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Electrochemical detection of the disease marker human chitinase-3-like protein 1 by matching antibody-modified gold electrodes as label-free immunosensors

Wethaka Chaocharoen [a], Wipa Suginta [a], Warakorn Limbut [b,c,d], Araya Ranok [a], Apon Numnuam [b,c,e], Panida Khunkaewla [a], Proespichaya Kanatharana [b,c,e], Panote Thavarungkul [b,c,f,**], Albert Schulte [a,*]

[a] Biochemistry–Electrochemistry Research Unit, Schools of Chemistry and Biochemistry, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[b] Trace Analysis and Biosensor Research Center, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[c] Center of Excellence for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[d] Department of Applied Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[e] Department of Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[f] Department of Physics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

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ABSTRACT

Tissue inflammation, certain cardiovascular syndromes and the occurrence of some solid tumors are correlated with raised serum concentrations of human chitinase-3-like protein 1 (YKL-40), a mammalian chitinase-like glycoprotein, which has become the subject of current research. Here we report the construction and characterization of an electrochemical platform for label-free immunosensing of YKL-40. Details of the synthesis of YKL-40 and production of anti-YKL-40 immunoglobulin G (IgG) are provided and cross-reactivity tests presented. Polyclonal anti-YKL-40 IgG was immobilized on gold electrodes and the resulting immunosensors were operated in an electrochemical flow system with capacitive signal generation. The strategy offered a wide linear detection range (0.1 μg/L to 1 mg/L) with correlation coefficients (R^2) above 0.99 and good sensitivity (12.28 ± 0.27 nF/cm^2 per decade of concentration change). Additionally, the detection limit of 0.07 ± 0.01 μg/L was well below that of optical enzyme-linked immunosorbent assays (ELISAs), which makes the proposed methodology a promising alternative for YKL-40 related disease studies.

1. Introduction

Molecular disease markers are important tools for mechanistic disease studies and also key components of diagnostic or prognostic systems for the clinical analysis of critical body conditions. Recently, human chitinase-3-like-protein 1 (YKL-40), a human chitinase-like protein and family-18 glycosyl hydrolase, was identified as a promising biomarker, with elevated concentrations associated with pathologies such as infection, inflammation, fibrosis and cancer [1–7]. Until now YKL-40 analysis has relied exclusively on optical enzyme-linked immunosorbent assays (ELISAs) with detection limits of about 20 μg/L [8], and to the best of our knowledge no alternative YKL-40 immunosensing scheme has been reported.

For clinical applications, immunosensing needs to be cheap, simple and efficient. It therefore requires a supply of high-grade antibodies and antigens, together with a sensitive and robust method for detecting antibody–antigen binding in sample solutions. Electroanalysis of antibody–antigen (Ab–Ag) interaction is a promising approach, offering high sensitivity detection with compact equipment that is compatible with economical, mass-produced sensor platforms. Usually, the antibody is immobilized on the electroactive surface of the working electrode (the "immunosensor") of a three-electrode electrochemical cell and antigen binding detected by amperometry, voltammetry or electrochemical impedance spectroscopy (EIS) [9]. Amperometry and voltammetry measure the protein interaction indirectly, using enzyme-labeled secondary antibodies that generate a concentration-dependent Faradaic current through catalytic formation of a redox product. EIS, on the other hand, is a label-free screening method that measures electrode capacitance changes produced by restructuring of the interfacial Helmholtz double-layer on antigen binding [9,10]. EIS-based immunosensing with modern electrochemical workstations is quite effective; however, special hardware and software are needed and data analysis depends upon a rather complex treatment of alternating current signals. A simpler method of detecting antigen-induced electrode capacitance changes is the analysis of the current response on application of a potential pulse [11,12].

* Corresponding author. Tel.: +66 44 22 6187; fax: +66 44 22 4185.
** Corresponding author. Tel.: +66 74 28 8008; fax: +66 74 44 6657.
E-mail addresses: panote.t@psu.ac.th (P. Thavarungkul), schulte@sut.ac.th (A. Schulte).

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In developing a novel electrochemicalYKL-40 immunosensor with a detection limit significantly lower than that of optical ELISA, we report procedures for the preparation of the YKL-40 antigen and of YKL-40-specific polyclonal IgG, characterization of the antibody and antigen and some promising results from a flow injection analysis system, with antigen binding monitored by electrode capacitance measurements through potential pulsing.

2. Experimental

2.1. Chemical reagents and other materials

Human cDNA, a protein A agarose column and HRP-conjugated-anti-rabbit IgG were obtained from GenScript Inc., Piscataway, NJ, USA. The pQETri System expression vector, a Ni-nitrilotriacetic acid (NTA) agarose column and *Escherichia (E.) coli* M15 (pREP) were purchased from Qiagen Ltd., Manchester, UK. The restriction enzymes *Sac*I and *Xho*I were from Promega, Madison, USA. The PCR primer came from GENEBIODESIGN Co., Ltd., Taipei, Taiwan and related verification of the recombinant plasmid by automated DNA sequencing was carried out by First BASE Laboratories Sdn Bhd, Seri Kembangan, Malaysia. Ampicillin, phenylmethylsulphonyl fluoride (PMSF), imidazole and Tritron X-100 were products of USB Corporation, Cleveland, OH, USA. Isopropyl thio-β-D-galactoside (IPTG), Freund's complete adjuvant, Freund's incomplete adjuvant, Tris–HCl, Tris-base and lysozyme were from Sigma Aldrich, St. Louis, MO, USA. 3,3′,5,5′-tetramethylbenzidine (TMB), the substrate for ELISA assays, was purchased from Invitrogen, Carlsbad, CA, USA. Glutaraldehyde, 1-dodecanethiol, potassium ferricyanide ($K_3[Fe(CN)_6]$), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Acros Organic, Bridgewater, NJ, USA. Protein concentrations were measured with a Pierce® BCA Protein Assay kit from Thermo Scientific, Rockford, IL, USA. All other basic chemical reagents (e.g. NaCl, KCl, Na_2HPO_4, KH_2PO_4, Urea, HCl, H_2SO_4 and thiourea) were from Carlo Erba Reagents, Cornaredo (MI), Italy and unless otherwise mentioned were of analytical grade.

Alumina slurries with a particle size of 1 and 5 μm, as used for gold sensor surface polishing, were obtained from Metkon Instruments Ltd., Bursa, Turkey. A Hiprep 26/10 desalting column was supplied by GE HealthCare, Hatfield, UK and Vivaspin-20 ultrafiltration membranes came from Vivascience AG, Hannover, Germany. All aqueous solutions were prepared with purified water from a reverse osmosis-deionizing system (Cascada™ Lab Water Systems, PALL Life Science, Ann Arbor, MI, USA). Buffers were filtered through Whatman® cellulose nitrate membrane with pore size 0.20 μm from GE HealthCare, Hatfield, UK.

2.2. Instrumentation

ELISA analysis was performed using a Biochrom® Anthos MultiRead 400 Microplate Reader (Biochrom Ltd., Cambridge, UK). All electrochemical immunosensor recordings were made with an EA163 potentiostat and e-corder 410 data acquisition device, from eDAQ, Denistone East, Australia. The system for flow injection-based sample measurements used a peristaltic pump (Miniplus® 3 from Gilson, Middleton, WI, USA), a manual injection valve (Biologic MV-6® from Bio-Rad, Hercules, CA, USA) and a three-electrode radial flow cell with customized 3-mm-diameter gold (Au) disk electrodes (ALS Co., Ltd., Tokyo, Japan). Supplementary Fig. 1S is a schematic representation of the entire YKL-40 detection system used in this study.

2.3. Preparation of recombinant human YKL-40 antigen

Human cDNA was used as the template for PCR amplification. Two flanking primers were designed according to the YKL-40 sequence, retrieved from the GENBANK database (Accession no. AAA16074). The oligonucleotides used for amplification of full-length YKL-40 DNA were 5′-AGAGCTCGGTGTGAAGGCGTCTCAAAC-3′ for the forward and 5′-TCTCGAGCGTTGCAGCGAGTGCATC-3′ for the reverse primer. The PCR product of expected size (1.1 kbp) was cloned in the pQETri System expression vector using *Sac*I and *Xho*I cloning sites (sequences underlined) following the manufacturer's protocol. The nucleotide sequences of both sense and anti-sense strands of the YKL-40 DNA fragment were confirmed by automated DNA sequencing.

Recombinant YKL-40 was expressed in *E. coli* M15(pREP) as a *C*-terminally $(His)_6$-tagged polypeptide. Cells were grown at 37° in Luria Bertani (LB) medium containing 100 μg/mL ampicillin until the OD_{600} of the cell culture reached 0.6. Expression was induced by the addition of isopropyl thio-β-D-galactoside (IPTG) to a final concentration of 0.5 mM. After 16 h of induction at 25°, the cell pellet was collected by centrifugation and resuspended in lysis buffer (50 mM Tris–HCl buffer, pH = 8.0, containing 300 mM NaCl, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1.0 mg/mL lysozyme and 1% (v/v) triton X-100), and then lysed on ice using a Sonopuls Ultrasonic homogenizer with a 6-mm-diameter probe (50% duty cycle; amplitude setting, 20%; 30 s, 6–8 times). After centrifugation, the pellet containing insoluble YKL-40 was solubilized in 8 M urea before purification of the dissolved protein by affinity chromatography on a Ni-NTA agarose column run under gravity. YKL-40 was eluted with 250 mM imidazole. Fractions containing YKL-40 were pooled and then subjected to several rounds of membrane centrifugation using Vivaspin-20 ultrafiltration membrane concentrators (M_r 10000 cut-off) for complete removal of

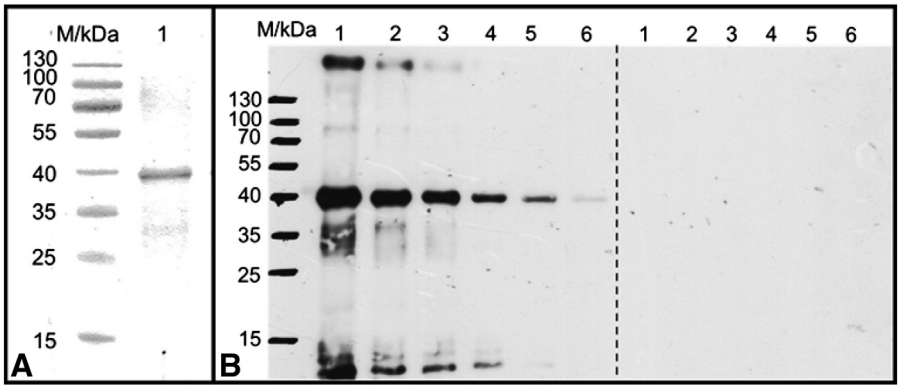

Fig. 1. Purification of recombinant YKL-40. (A) SDS-PAGE of affinity-purified YKL-40 (lane 1, 2 μg): Coomassie-blue stained 12% polyacrylamide gel. (B) YKL-40 protein detected by immunoblotting with the anti-YKL-40 serum (left-hand panel). Lane: M, standard protein markers; 1–6, purified YKL-40 protein (2 μg) detected with various dilutions (1:5000, 1:10000, 1:20000, 1:40000, 1:80000 and 1:160000) of antiserum. The same dilutions of pre-immune serum were used as a negative control (right-hand panel).