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Production and characterization of anti-*Salmonella* polyclonal antibodies as bio-recognition element for developing a microbial monitoring method

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미생물학적 모니터링 분석방법 개발을 위한 생물학적 수용체로서 살모넬라에 특이적인 다중클론 항체의 생산 및 특성 검토

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Abstract

For the construction of the microbial monitoring method, anti-*Salmonella* polyclonal antibodies (pAbs) were produced from a rabbit and purified by saturated ammonium sulfate precipitation and protein A affinity column. The reactivity of anti-*Salmonella* pAbs was compared to that of commercial ones by using an indirect ELISA. The specificity of anti-*Salmonella* pAbs was investigated using 20 *Salmonella* serotypes and 20 non-*Salmonella* strains. A capturing ability of anti-*Salmonella* pAbs was investigated by exposing antibody-immobilized gold biosensor to different concentration of *Salmonella* mixture. Anti-*Salmonella* pAbs were successfully produced and purified with an antibody concentration of 2.0 mg/mL The reactivity of purified anti-*Salmonella* pAbs was greater than that of commercial one at all tested concentrations. All *Salmonella* serotypes, except *S.* Diarizonae, showed excellent binding efficiency with purified anti-*Salmonella* pAbs. Moreover, the purified anti-*Salmonella* pAbs showed excellent specificity against all non-*Salmonella* strains. The anti-*Salmonella* pAbs immobilized on the gold biosensor demonstrated the successful capturing capability against *Salmonella* with a dose-response manner. Therefore, the anti-*Salmonella* pAbs exhibited sufficient reactivity, specificity, as well as capturing capability against Salmonella to be considered as a bio-recognition element.

Key words : *Salmonella*, polyclonal antibody, bio-recognition, quality control, gold biosensor

Introduction

A demand for high quality but safe food has been on the rise for both food industry and consumers worldwide. Although several intervention strategies have been introduced and implemented, the number of foodborne outbreaks is either increasing and/or steady, depending on the causative foodborne pathogens (1). *Salmonella*, along with *Escherichia coli* O157:H7, has been the most prevalent foodborne pathogen (2-3). Approximately 1.4 million cases of *Salmonella* infections are reported in the United States every year, and the annual cost for medical treatment and lost productivity has been estimated to be 0.5 to 2.3 billion dollars (1). The general symptoms of *Salmonella* infections are characterized by fever, headache, abdominal pain, diarrhea or constipation, vomiting, and nausea (4-5). So far, out of more than 2,500 serotypes of *Salmonella*, *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, *S.* Javiana, and *S.* Heidelberg are

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the most frequently reported (3,6,7). *Salmonella* outbreaks have mainly been associated with the consumption of meat, poultry, milk, and dairy products contaminated with *Salmonella*. In the past two decades, *Salmonella* outbreaks have been increasing associated with minimally processed fruits and vegetables, such as seed sprouts, unpasteurized fruit juices, mangoes, tomatoes, and spinach (3,7,8).

Considerable studies have been performed for monitoring and controlling *Salmonella* contamination by using PCR, ELISA, and various biosensor methods (9-10). Since the last two decades, biosensor method has been recognized as an on-site applicable rapid detection method for food industry and its consumers (10-11). Biosensor is defined as an analytical device that converts recognition of a target analyte into a measurable signal by using a physicochemical transducer (12). Our research group has already developed a gold biosensor combined with a light microscopic imaging system (LMIS), for the detection of *Listeria monocytogenes* and *E. coli* O157:H7 in food (13-15). In the detection system, the specific and reactive antibody is immobilized on the gold sensor platform for the specific recognition of the target pathogens. When the antibody-immobilized gold sensor is placed on the surface of a food, specific binding of target

pathogens to the antibody occurs on the gold sensor. Finally, the bound target pathogen is visualized and enumerated by a light microscope connected with a charge-coupled device (CCD) camera (13). Using the same principle and methodologies, the current system is expected to detect *Salmonella* in food as a on-site applicable method.

There are several bio-recognition elements available including tissues, cells, enzymes, antibodies, and nucleic acids. Among them, the antibody has been chosen most popularly due to its excellent reactivity (sensitivity) and specificity (16-17). Since antibodies are produced by lymphocytes as defense mechanism in response to antigens in vertebrates, they show excellent reactivity and specificity against their targets. The overall goal of our study was to develop a rapid, on-site applicable gold biosensor method combined with LMIS for *Salmonella* detection. In order to construct the biosensor method, anti-*Salmonella* polyclonal antibodies were produced and purified from a rabbit. The characteristics of purified antibodies including reactivity and specificity, and capturing capability against *Salmonella* were investigated as a bio-recognition element in this study.

Table 1. Inclusive and exclusive specificity of purified anti-*Salmonella* polyclonal antibodies (n=3)

Salmonella serotypes	Absorbance	non-*Salmonella* strains	Absorbance
Salmonella Arizonae (SA 4407)	0.852±0.036	*Bacillus cereus* ATCC 13061	0.473±0.218
S. Bongori (SA 4410)	1.008±0.123	*B. cereus* ATCC 1611	0.325±0.115
S. Cerevisae (SA 3004)	1.175±0.007	*Campylobacter jejuni*	0.298±0.187
S. Diarizonae (SA 4408)	0.469±0.012	*Listeria monocytogenes* (H7757)	0.139±0.095
S. Dublin	0.846±0.020	*L. monocytogenes* (H7738)	0.185±0.014
S. Enteritidis	1.621±0.042	*L. monocytogenes* ATCC 19111	0.270±0.011
S. Gallinarum (SA 4404)	0.997±0.001	*L. innocua* ATCC 33090	0.183±0.059
S. Heidelberg (SARA 36)	1.166±0.194	*Shigella boydii* NCCP 11190	0.256±0.068
S. Houtenae (SA 4409)	1.002±0.153	*S. sonnei* ATCC 9290	0.269±0.138
S. Indica (SA 4411)	1.082±0.050	*Staphylococcus aureus* ATCC 12600	0.121±0.150
S. Javiana	1.072±0.013	*S. aureus* ATCC 6538	0.259±0.146
S. Montevideo	0.893±0.014	*S. aureus* ATCC 25923	0.342±0.080
S. Newport	0.988±0.100	*Escherichia coli* (GM 2163)	0.482±0.135
S. Panama	1.190±0.090	*E. coli* ATCC BAA-2192	0.370±0.246
S. Paratyphi A (R737)	0.874±0.130	*E. coli* ATCC BAA-2196	0.324±0.203
S. Paratyphimurium UF	1.452±0.221	*E. coli* O157:H7 204p	0.309±0.163
S. Pullorum (SARB 52)	1.288±0.054	*E. coli* O157:H7 505B	0.312±0.056
S. Salamae (SA 4406)	0.803±0.103	*E. coli* O157:H7 700599	0.264±0.073
S. Typhi	1.257±0.122	*Vibrio parahaemolyticus* ATCC 17802	0.102±0.036
S. Typhimurium	1.721±0.042	*Yersinia enterocolitica* ATCC 23715	0.152±0.101

Materials and Methods

Bacteria and culture condition

All the foodborne pathogens listed in Table 1, except for bacteria ending with ATCC, were obtained from the Food Microbiology Laboratory at Auburn University Auburn, AL, USA. The other foodborne pathogens with ATCC label were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Each bacterial strain was cultivated in 20 mL of Tryptic Soy Broth (TSB, Difco Laboratories, Sparks, MD, USA) while *L. monocytogenes* was cultivated in TSB with 0.6% yeast extract. After incubation at 37℃ at 200 rpm for 16 h, each culture was washed three times with phosphate buffered saline (PBS, pH 7.2, Sigma-Aldrich Co., St. Louis, MO, USA) by centrifugation at 5,000 $\times g$ for 5 min. The collected bacterial cells were re-suspended in PBS and the bacterial concentration was adjusted to 10^8 CFU/mL using a pre-constructed standard curve, determined by optical density at 640 nm. *Salmonella* mixture was prepared by mixing equal amounts of *S.* Typhimurium and *S.* Enteritidis.

Purification of polyclonal antibodies (pAbs) against *Salmonella*

A New Zealand white rabbit (approximately 3 kg) was used for the production of pAbs against *Salmonella*. The purification of pAbs was performed following a modified Kohler and Milstein's method (1975). For the immunization of the rabbit, *Salmonella* mixture (10^8 cells), inactivated with 1% formalin, was emulsified with Ribi's adjuvant system at 285 g/mL. The rabbit was immunized with 1 mL of the adjuvant emulsion through an intradermal injection of 50 µL into each of eight sites on the back, close to the spinal cord, and 300 µL aliquot intramuscular into each hind leg. The same immunization procedure with the adjuvant emulsion was performed to boost the antibody production in the rabbit at 4 week intervals after the initial immunization. Seven days later, the rabbit blood containing anti-*Salmonella* pAbs was collected from the central ear artery and centrifuged at 5,000 $\times g$ for 20 min at 4℃. Solid ammonium sulfate was added slowly into 1 mL of the collected anti-*Salmonella* rabbit serum until a final concentration of 0.5 g/mL. After placing on ice for 5 min, the serum was centrifuged at 5,000 $\times g$ for 5 min at 4℃ and the precipitate was washed twice with 1 mL of saturated ammonium sulfate. The precipitate was then suspended in PBS and dialyzed in a 20 mM sodium phosphate buffer (pH 7.0) at 4℃ overnight. The suspension

containing anti-*Salmonella* pAbs was applied to protein A affinity column (Sigma-Aldrich Co., St. Louis, MO, USA) and eluted with 0.1 M citric acid (pH 3.45). After adjusting pH of the eluates with 2 M Tris (pH 9), the protein content of each fraction was estimated by measuring adsorption at 280 nm. Finally, all the eluates were dialyzed with PBS at 4℃ overnight, and the protein content of purified anti-*Salmonella* pAbs was determined by Bradford method.

Reactivity and specificity of purified anti-*Salmonella* pAbs by indirect ELISA method

Salmonella mixture (*S.* Typhimurium and *S.* Enteritidis) and forty foodborne pathogens (20 *Salmonella* serotypes and 20 non-*Salmonella* strains) were used for the investigation of the reactivity and specificity of anti-*Salmonella* pAbs, respectively. A 96-well polystyrene assay plate (Costar, Cambridge, MA, USA) was coated with 100 µL of *Salmonella* mixture for the reactivity test and each bacterial suspension (10^8 CFU/mL) for specificity test at 37℃ for 1 h. After washing three times with 200 mL PBS containing 0.1% Tween-20 and 0.02% sodium azide (PBST), the plate was blocked with 200 µL of 1% bovine serum albumin (BSA, Equitech-Bio Inc., Kerrville, TX, USA) for 1 h at 22℃, followed by washing with 200 mL PBST. An aliquot of 100 µL of purified anti-*Salmonella* pAbs was added and the plate was incubated at 22℃ for 2 h, prior to washing with PBST. Commercial anti-*Salmonella* pAbs (Abcam Co., Cambridge, MA, USA) were used for the comparison of reactivity with that of purified anti-*Salmonella* pAbs. An aliquot of 100 µL of alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma-Aldrich Co.) was added to each well for incubation at 22℃ for 1 h. Finally, 100 µL of p-nitrophenyl phosphate (p-npp, Sigma-Aldrich Co.) in 10 mM diethanolamine buffer (pH 9.5) was added to the wells for color development. Finally, the absorbance at 405 nm was measured at 0 min and 15 min using a microplate reader (Thermo Labsystems Inc., Helsinki, Finland).

Salmonella binding to the purified anti-*Salmonella* pAbs immobilized on a gold biosensor

A glass sensor platform was prepared by cutting a microscopic cover glass (0.17 mm thickness) into 5×5 mm with micro-dicing saw (MPE Inc., Grass Valley, CA, USA). After cutting, it was ultrasonically cleaned with an acetone solution, and rinsed with alcohol and deionized (DI) water. The cleaned sensor was sputtered with 140 nm gold by using a Pelco SC-6 sputter coater (Ted Pella Inc., Redding, CA,

USA). For antibody immobilization, 100 μL of purified anti-*Salmonella* pAbs were added on the gold sensor surface for incubation at 37℃ for 1 h. After washing three times with PBS, the pAbs-immobilized gold sensor was blocked with 100 μL of 1% BSA at 22℃ for 1 h. Finally, the sensor was incubated with *Salmonella* mixture at 37℃ for 1 h and washed four times with DI water, prior to SEM observation (S-4800, Hitachi Ltd., Tokyo, Japan).

Statistical analysis

All experiments were performed at least in triplicates and the data are expressed as mean±SD. Student's paired t-test, for two groups, and one-way analysis of variance (ANOVA), for more than two groups, were used to compare the means by using GraphPad and InStat V.3 software (GraphPad Co., San Diego, CA, USA). Differences were considered to be statistically significant at p<0.05.

Results and Discussion

Purification of anti-*Salmonella* pAbs

Unlike the preparation of monoclonal antibodies (mAbs), the preparation of pAbs is less complicated since it does not require an immortal hybridoma (18-19). Anti-*Salmonella* pAbs were produced from a rabbit and purified by saturated ammonium sulfate precipitation and protein A affinity column. The final antibody concentration was determined to be 2.0 mg/mL, which was greater than that of commercial anti-*Salmonella* pAbs (1.0 mg/mL).

Comparative reactivity of purified anti-*Salmonella* pAbs

Reactivity of purified anti-*Salmonella* pAbs was tested and compared with that of commercial ones by indirect ELISA (Fig. 1). Since the original concentration of purified anti-*Salmonella* pAbs was two-fold greater than that of commercial anti-*Salmonella* pAbs, its concentration was adjusted with that of commercial ones to enable the direct comparison between both antibodies. The reactivity of purified anti-*Salmonella* pAbs was greater than those of commercial anti-*Salmonella* pAbs over the entire range of pAbs concentration, although there were no significant differences between the two groups at the same dilution of anti-*Salmonella* pAbs (p>0.05). Higher the antibody concentration, less differences of absorbance was observed for both anti-*Salmonella* pAbs. However, the purified

anti-*Salmonella* pAbs obviously had greater binding efficiency against *Salmonella* mixture at relatively lower concentration of pAbs, ranging from 1/3,200 to 1/6,400 dilution of the original concentration of pAbs (1.0 mg/mL). The greater binding efficiency of purified anti-*Salmonella* pAbs over commercial pAbs can presumably be attributed to targeting *S.* Typhimurium and *S.* Enteritidis and relatively delicate purification procedures. Based on our results, the concentration of purified anti-*Salmonella* pAbs was determined to be 0.00125 mg/mL (1,600 times dilution of original concentration of purified anti-*Salmonella* pAbs) considering both reactivity and economic aspects. Finally, this study confirmed that the purified anti-*Salmonella* pAbs had a competitive reactivity over commercial anti-*Salmonella* pAbs.
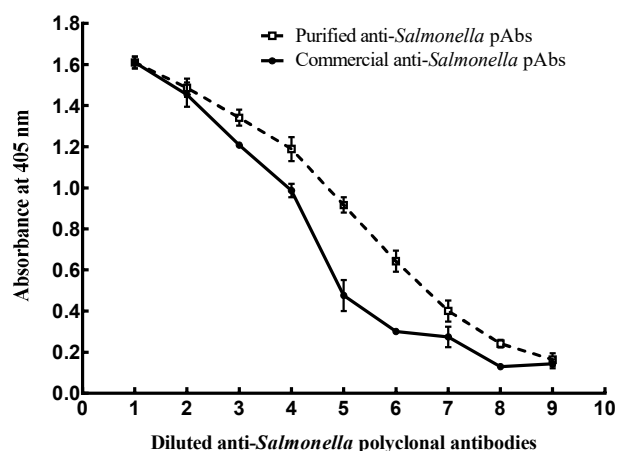

Fig. 1. Comparative reactivity of purified anti-*Salmonella* pAbs and commercial anti-*Salmonella* pAbs.

The x-axis numbers, corresponding dilutions are as follows: 1, 200 times dilution; 2, 400 times dilution; 3, 800 times dilution; 4, 1,600 times dilution; 5, 3,200 times dilutions; 6, 6,400 times dilution; 7, 12,800 times dilution; 8, 25,600 times dilution; 9, 51,200 times dilution of anti-*Salmonella* pAbs (1.0 mg/mL). Vertical bars represent SD and paired t-test was performed at p<0.05 (N=3).

Specificity of purified anti-*Salmonella* pAbs

Besides reactivity, the other desired property, for the antibody to be useful as a bio-recognition element, is its specificity against all pathogenic *Salmonella* serotypes. So far, 20 most frequent serotypes have been held responsible for approximately 70% of *Salmonella* outbreaks (approximately 13% unknown serotypes and 15% other serotypes) (7). As shown in Table 1, all *Salmonella* serotypes, except *S.* Diarizonae, showed significantly higher binding efficiencies with purified anti-*Salmonella* pAbs (p<0.05). Among the 20 *Salmonella* serotypes, purified anti-*Salmonella* pAbs showed the best binding reactivity with *S.* Typhimurium and *S.* Enteritidis, which exhibited maximum color change during

incubation. However, the purified anti-*Salmonella* pAbs showed no cross-reactivity against other exclusive foodborne pathogens including major strains of *Bacillus*, *Campylobacter*, *E. coli/E. coli* O157:H7, Listeria, Shigella, Staphylococcus, *Vibrio*, and *Yersinia*. Their absorbance changes were obviously lower than those of *Salmonella* serotypes, except for *S.* Diarizonae.

Based on the recent annual report of *Salmonella* outbreaks, there were 45,735 cases recognized by CDC in 2013 (7). Among them, *S.* Typhimurium and *S.* Enteritidis accounted for 12.8% and 15.1%, respectively. Our purified antibodies showed the best reactivity with these two major serotypes of *Salmonella*. Furthermore, our pAbs also showed binding efficiency with some of the recent top 20 major serotypes of *Salmonella* including *S.* Newport, *S.* Javiana, *S.* Heidelberg, *S.* Montevideo, and *S.* Typhi. Overall, the purified anti-*Salmonella* pAbs showed an excellent specificity against pathogenic *Salmonella*.

Salmonella binding to the purified anti-*Salmonella* pAbs immobilized on a gold biosensor

For the efficient performance of purified anti-*Salmonella* pAbs, the antibodies need to bind with *Salmonella* after their immobilization on the gold sensor surface (15). If the purified antibodies are immobilized on gold sensor properly, their binding with *Salmonella* will be increased so that numerous *Salmonella* can bind on the antibody-immobilized sensor surface. As seen in Fig. 2B and 2C, there were numerous bindings of *Salmonella* observed, corresponding with the increase in *Salmonella* concentration. Meanwhile, there are a few non-specific binding of *Salmonella* on the sensor surface devoid of antibody, but they are very weak (p<0.05). Capture capacity of the pAbs against *Salmonella* could be enhanced by using various immobilization methods, such as

a covalent attachment with cross-linker and the application of protein A, G, and other derivatives on the sensor platforms (13,14,20,21). However, such studies will be reserved for the future, and our focus for this report will remain on developing new bio-recognition element for *Salmonella* detection with excellent reactivity and specificity.

In summary, anti-*Salmonella* pAbs were successfully isolated and purified from a rabbit and demonstrated competitive reactivity over commercial anti-*Salmonella* pAbs. In addition, it showed excellent specificity against pathogenic *Salmonella* serotypes and sufficient binding capability with *Salmonella* on the gold sensor. Therefore, the purified anti-*Salmonella* pAbs could be a novel bio-recognition element for application in a gold biosensor combined with LMIS for *Salmonella* monitoring.

요   약

살모넬라는 주요한 식중독균의 하나로 미생물학적 식품 품질을 보증하기 위해 신속·정확하게 현장에서 관리되고 모니터링되어야 한다. 본 연구팀에서는 광학 현미경 기반의 이미징 시스템과 바이오센서가 결합된 모니터링 시스템을 개발하였고, 이를 살모넬라에 적용하기 위해서는 바이오센서에 적용되는 생물학적 수용체의 개발이 절대적으로 필요한 실정이다. 따라서 본 연구에서는 살모넬라에 반응하는 특이적인 anti-*Salmonella* 다중클론 항체를 토끼로부터 성공적으로 생산·정제하였으며, 최종 항체의 농도는 2 mg/mL로 결정되었다. 또한, 정제된 anti-*Salmonella* 다중클론 항체의 역가가 상업용 anti-*Salmonella* 다중클론 항체보다 전 항체 농도 범위에서 우수함을 알 수 있었다. 정제된 항체 특이성 검토를 위해, 정제된 anti-*Salmonella* 다중클론 항체를 20 종의 살모넬라 serotypes과 20 종의 non-*Salmonella* strains과 반응시킨 결과, 20 종의 non-*Salmonella* strains과
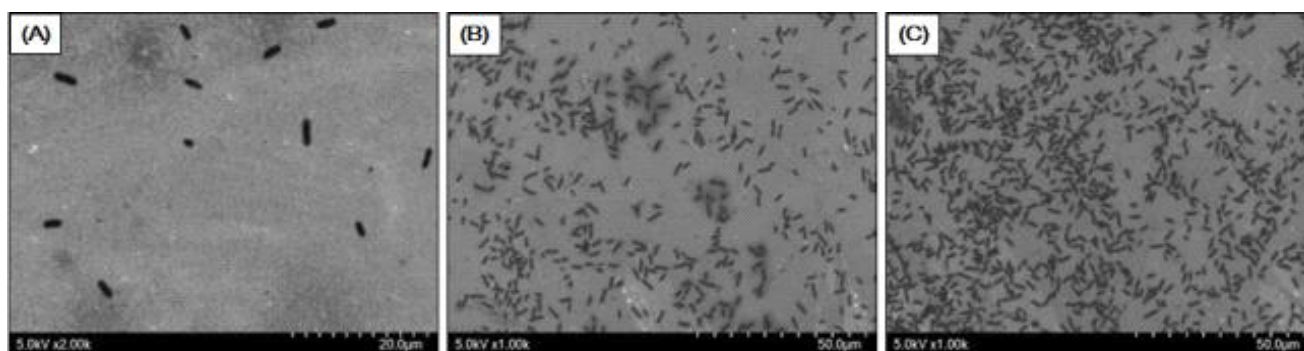

Fig. 2. SEM images of *Salmonella* captured on the surface of (A) antibody-devoid gold biosensor after exposure to *Salmonella* mixture with the concentration of 10^8 CFU/mL, and of antibody-immobilized gold biosensor after exposure to *Salmonella* mixture with the concentrations of (B) 10^6 CFU/mL and (C) 10^8 CFU/mL.

는 특이성을 보여주지 않은 반면 19 종의 살모넬라 serotypes와 유의적으로 높은 반응성으로 보여 주었다. 최종적으로, anti-*Salmonella* 다중클론 항체가 고정화된 골드 센서를 살모넬라 현탁액에 노출시켰을 때, 수 백여 개의 살모넬라가 센서 표면에 capture되어 있음을 확인할 수 있었다. 따라서, 본 연구를 통해 생산·정제된 anti-*Salmonella* 다중클론 항체는 새로운 생물학적 수용체로서 충분한 역가와 특이성 그리고 센서에 적용 시 살모넬라와의 높은 결합력을 보여주었으므로 광학현미경 기반 이미징 시스템을 활용한 바이오센서 모니터링 시스템에 새로운 생물학적 수용체로 적용될 수 있으리라 판단된다.

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