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Cascade enzyme-linked immunosorbent assay (CELISA)

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ABSTRACT

Immunoassays are representative biochemical detection methods. Among them, sandwich-type immunoassays, typified by sandwich ELISA, have used in disease diagnosis or biochemical detection with high target selectivity. Horseradish peroxidase and alkaline phosphatase have been typically used for signal amplification in ELISA. Recently developed sandwich-type immunoassays such as biobarcode immunoassays, immuno-PCR, and immuno-RCA have improved sensitivity by changing mainly the signal amplification method. To develop a novel amplification method in ELISA, an enzyme-cascading system was incorporated into an ELISA, and the new assay is termed a cascading enzyme-linked immunosorbent assay (CELISA). This CELISA includes a trypsinogen–enterokinase combination as the cascading enzyme system, and was used to detect alpha-fetoprotein (AFP), which is a liver cancer marker, and prostate-specific antigen (PSA). Using a colorimetric reagent for signal generation, CELISA had 0.1–10 pM limits-of-detection for AFP and PSA in whole human serum and assay buffers, depending on the platform, well plate, or microbead type used. This study represents the first example that incorporated an enzyme cascading step in an ELISA system, resulting in successful signal amplification with sensitive detection of pathogenic antigens in serum.

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1. Introduction

Immunoassays are widely used in the clinic and in medical research (Butler, 2000; Hage, 1999; Yalow and Berson, 1959). In particular, sandwich-type immunoassays, which use two antibodies directed against two different epitopes of the same target, are commonly used in disease diagnosis owing to high selectivity. The sandwich ELISA (enzyme-linked immunosorbent assay) has been, however, continuously challenged by other immunoassays with increased detection sensitivities (Ekins, 1980; Porstmann and Kiessig, 1992). The sensitivity problem is becoming a major issue not only in immunoassays, but also for all biosensors, because the detection of low-abundance biomarkers has been recognized to be of major importance for early diagnosis of lethal diseases (Kozlowski et al., 2003; Rao et al., 2006; Wang et al., 2005). Therefore, recent efforts in immunoassay technology have focused mainly on the development of new signal amplification methods, as well as new antibodies, resulting in novel techniques such as biobarcode immunoassays, immuno-PCR (polymerase chain reaction), and immuno-RCA (rolling-circle amplification) (Brakmann, 2004; Hage, 1999; Nam et al., 2003; Niemeyer et al., 2005; Schweitzer et al., 2000). Although the newly developed methods have improved sensitivity, they suffer from poor reproducibility, high cost, and time-consuming or complicated protocols.

Here we report a novel immunoassay method termed "cascading enzyme-linked immunosorbent assay" (CELISA), which involves incorporation of an enzyme-cascading step into an ELISA system, in place of the use of enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) in conventional ELISA (Porstmann et al., 1985; Porstmann and Kiessig, 1992; Regalado et al., 2004). CELISA consists of a capture antibody immobilized on a solid support such as nano/microparticles or well plates, a detection antibody accompanied by a cascading enzyme, a proenzyme, and a substrate. Fig. 1 compares CELISA with conventional sandwich ELISA. The major difference between CELISA and ELISA is in an enzyme which accompanies the detection antibody. In ELISA, HRP catalyzes a substrate change, resulting in an altered substrate absorption or fluorescence emission. In CELISA, enterokinase (EK) converts catalytically inactive trypsinogen (the trypsin precursor protein) to active trypsin, which in turn catalyzes change of a chromogenic substrate. In this context, EK and trypsinogen are termed a cascading enzyme and a proenzyme, respectively. The turnover number (k_{cat}) of trypsinogen activation by EK is 414 min^{-1} (Nemoda and Sahin-Toth, 2005), meaning that EK can activate 414 molar equivalents of trypsinogen per minute in the presence of sufficient trypsinogen. This means that every EK molecule trapped *via* antigen–antibody

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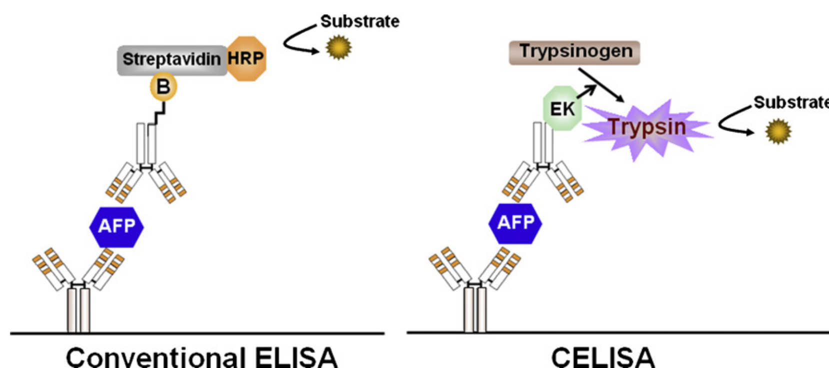

Fig. 1. Comparison of CELISA with conventional ELISA. Instead of using HRP as in ELISA, CELISA employs enterokinase, which converts inactive trypsinogen to catalytically active trypsin. In turn, trypsin acts on a substrate to generate a signal.

interaction can activate 10^4 trypsinogen molecules every 25 min, thereby amplifying the signal by 10^4.

The first CELISA was developed to detect alpha-fetoprotein (AFP) and prostate-specific antigen (PSA) on well plates and microbeads, and shows limits-of-detection (LODs) of 0.1–10 pM (MacDougall, 1980).

2. Materials and methods

2.1. Materials

Magnetic microparticles (MMPs) and silica microparticles (SMPs) with carboxylate surfaces were purchased from Invitrogen (1 μm Dynabeads® Myone™) and Polysciences Inc. (0.5 μm Silica Microspheres), respectively. Alpha-fetoprotein (AFP) was purchased from Biodesign. Monoclonal mouse anti-AFP antibodies 14C3 and 11C3 were purchased from BoditechMed (Chuncheon, Korea). Monoclonal mouse anti-1/PSA and anti-3/PSA were purchased from R&D. Sulfo-NHS-biotin and Sulfo-SMCC were purchased from PIERCE. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) was the product of TCI. Bovine trypsinogen, N-hydroxysuccinimide (NHS), N_α-benzoyl-L-Arg-*p*-nitroanilide hydrochloride (BAPNA), horseradish peroxidase (HRP), and prostate-specific antigen (PSA) were purchased from Sigma. Suc-AlaAlaProArg-*p*-nitroanilide (Suc-AAPR-*p*NA) was a product of Bachem. ELISA kit was purchased from BD Biosciences. Calf intestinal enterokinase was a Roche product. Immobilizer™ carboxyl plates and PD-10 desalting columns were purchased from NUNC and GE Healthcare, respectively. Acryl-Cuvettes for UV–vis spectrophotometry were purchased from SARSTEDT (Germany). MagnaRack™ was purchased from Invitrogen. Frequently used buffers were Buffer A (25 mM MES; pH 6.0), Buffer B (5 mM EDTA, 50 mM triethanolamine; pH 7.3), Buffer C (25 mM HEPES; pH 7.5), Buffer D (0.5% [w/v] BSA in TBST), and Buffer E (250 mM ethanolamine; pH 8.0).

The data in Figs. 2, 3, 5 and 6 were fitted to a four parameter logistic equation ($y = d + (a - d)/[1 + (x/c)^b]$; a: estimated response at zero concentration, b: slope factor, c: mid-range concentration, d: estimated response at infinite concentration, y: response in the enzyme reaction rate, x: antigen concentration) (Finney, 1983) using KaleidaGraph 4.0 (Synergy Software).

2.2. Conjugation of capture antibody to MMPs and wellplates

MMP surfaces were activated for protein conjugation according to the manufacturer's protocol with a slight modification. Briefly, 100 μL of a suspension (10 mg/mL in water) of MMPs was added to 1 mL of Buffer A. After magnetic pulldown of MMPs using a MagnaRack, supernatants were removed. The procedure was repeated twice to ensure complete washing of MMPs. The resuspended MMPs, in 100 μL Buffer A, were mixed with 25 mg EDC and 25 mg NHS, in 1 mL Buffer A, and incubated for 30 min at room temperature, to form an NHS-activated MMP surface. MMPs were collected by magnetic pulldown and washed twice with Buffer A.

Monoclonal mouse anti-AFP 11C3 or anti-3/PSA antibodies were covalently immobilized to MMPs, generally according to the manufacturer's instructions, but with a slight modification. To 1 mg NHS-activated MMPs in 1 mL of buffer A, 4 μL of a solution (12.5 mg/mL) of antibody in PBS, pH 7.4, was added, and the mixture was incubated with slow tilt rotation at room temperature for 5 h. After removal of supernatant, the remaining NHS groups on MMP were blocked by successive treatment with Buffer D and Buffer E at room temperature, each for 1 h. The Ab–MMP conjugates were washed three times with TBST (0.05% Tween-20, 135 mM NaCl, 50 mM Tris; pH 7.4) and resuspended in 1 mL TBST. The Ab–MMP conjugate was stable for several months at 4 °C.

Antibody-coated wellplates were prepared just before use. Each well was filled with 100 μL of 20 μg/mL anti-AFP 11C3 or anti-3/PSA in 100 mM sodium phosphate buffer, pH 8.0, and incubated with

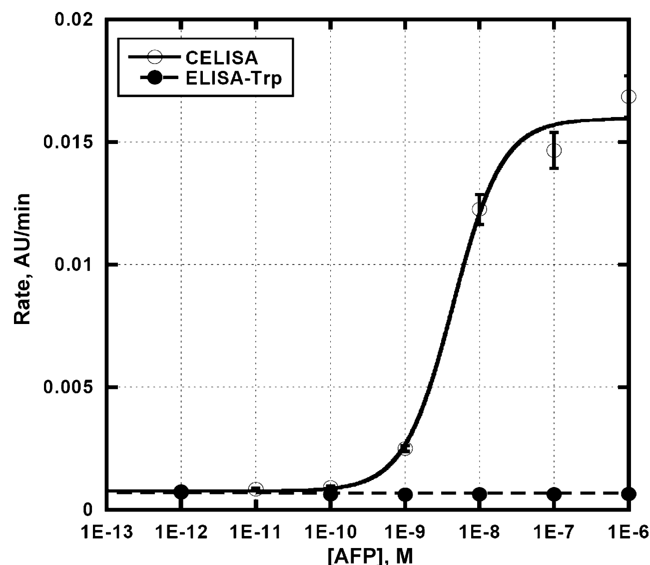

Fig. 2. Signal amplification using an enzyme cascading system. Closed circles represent AFP detection signals from ELISA, where trypsin conjugated to the detection antibody hydrolyzes the substrate. Open circles represent AFP detection signals from CELISA, where enterokinase conjugated to the detection antibody activates trypsinogen to trypsin which, in turn, hydrolyzes a substrate. Whereas CELISA detected a limit of 0.1 nM of AFP, ELISA using trypsin failed to detect 1000 nM AFP, showing that CELISA employing EK-trypsinogen is at least 10,000 times more sensitive than is ELISA with trypsin as the antibody-linked enzyme.

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