Zeptomole detection of an enzyme by a simple colorimetric method

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Abstract

An enzyme immunoassay, in which an enzyme (e.g., alkaline phosphatase, ALP) is conjugated with an antibody, is a precise and simple protein detection method. Precise measurements of enzymes at low concentrations allows for ultrasensitive protein detection. The application of a phosphorylated substrate to ALP followed by use of the dephosphorylated substrate in thionicotinamide-adenine dinucleotide cycling provides simple and precise quantification of ALP. We describe a protocol for detecting ALP at the zeptomole level using a simple colorimetric method.
Introduction

Precise and simple measurement of proteins at an ultrasensitive level is required in the fields of biology, medicine, and industry.\textsuperscript{1,2} Various methods have been proposed for achieving this aim, including western blotting, mass spectrometry, and enzyme immunoassay.\textsuperscript{3} Among these, western blotting is an easy technique to perform, but lacks precision. Mass spectrometry provides precision, but requires an expensive and complicated apparatus. Enzyme immunoassays are both precise and simple. Although some enzyme immunoassays employ detection techniques using radioisotopes and fluorescence, many enzyme immunoassays use a colorimetric method.\textsuperscript{4,5} Increasing the detection sensitivity of colorimetric enzyme immunoassays will provide the simplest and most precise protein detection method.\textsuperscript{4}

One type of enzyme immunoassay is an enzyme-linked immunosorbent assay (ELISA). A sandwich ELISA has a higher specificity than other ELISA types, such as direct ELISA, indirect ELISA, and competitive ELISA.\textsuperscript{4} Sandwich ELISA combines 2 different antibodies recognizing different epitopes on a target protein; one antibody is used for immobilization on the surface of microplate wells, and the second antibody is labeled with an enzyme (\textit{e.g.}, alkaline phosphatase; ALP, EC. 3.1.3.1) and used to capture specific proteins. Ultrasensitive measurement of the enzyme used in the ELISA allows for easy and precise protein detection. The difficulty for researchers is finding 2 sets of specific antibodies for the target protein, and this issue should be considered separately.

On the other hand, even a trace amount of molecules can be measured with an enzyme cycling method.\textsuperscript{6,7} In particular, thionicotinamide-adenine dinucleotide cycling (thio-NAD cycling) is useful.\textsuperscript{8,9} In the presence of an excess amount of NADH and
thio-NAD, a single dehydrogenase, such as 3α-hydroxysteroid dehydrogenase (3α-HSD, EC. 1.1.1.50), catalyzes substrate cycling between 3α-hydroxysteroid and its corresponding 3-ketosteroid. In each turn of the cycle, one molecule of thio-NAD is reduced to thio-NADH, which can be measured directly as an increase in the absorbance at 400 nm (11,900 M⁻¹ cm⁻¹), e.g., 405 nm, using a commercially available microplate reader, without any interference from the other cofactors, such as thio-NAD, NAD, and NADH. For example, androsterone or its derivative can be used as a 3α-hydroxysteroid for 3α-HSD. Let us suppose that we would like to count ALP molecules. For this purpose, we add phosphorylated androsterone and ALP into a thio-NAD cycling system including 3α-HSD, NADH, and thio-NAD (Fig. 1), and then measure the absorbance of the accumulated thio-NADH at 405 nm to obtain the number of ALP molecules. This idea was first described by Watabe and Ito, and the method has been applied to various sandwich ELISAs.

In the present study, we re-examined the optimal conditions for counting ALP molecules using a new combination of commercially available chemicals. We used another commercially available 3α-HSD different from that used previously, adjusted the concentrations of enzymes, co-enzymes and a substrate, and examined the reaction time. The results demonstrated zeptomole detection of ALP in a 100-μL volume by only measuring the absorbance at 405 nm. Thus, we developed a simple colorimetric method with detection sensitivity comparable to that of real-time polymerase chain reaction (PCR).

**Experimental**
Reagents and chemicals

ALP and NADH were purchased from Roche (Basel, Switzerland). 3α-HSD was purchased from Asahi Kasei Pharma (Tokyo, Japan). Thio-NAD was from Oriental East (Tokyo, Japan). 17β-methoxy-5β-androstan-3α-ol 3-phosphate was synthesized by one of the authors (T.Y.). The other chemicals were commercial grade.

Apparatus

We used a Corona Electric SH-1000 microplate reader (Hitachinaka, Ibaraki, Japan).

Procedures

The thio-NAD cycling solution contained 1.0 mM NADH, 2.0 mM thio-NAD, 0.4 mM 17β-methoxy-5β-androstan-3α-ol 3-phosphate, and 10 U/mL 3α-HSD in 100 mM Tris-HCl (pH 9.0). The ALP solution was prepared at concentrations of 5.0 × 10^{-16} to 1.0 × 10^{-21} moles/mL diluted with 100 mM Tris-HCl (pH 9.0). Both solutions, 50 µL of 2×thio-NAD cycling solution and 50 µL of 2×ALP solution, were applied into each well of 96-well microplates. Absorbance was measured with a microplate reader at 37°C for a designated time. Detectable thio-NADH signals were measured with a grating microplate reader at 405 nm. The 405-nm signals were normalized to those of 660-nm signals.

The experimental data were obtained by subtracting the mean value of blank signals from each of the corresponding measured data. The limit of detection (LOD) was estimated from the standard deviation of the blanks and a confidence factor of 3. We used 2 different approaches for this estimation. In the first approach, the estimate was obtained by direct measurement using a trace amount of ALP, and in the second
approach, the estimate was obtained on the basis of calibration curves. The limit of quantitation (LOQ) was estimated by the same method used to estimate the LOD, but with a confidence factor of 10. The coefficient of variation (CV) was obtained in the examinations of inter-assay and intra-assay reproducibility.

Results and Discussion

Five different experimenters attempted to detect LOD using a trace amount of ALP (Fig. 2). In the experiments using 100 mM Tris-HCl with or without ALP, the absorbance of thio-NADH was measured after 60 min of cycling, and the signal-to-blank ratios were determined. At a concentration over $1.0 \times 10^{-21}$ moles/assay, the 5 experimenters ‘consistently’ obtained signal-to-blank ratio > 1.0. Thus, our procedure succeeded in detecting ALP at zeptomole levels.

Next, the 3 different experimenters obtained 3 different linear calibration curves for ALP provided with a thio-NAD cycling reaction (Fig. 3). The curves were obtained from the absorbance of thio-NADH after 100 min of cycling reaction. One linear calibration curve was expressed as $y = 1.00 \times 10^{16}x$, $R^2 = 0.99$ (Fig. 3A). The LOD of ALP obtained statistically using the standard deviation of the blanks and a confidence factor of 3 was $2.24 \times 10^{-20}$ moles/assay. In one assay, a 100-μL volume was used. The minimum LOQ of ALP calculated statistically using the standard deviation of the blanks and a confidence factor of 10 was $7.45 \times 10^{-19}$ moles/assay. The inter-assay CV was 0.6% for $5.0 \times 10^{-19}$ moles/assay ($n = 3$).

The linear calibration curve obtained by the second experimenter was expressed as $y = 2.00 \times 10^{16}x$, $R^2 = 0.99$ (Fig. 3B). The LOD was $6.78 \times 10^{-20}$ moles/assay, the
minimum LOQ was $2.26 \times 10^{-19}$ moles/assay, and the inter-assay CV was 1.6% for $5.0 \times 10^{-19}$ moles/assay ($n = 3$). Finally, the linear calibration curve obtained by the third experimenter was expressed as $y = 1.00 \times 10^{16}x$, $R^2 = 0.99$ (Fig. 3C). The LOD was $1.50 \times 10^{-19}$ moles/assay, the minimum LOQ was $5.00 \times 10^{-19}$ moles/assay, and the inter-assay CV was 0.5% for $5.0 \times 10^{-19}$ moles/assay ($n = 3$). The intra-assay CV for these 3 experimenters was 9.0% for $5.0 \times 10^{-19}$ moles/assay that was obtained from the results by these 3 different experimenters. Therefore, the LOD calculated statistically was one order of magnitude worse than that obtained by direct measurement.

As described above, we achieved zeptomole sensitivity for an enzyme by the direct measurements in colorimetric-based assays. To date, only complicated methods, such as fluorescent, chemiluminescent and radio isotope detection systems, have provided such high sensitivity. Our measurement system is easy to use and will contribute greatly to advance scientific inquiry. As can be seen in Figs. 2 and 3, the linear response of 405-nm absorbance against the ALP amounts was kept in the range over the $10^{-19}$ moles. Thus, we pursued the LOD by the direct measurements and reached the zeptomole detection.

**Conclusions**

When absorbance is measured at 405 nm (if necessary, absorbance is measured at 660 nm for normalization), ALP can be detected at the zeptomole level. Our method is very versatile, and it is applicable to ELISA with suitable antibodies specific to the target proteins. For example, the proteins included in human immunodeficiency virus (HIV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and tubercle bacillus
for infectious diseases\textsuperscript{15-17} and the peptides and proteins like insulin and adiponectin for lifestyle diseases\textsuperscript{11,18-20} can be measured precisely. The LOD of our ultrasensitive ELISA has been so far the attomole or subattomole level,\textsuperscript{11,15-20} but from now on this ultrasensitive ELISA will challenge us to achieve a zeptomole-level detection. In one assay using our method, we used a volume of 100 μL. This value corresponds to $10^{-23}$ moles (\textit{i.e.}, a few molecules) per 1 μL. Our method has sensitivity comparable to that of real-time PCR.\textsuperscript{14}

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**Figure Captions**

Fig. 1 Ultrasensitive detection of ALP by thio-NAD cycling using androsterone derivatives, 3α-HSD and its coenzymes. During the cycling reaction, thio-NADH accumulates in a triangular number with time. Accumulated thio-NADH can be measured directly as the absorbance at 405 nm without any interference from the other cofactors.

Fig. 2 Direct detections of ALP at low concentrations. The ALP solutions of $1.0 \times 10^{-22}$ to $5.0 \times 10^{-20}$ moles/assay were used for the cycling reaction. The 5 different bluish color bars indicated the data obtained by the 5 different experimenters. The absorbance was obtained from a cycling reaction time of 60 min and is shown as the ratio to the absorbance values of the blank. The horizontal dotted line indicates the absorbance values of the blank (signal-to-blank = 1), and the vertical dotted line indicates the LOD (signal-to-blank > 1). We could consistently obtain the signal-to-blank > 1 at a concentration over $1.0 \times 10^{-21}$ moles/assay.

Fig. 3 Linear calibration curves for ALP provided with the thio-NAD cycling reaction. Three datasets measured by 3 different experimenters are presented as (A), (B), and (C). The ALP solutions of $5.0 \times 10^{-19}$ to $5.0 \times 10^{-17}$ moles/assay were used for the cycling reaction. The absorbance of thio-NADH was measured after a cycling reaction time of 100 min in the 3 cases. The number of experiments was 3 each.
Fig. 2

![Bar graph showing signal-to-blank ratio vs. ALP concentration.](image)

- **Y-axis**: Signal-to-blank ratio
- **X-axis**: ALP concentration (moles/assay)
- Data points at varying ALP concentrations from 0 to 5.0x10^{-23} moles/assay.
Fig. 3

A

\[ y = 1.00 \times 10^{16}x \]
\[ R^2 = 0.99 \]
\[ \text{LOD} = 2.24 \times 10^{-20} \text{ moles/assay} \]
\[ \text{LOQ} = 7.45 \times 10^{-19} \text{ moles/assay} \]

B

\[ y = 2.00 \times 10^{16}x \]
\[ R^2 = 0.99 \]
\[ \text{LOD} = 6.78 \times 10^{-20} \text{ moles/assay} \]
\[ \text{LOQ} = 2.26 \times 10^{-19} \text{ moles/assay} \]

C

\[ y = 1.00 \times 10^{16}x \]
\[ R^2 = 0.99 \]
\[ \text{LOD} = 1.50 \times 10^{-19} \text{ moles/assay} \]
\[ \text{LOQ} = 5.00 \times 10^{-19} \text{ moles/assay} \]
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