Ultrasensitive enzyme-linked immunosorbent assay (ELISA) of proteins by combination with the thio-NAD cycling method

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An ultrasensitive method for the determination of proteins is described that combines an enzyme-linked immunosorbent assay (ELISA) and a thionicotinamide-adenine dinucleotide (thio-NAD) cycling method. A sandwich method using a primary and a secondary antibody for antigens is employed in an ELISA. An androsterone derivative, 3α-hydroxysteroid, is produced by the hydrolysis of 3α-hydroxysteroid 3-phosphate with alkaline phosphatase linked to the secondary antibody. This 3α-hydroxysteroid is oxidized to a 3-ketosteroid by 3α-hydroxysteroid dehydrogenase (3α-HSD) with a cofactor thio-NAD. By the opposite reaction, the 3-ketosteroid is reduced to a 3α-hydroxysteroid by 3α-HSD with a cofactor NADH. During this cycling reaction, thio-NADH accumulates in a quadratic function-like fashion. Accumulated thio-NADH can be measured directly at an absorbance of 400 nm without any interference from other cofactors. These features enable us to detect a target protein with ultrasensitivity (10−19 mol/assay) by measuring the cumulative quantity of thio-NADH. Our ultrasensitive determination of proteins thus allows for the detection of small amounts of proteins only by the application of thio-NADH cycling reagents to the usual ELISA system.

Key words: androsterone, enzyme cycling, 3α-hydroxysteroid dehydrogenase, insulin, thio-NAD

To diagnose and assess the progression of diseases, the quantitative examination of mRNA amounts in blood for target molecules of diseases is commonly performed1,2. The recent spread of real-time polymerase chain reaction (PCR) methods has enabled us to determine a few copies of mRNAs even in single cells3, as nucleic acids, i.e., RNA and DNA, can be amplified by PCR even when they exist in very small quantities. However, to determine the principal cause of a disease requires the identification of both quantitative and qualitative changes in proteins or peptides in the body. That is, the exact determination of proteins is required for the diagnosis, as opposed to just the quantitative determination of mRNAs. For this purpose, we must develop a novel determination method for trace amounts of proteins, because proteins consisting of 20 kinds of amino acids cannot be amplified.

Generally, the concentrations of the majority of proteins that are used for the diagnosis and judgments regarding the disease’s progression of cancer, neurological disorders and early stage of infection are thought to circulate in the range of 10−12 to 10−16 M4,7. When we use a 100 μL sample isolated from the body (such as blood) for an assay, we must detect...
is referred to as an enzyme cycling method\textsuperscript{20,21}. In general, amplification is achieved by two enzyme reaction systems in which each enzyme independently and cooperatively acts on the same substrate in a different way. In addition, there is another substrate cycling reaction conducted by a single dehydrogenase such as 3α-hydroxysteroid dehydrogenase (3α-HSD, EC. 1.1.1.50; Fig. 1B)\textsuperscript{22–28}. In this cycling reaction, 3α-HSD catalyzes a substrate cycling between 3α-hydroxysteroid and its corresponding 3-ketosteroid in the presence of an excess amount of NADH and thionicotinamide-adenine dinucleotide (thio-NAD), because 3α-HSD utilizes both NADH and thio-NAD as cofactors\textsuperscript{29}. In each turn of the cycle, one molecule of thio-NAD is reduced to thio-NADH, which can be measured directly by an increase in the absorbance at 400 nm (11,900 M\textsuperscript{–1} cm\textsuperscript{–1}), e.g., 405 nm with a commercially available microplate reader, without any interference from other cofactors such as thio-NAD, NAD and NADH, the absorbance maximums of which are all under 340 nm. These features make it possible to determine the amount of 3α-hydroxysteroids with high sensitivity by measuring the cumulative quantity of thio-NADH. This detectable signal also changes linearly with time.

In the present study, we propose the combination of a sandwich ELISA and a thio-NAD cycling method for an ultra-sensitive determination of trace amounts of proteins (Fig. 2). A suitable method may be a ‘sandwich’ enzyme-linked immunosorbent assay (ELISA)\textsuperscript{19}. ELISA is an easy, rapid, specific and highly sensitive detection method and thus has been widely used as a diagnostic tool in medicine and for quality-control checks in various industries. In this assay, an enzyme linked to a secondary antibody converts its substrate to another form. Most commonly, this produces a color change in the substrate, that is, a detectable signal. We usually detect this visible signal that indicates the quantity of antigen, i.e., proteins, with a microplate reader (Fig. 1A). This detectable signal changes linearly with time.

For the exact quantification of trace amounts of proteins, there is another assay for the determination of trace amounts of substrates by amplification techniques as a result of the continuous reaction of enzyme function. This is referred to as an enzyme cycling method\textsuperscript{20,21}. In general, amplification is achieved by two enzyme reaction systems in which each enzyme independently and cooperatively acts on the same substrate in a different way. In addition, there is another substrate cycling reaction conducted by a single dehydrogenase such as 3α-hydroxysteroid dehydrogenase (3α-HSD, EC. 1.1.1.50; Fig. 1B)\textsuperscript{22–28}. In this cycling reaction, 3α-HSD catalyzes a substrate cycling between 3α-hydroxysteroid and its corresponding 3-ketosteroid in the presence of an excess amount of NADH and thionicotinamide-adenine dinucleotide (thio-NAD), because 3α-HSD utilizes both NADH and thio-NAD as cofactors\textsuperscript{29}. In each turn of the cycle, one molecule of thio-NAD is reduced to thio-NADH, which can be measured directly by an increase in the absorbance at 400 nm (11,900 M\textsuperscript{–1} cm\textsuperscript{–1}), e.g., 405 nm with a commercially available microplate reader, without any interference from other cofactors such as thio-NAD, NAD and NADH, the absorbance maximums of which are all under 340 nm. These features make it possible to determine the amount of 3α-hydroxysteroids with high sensitivity by measuring the cumulative quantity of thio-NADH. This detectable signal also changes linearly with time.

In the present study, we propose the combination of a sandwich ELISA and a thio-NAD cycling method for an ultra-sensitive determination of trace amounts of proteins (Fig. 2). In a sandwich ELISA, we use 17β-methoxy-5β-androstan-3α-ol 3-phosphate as a synthetic substrate for alkaline phos-
Japan) and purified by BL (Numazu, Japan). 5β-Androsterone was purchased from Steraloids (Newport, RI, USA). 17β-methoxy-5β-androstan-3α-ol 3-phosphate was synthesized according to Iwai et al. (unpublished data). Briefly, 17β-methoxy-5β-androstan-3α-ol 3-phosphate was synthesized from 5β-androsterone (5β-androstan-3α-ol-17-one) via 5 steps including (1) protection of 3α-hydroxyl group by converting into 3-(2’-tetrahydropyranyl) ether, (2) reduction of 17-keto group to 17β-hydroxyl group with NaBH₄, (3) methylation of 17β-hydroxyl group with CH₃I/NaH, (4) elimination of 3-(2’-tetrahydropyranyl) group with HCl, and (5) phosphorylation of 3α-hydroxyl group with POCl₃ to give 17β-methoxy-5β-androstan-3α-ol 3-phosphate. Para-Nitrophenylphosphate (p-NPP) was purchased from KPL (Gaithersburg, MD, USA). Absorption measurements were made with a Corona Electric MTP-500 microplate reader (Hitachinaka, Japan) thermostated at 37°C.

Materials and Methods

Chemicals and equipment

The primary antibody was monoclonal mouse anti-human insulin 7F8 from HyTest (Turku, Finland). The secondary antibody was monoclonal mouse anti-insulin D4B8 from HyTest (Turku, Finland). Alkaline phosphatase (ALP) was purchased from Roche (origin from calf intestine, recombinant by Pichia pastoris; Mannheim, Germany). Sulfo-EMCS was purchased from Dojindo (Kumamoto, Japan). Human insulin was purchased from MP Biomedicals (recombinant by yeast; MP Bio Japan, Tokyo, Japan). Blocking One-P was purchased from Nacalai Tesque (Kyoto, Japan). Thio-NAD and NADH were purchased from Roche (Mannheim, Germany). 3α-hydroxysteroid dehydrogenase (3α-HSD) was purchased from Kikkoman Biochemifa (origin from Comamonas testosteroni, recombinant by E. coli; Tokyo, Japan) and purified by BL (Numazu, Japan). 5β-Androsterone was purchased from Steraloids (Newport, RI, USA). 17β-methoxy-5β-androstan-3α-ol 3-phosphate was synthesized according to Iwai et al. (unpublished data). Briefly, 17β-methoxy-5β-androstan-3α-ol 3-phosphate was synthesized from 5β-androsterone (5β-androstan-3α-ol-17-one) via 5 steps including (1) protection of 3α-hydroxyl group by converting into 3-(2’-tetrahydropyranyl) ether, (2) reduction of 17-keto group to 17β-hydroxyl group with NaBH₄, (3) methylation of 17β-hydroxyl group with CH₃I/NaH, (4) elimination of 3-(2’-tetrahydropyranyl) group with HCl, and (5) phosphorylation of 3α-hydroxyl group with POCl₃ to give 17β-methoxy-5β-androstan-3α-ol 3-phosphate. Para-Nitrophenylphosphate (p-NPP) was purchased from KPL (Gaithersburg, MD, USA). Absorption measurements were made with a Corona Electric MTP-500 microplate reader (Hitachinaka, Japan) thermostated at 37°C.

Enzyme-linked secondary antibody

The secondary antibody was digested to F(ab’)₂ by pepsin, and reduced to Fab by 2-mercaptoethanolamine. A maleimide terminal was then introduced into ALP by sulfo-EMCS. Finally, a SH group of Fab and a maleimide terminal of ALP were joined.

![Figure 2](image_url) Ultrasensitive determination of proteins by combination of ELISA and enzyme cycling method. Signals can be obtained as a quadratic function-like response occurring over time.
Ultrasensitive determination

1. Coat a primary antibody.
   Dilute the primary antibody with 50 mM Na₂CO₃ (pH 9.6) to a concentration of 20 μg/mL. Add 50 μL of the antibody into each well of 96-well microplates. Incubate for 1 h at room temperature.

2. Wash microplates.
   Wash the microplates 3 times with TBS including 0.05% Tween 20.

3. Block nonspecific binding sites.
   Dilute Blocking One-P 5 times with distilled water. Block nonspecific binding sites by filling wells this solution at 150 μL/well. Incubate for 45 min at room temperature.

4. Wash microplates.
   Wash the microplates 3 times with TBS including 0.05% Tween 20. Repeat this procedure 3 times.

5. Add an antigen.
   Dilute insulin with TBS including 5% Blocking One-P to 0.1–1.0 pg/mL. Add 50 μL of this antigen solution to each well. Shake the microplates for 1 h at room temperature.

6. Wash microplates.
   Wash the microplates 3 times with TBS including 0.05% Tween 20. Repeat this procedure 3 times.

7. Add an enzyme-linked secondary antibody.
   Dilute an enzyme-linked secondary antibody with TBS including 5% Blocking One-P and 0.05% Tween 20 to 10 pmol/mL. Add 50 μL of this antibody solution to each well. Incubate overnight at 4°C.

8. Wash microplates.
   Wash the microplates 3 times with TBS including 0.05% Tween 20. Repeat this procedure 3 times.

9. Add a thio-NAD cycling solution.
   Dissolve 1 mM NADH, 1.5 mM thio-NAD, 0.25 mM 17β-methoxy-5β-androstan-3α-ol 3-phosphate and 5 U/mL 3α-HSD into 0.1 mM Tris-HCL (pH 9.0). This is referred to as a thio-NAD cycling solution. Add 50 μL of this thio-NAD cycling solution to each well.

10. Measure absorbance.
    Measure the absorbance at 405 nm with a microplate reader every 5 min for 1 h at 37°C.

Limit of detection and coefficient of variation

The experimental data were obtained by subtracting the mean value of blank signals from each of the corresponding measured data. The limit of detection was estimated from the mean of the blank, the standard deviation of the blank and a confidence factor of 3. The coefficient of variation calculated from 3 data points was obtained for 0.1 pg/mL human insulin.

Results and Discussion

We developed an ultrasensitive ELISA for the determination of human insulin based on 3α-HSD-catalyzed enzyme cycling (Fig. 3). In an ELISA, we employed a sandwich method using the primary and the secondary antibody for human insulin. ALP-linked to the second antibody hydrolyzed 17β-methoxy-5β-androstan-3α-ol 3-phosphate to 17β-methoxy-5β-androstan-3α-ol. 17β-methoxy-5β-androstan-3α-ol was then oxidized to 17β-methoxy-5β-androstan-3-one under a catalytic reaction of 3α-HSD with a cofactor thio-NAD. By the opposite reaction, 17β-methoxy-5β-androstan-3-one was reduced to 17β-methoxy-5β-androstan-3α-ol with a cofactor NADH. During this cycling reaction, thio-NADH accumulated in a quadratic function-like fashion. More specifically, because the thio-NADH signaling intensity depends on the number of thio-NADH molecules accumulated by the enzyme reactions, the thio-NADH signaling intensity = a × b × Σ k = a × b × n(n+1) / 2. Here a is the turnover rate of ALP per min; b is the cycling rate of 3α-HSD per min; n = min of measuring time. The amount of insulin was calculated from the increase in absorbance at 405 nm.

Our ultrasensitive method gave a linear calibration curve (y = 0.33x – 0.009, R² = 1.00) for human insulin in the range of 0.1–1.0 pg/mL (Fig. 4). This curve was obtained from the absorbance of thio-NADH at a cycling reaction of 60 min. The limit of detection was 0.0047 pg/assay (i.e., 8.0 × 10⁻¹⁹ mol/assay), and the coefficient of variation was 4% for 0.1 pg/mL. Here we note that the molecular mass of human insulin is 5.8 kDa.

For comparison with a conventional ELISA, p-NPP was
used as a chromogenic substrate for ALP. p-NPP has been widely used in an ELISA. Under their influence, the decay to p-nitrophenol is catalyzed and can also be measured with a 405 nm spectrophotometer. For the use of p-NPP, the limit of detection was 0.28 pg/assay (i.e., 4.9 × 10⁻¹⁷ mol/assay). These results showed that our ultrasensitive determination is at least 2 orders of magnitude more sensitive than conventional ELISA.

Although we only noted the results of the thio-NAD cycling reaction, the combination of 3α-hydroxysteroid 3-phosphate, 3α-HSD, thio-NAD and NADH has been found to detect ALP at 10⁻²⁰ mol/assay (Iwai et al., unpublished data). That is, our method also has the potential to reach limits of detection of 10⁻²⁰ mol/assay. Ishikawa and Hashida 30,31 have achieved the limit of detection of 10⁻²⁰ mol/assay for HIV-1 p24 by their own method, the ‘immune complex transfer enzyme immunoassay’. This method, however, requires time and effort for the detection compared with our method. If their method would be combined to ours, we might obtain further sensitivity.

We can apply our ultrasensitive method to the measurement of insulin in human blood. Because our method is based on an ELISA, the procedure includes the terms of ‘washing microplates’. Thus the debris contaminated in blood will be washed out. The study using blood will be reported in the near future. Further, we can use another enzyme, which catalyzes the formation of 3α-hydroxysteroids or 3-ketosteroids as products, instead of ALP in our ultrasensitive measurement. For example, β-galactosidase can give 3α-hydroxysteroid by using β-galactoside of 3α-hydroxysteroid as its synthetic substrate.

In conclusion, our ultrasensitive determination of proteins by a combination of ELISA and the thio-NAD cycling method is a powerful tool for early disease detection because of its ultrasensitivity. Our method is very convenient because it only requires adding an enzyme cycling solution to a usual ELISA. Our next target is to reduce the procedure time.

Figure 4 Linear calibration curve for human insulin in our ultrasensitive determination. The absorbance of thio-NADH was obtained at the cycling reaction of 60 min. y=0.33x−0.009, R²=1.00.

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Conflict of Interest

H.K., M.K., T.Y., A.I., T.M. and E.I. declare that they have no conflict of interest. S.W. is an employee of BL Co. Ltd.; and M.M. and K.N. are the employees of TAUNS Co. Ltd.

Author Contributions

S.W., T.M. and E.I. directed the entire project. H.K., M.K., M.M., K.N. and A.I. performed the experiments. T.Y. synthesized the androsterone derivatives. S.W., K.N., T.Y., T.M. and E.I. co-wrote the manuscript.

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