions the labeled antibody bound by the lowest detectable rabbit IgG standard sufficed to produce a phenol concentration in the 10 to 100 nmol/L range. Phenol concentrations in these ranges did not strain the detection capabilities of the electrochemical technique. For both the optimum and compromise assays the detection limit was not set by the ability to quantitate the phenol produced by the enzyme, but rather by the amount of nonspecific adsorption of the antibody–enzyme conjugate demonstrated by the 0 ng/mL standard. This nonspecific binding corresponded to a phenol concentration in the 10 nmol/L region.

**Competitive Enzyme Immunoassay**

The development of a sensitive competitive assay required the optimization of the antigen coating concentration, the antibody–enzyme conjugate dilution, and the antigen/antibody incubation interval.

The antigen coating concentrations (0.1, 1.0, and 10 g/L) and the antibody–enzyme conjugate dilutions (1000-, 10000-, and 10 0000-fold) were investigated, as described above, in checkerboard fashion. Assay sensitivity was best with a 0.1 g/L antigen coating concentration and a 100-fold antibody–enzyme conjugate dilution.

Competitive heterogeneous assays are most sensitive when the antigen/antibody reaction is allowed to proceed to equilibrium (9). The time required for the solid-phase antigen/labeled-antibody reaction to reach equilibrium was determined by incubating the 0 \( \mu \)g/L rabbit IgG standard and the 100-fold diluted antibody–enzyme conjugate with the
The determination of a substance at this concentration by a competitive assay necessitates the use of an antibody–enzyme conjugate of similar concentration, as well as antibodies with large affinity constants, given that the detection limit of competitive assays is inversely related to the binding constant (10). The implementation of such assays will require a sensitive detection scheme for measuring the enzyme-generated product within a reasonable time. The present electrochemical methodology would be ideally suited to such determinations.

A heterogeneous sandwich-type immunoassay, as discussed above, can achieve an even lower detection limit (10 ng/L), which should be capable of being decreased even further. However, this will depend on the development of ways to eliminate nonspecific binding of the antibody–enzyme conjugate.

Even in its present state, however, this approach we discuss offers an attractive alternative to the conventional RIA methods of detection.

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References