0273-2289/94/4802—0117$05.40

Immunoassay for Native Enzyme Quantification in Biological Samples

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Received October 16, 1993; Accepted December 2, 1993

ABSTRACT

In order to detect low levels of enzyme activity, specifically glucose oxidase, in biological samples, an immunoenzymatic assay was developed since currently available methods could not be used because of either their lack of sensitivity or the conditions prevailing in our samples: turbidity of the medium, presence of redox systems other than glucose oxidase, and high concentration of proteins.

The principle of the method is to coat a polystyrene surface with a fragment Fc-specific anti-IgG, then with an antibody directed against the looked-for enzyme, which is simultaneously the antigen and the enzyme activity required for immunoenzymatic detection. We applied this concept to biological samples after glucose oxidase administration to mice. This method achieves specificity and sensitivity (20 ng/mL or 1 ng) with samples of biological origin. No marker is needed since the antigen itself possesses an enzyme activity. This method, which requires a small sample volume (50 μL, 20 μL, if necessary), can be extended easily to the many enzymes currently used as markers. It could also be applied to the native enzymes of medical interest for which antibodies and a colorimetric reaction are available.

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Index Entries: Immunoglobulin; rabbit anti-mouse fragment Fc-specific; phosphate buffered saline PBS-Tween; *o*-phenylenediamine; ABTS.

Abbreviations: IgG, immunoglobulin; anti-Fc IgG, rabbit anti-mouse fragment Fc-specific IgG; PBS, phosphate buffered saline; PBS-Tween, Tween 20 0.1% dissolved in PBS; OPD, *o*-phenylenediamine; ABTS, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt.

INTRODUCTION

Drug targeting is a rapidly expanding domain. Applications are considered in such fields as inherited metabolic disease treatment (*1*), cancer therapy (*2,3*), or intracellular infections (*4,5*). For the study of the efficiency of different moieties as homing devices, we chose to use an enzyme as a marker and elected glucose oxidase as a first model enzyme because such an activity is absent in mammals.

In order to study the in vivo distribution of the glucose oxidase-homing device conjugates, we needed a detection method more sensitive than any currently available, that was also able to quantify glucose oxidase activity in crushed tissues, i.e., in turbid media.

When oxygen is used as the cosubstrate, glucose oxidase activity can be measured through the production of hydrogen peroxide using peroxidase and a chromogen. Unfortunately, turbid samples prevent absorbance measurements. It can also be measured through oxygen consumption, which implies the necessity to control the transfer of oxygen from the environment to the sample and the solubility of oxygen in the different samples.

Glucose oxidase accepts cosubstrates other than oxygen. Phenazine methosulfate and a tetrazolium salt can be used as the intermediate and the final electron acceptors, respectively. The tetrazolium salt is transformed into a colored formazan. Beside the perturbation of absorbance measurements by turbid samples, the specific limit of this method is the collection by phenazine methosulfate of electrons originating from biological redox reactions other than glucose oxidase activity.

Oxygen can also be replaced by an electron acceptor, benzoquinone, whose reduced form, hydroquinone, is electroactive. The detection of the reduced cosubstrate by controlled-potential amperometry allows determination of the enzymatic reaction rate (*6*). When this electrochemical method was applied to crushed organs, the detection was perturbed by protein adsorption on the electrode surface below a certain threshold of enzyme activity.

Another possibility is to quantify the gluconic acid produced during the reaction. The published methods (*7,8*) required gluconic acid isolation. Based on the acid release consecutive to the spontaneous hydrolysis of gluconolactone into gluconic acid, a pH control method using a pH-stat

was developed (9). The detection threshold is about 100 μg/mL, too high for our samples.

Since we needed a method that took into account all the constraints induced by our biological samples, we applied an immunoenzymatic concept to glucose oxidase detection in mouse organs. The principle is to coat a polystyrene surface with a fragment Fc-specific anti-IgG for a better orientation of the specific antibody, then with an antibody directed against glucose oxidase, the latter being simultaneously the antigen and the enzyme activity required for immunoenzymatic detection. The sample in which glucose oxidase is to be detected is incubated. A chromogen is then added and glucose oxidase activity evaluated. Applying this method to glucose oxidase detection in animal organs, we noticed that the sample nature had no influence owing to the washings performed between consecutive steps. Thus, the threshold of glucose oxidase activity detection is lowered to 20 ng/mL and the sample volume needed remains very low.

MATERIALS AND METHODS

Chemicals were of the highest available grade.

Immunoenzymatic Assay

A 10 μg/mL solution of rabbit antimouse fragment Fc-specific IgG (anti-Fc IgG, Jackson Immunoresearch Labs [West Grove, PA]) was prepared in phosphate buffered saline (PBS) 10 m*M* pH 7.5. Two hundred microliters of this solution were incubated in each microtiter plate well for 2 h at 37°C. After three washings with Tween 20 0.1% dissolved in PBS 10 m*M*, pH 7.5, 200 μL of 0.5% ovalbumin (Sigma [St. Louis, MO], grade V) were incubated overnight at 4°C in the wells to avoid nonspecific interactions. After three PBS-Tween washings, 50 μL of 1 μg/mL mouse monoclonal antiglucose oxidase antibody (Clonatec [Paris, France]) were added to each well and incubation was allowed for 1 h at 37°C. After three PBS-Tween washings, 50 μL of a 10 μg/mL solution of glucose oxidase (E.C. 1.1.3.4, Boehringer Mannheim [Mannheim, Germany] grade I) from *Aspergillus niger* were added to each well. Incubation was allowed for 1 h at 37°C.

After three PBS-Tween washings, the reaction was developed by adding 100 μL of a substrate solution consisting of 10 mg/mL of D-glucose, 25 μg/mL of horseradish peroxidase (Sigma, grade VI) and 350 μg/mL of *o*-phenylenediamine (OPD) dissolved in citrate-phosphate buffer 0.1*M* pH 5. After 1 h at room temperature, the reaction was stopped by adding 50 μL of 1*M* sulfuric acid. The absorbance was measured at 490 nm using a microtiter plate reader Dynatech (Chantilly, VA) MR 5000.

Biological Experiments

Glucose oxidase (3.5 μg/g body wt) was intramuscularly injected in the right gastrocnemius muscle of Swiss female mice. Eight hours later, the mice were killed and the following organs were sampled: right and left gastrocnemius muscles, right and left thigh muscles, right and left sciatic nerves, brain, bowels, lungs, spleen, liver, and blood.

After sampling, liver, muscles, brain, lungs, bowels, and spleen were weighed and homogenized in water using an Ultra-Turrax emulsifier. They were frozen, thawed, and centrifuged. Nerves were homogenized in distilled water using a glass potter homogenizer. The samples were sonicated, frozen, thawed, and centrifuged. Blood was centrifuged. For each sample except nerve, where 20 μL were used, 50 μL of supernatant were added into the coated wells.

The influence of the organs on the immunoenzymatic detection was tested. Each well was prepared as usual: 200 μL of an anti-Fc IgG solution (10 μg/mL) were incubated for 2 h at 37°C. After three PBS-Tween washings, 0.5% ovalbumin was incubated overnight at 4°C. The antiglucose oxidase antibody (1 μg/mL) was incubated in the wells for 1 h at 37°C. After washing the wells, 50 μL of a glucose oxidase solution (10–500 ng/mL) followed by 50 μL of organ samples from an untreated mouse were incubated for 1 h at 37°C. The colored reaction was performed as described previously.

To compare immunoenzymatic results to enzymic activity of glucose oxidase, we measured the glucose oxidase solution activity in the wells. Fifty microliters of a glucose oxidase solution (10–500 ng/mL) were deposited into the wells, followed by 50 μL of substrate (composition as above). Absorbance reading was performed as above.

RESULTS

Immunoenzymatic Assay

We first used anti-mouse immunoglobulin G (IgG, Jackson Immunoresearch Labs) as a preliminary coating. For spectrophotometric measurements, the optimal antimouse immunoglobulin G concentration for a satisfactory glucose oxidase activity detection was 60 μg/mL. The optimal antiglucose oxidase antibody concentration was 2 μg/mL. However, the anti-IgG was not adapted to this immunosandwich because the antiglucose oxidase antibody site might not be well oriented as far as enzyme coupling is concerned. This was confirmed by the comparison of glucose oxidase activity measured at 490 nm in coated wells and in solution at the same concentration. We obtained a 5% yield (glucose oxidase activity measured in coated wells × 100/glucose oxidase activity measured in solution). For this reason, we tried an anti-Fc IgG coating.

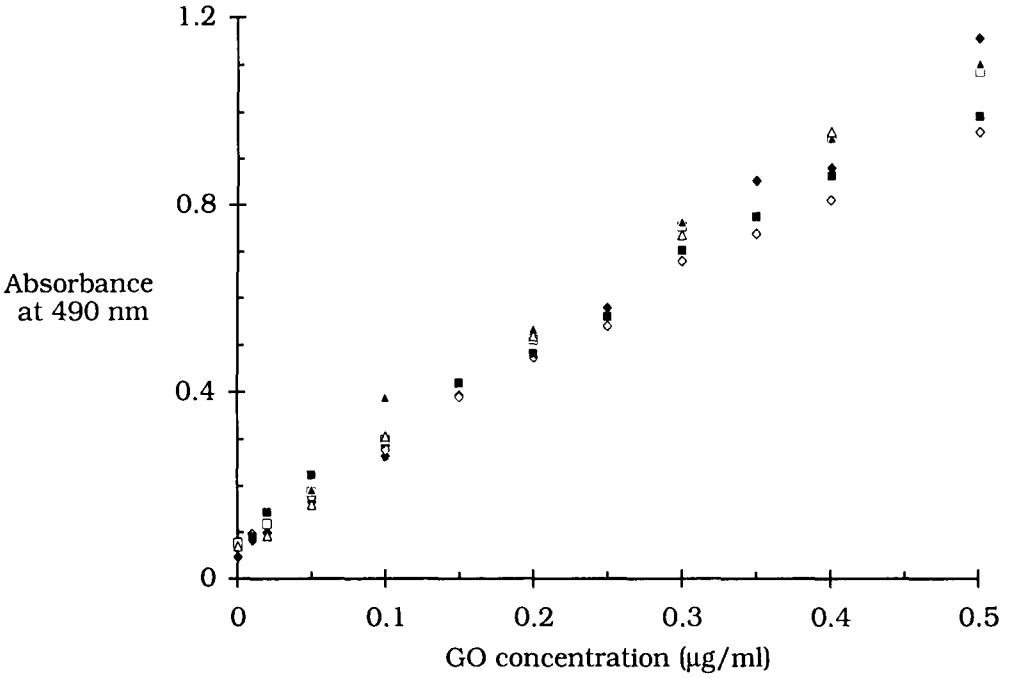

Fig. 1. Coating was made with 200 µL of a 10 µg/mL anti-Fc IgG solution and with 50 µL of a 1 µg/mL antiglucose oxidase solution. A 50–100 ng/mL glucose oxidase solution (◆) was added followed by 10 µL of organ supernatant (nerve [■], liver [◇], spleen [▲], muscle [△]). All were incubated for 1 h at 37°C. Glucose oxidase activity is demonstrated by OPD coloration. Glucose oxidase activity was measured in solution (□).

Under the same conditions, the optimal anti-Fc IgG concentration was 10 µg/mL while testing a 0.05–100 µg/mL range, and the optimal antiglucose oxidase concentration 1 µg/mL in a 0.5–2 µg/mL range. With these conditions, the curve obtained is linear and the yield (calculated as indicated previously) is about 90%.

For the detection of glucose oxidase activity, we assessed two chromogens. Since we get less sensitivity with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), we used OPD.

Biological Experiments

Figure 1 shows that biological samples did not influence the results of the immunoenzymatic assays. Glucose oxidase is recognized by antibodies with the same affinity in presence or absence of organ homogenates in the wells.

At predetermined times after glucose oxidase administration, the mice were killed by cervical elongation and organ samples were taken. The immunoenzymatic method described here allows the detection of glucose oxidase activity inside the organs (Table 1). The immunoenzymatic assay is linear between 1 ng (20 ng/mL) and 10 ng (200 ng/mL) of glucose oxidase.

Table 1
Glucose Oxidase Activity Detection by Immunoenzymatic Method After Injections of Glucose Oxidase in the Right Gastrocnemius Muscle of Three Mice[a]

Samples	Glucose oxidase, ng/mL
Right thigh muscle	400
Right gastrocnemius	167
Sciatic nerve, right	140
Liver	120
Blood	65
Spleen	42
Brain	40
Spinal cord	20
Lungs	0
Bowels	0

[a]Mice were killed after 8 h. The signal was considered positive when the absorbance was at least twice the background.

DISCUSSION

The immunoenzymatic assay described in this paper was elaborated because the various methods available for glucose oxidase detection were inadequate for our purposes: measurement of a low enzyme activity in biological turbid media.

The first advantage of the immunoenzymatic method described here is that the antigen itself is an enzyme (glucose oxidase, in the example presented) that produces the colored reaction if a suitable medium is added. Consequently, we do not have to use an enzyme-antibody complex as most ELISA tests do. Also, this method can be generalized for all enzymes usually utilized as markers and for which antibodies are available: β-galactosidase, alkaline phosphatase, and so on. We used it with the same success for β-galactosidase (data not presented). It could also be employed for the detection of enzyme activities of medical interest provided that antienzyme antibodies and a colorimetric method are available.

Second, it rules out interactions owing to complex samples, since the wells are washed between consecutive steps. We tested the coating of homogenized organs on anti-Fc IgG. The results showed that the signal on the microtiter reader was owing only to glucose oxidase coated on anti-glucose oxidase antibody. Also, no nonspecific fixation of glucose oxidase on anti-Fc IgG occurred. Moreover, the enzyme activity found in organs

after injection of glucose oxidase to mice is in agreement with the results measured by other methods when they can be applied.

Finally, this method is able to measure glucose oxidase activity in organs down to 20 ng/mL with a detection threshold lowered to 1 ng. If the detection threshold were to be lowered still, a fluorescent substrate of peroxidase could be used. This immunoenzymatic method allowed us to test small mouse samples in which glucose oxidase activity is low, such as sciatic nerve or blood samples.

ACKNOWLEDGMENT

The authors would like to thank J. Bresson from Clonatec Inc. (Paris, France) for the glucose oxidase MAbs.

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