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Detection of Sugar Process Contamination Using Dextran Binding Phages Produced by Batch Fermentation of *Escherichia coli*

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대장균의 회분식 발효에 의해 생산된 덱스트란 결합 파아지를 활용한 설탕 제조공정 오염 검출

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Abstract

Sequential passes through Sephadex™ columns were used to select phages that displays ligands for dextran (α-1,6 linked linear chains) from a phage antibody library. Those phages that bound to the Sephadex™ in each iteration were replicated in *E. coli*. A phage preparation isolated on the third round selection produced 5.4 nephelos turbidity units (NTU) in a dextran specific immunonephelometric assay, a 2.2 fold higher value than the phage preparation from the first round selection. This phage gave 72 ± 10 normalized intensity (N.I.) in a dip-stick assay against high molecular size dextran (T2000, 2×10^6) and significantly lower color (30 ± 6 N.I.) against low molecular size dextran (T10, 10^4). The presence of an Fab insert in each of these phages was confirmed using a β-galactosidase linked assay and polymerase chain reaction.

Key words : Sephadex, phage, dextran, dip-stick assay, fermentation

Introduction

Phage display antibody libraries are collections where each phage contains a different specific binding protein. All possible antibody binding fragments can be contained in the viral protein coats of the library. The antibody fragments maintain antigen binding affinity(1). Use of these libraries allows selection of antigen binding proteins without animal immunization or hybridoma production(2,3). A filamentous phage (Fd) displays Fab (antigen binding fragments) as fusion proteins with a minor coat protein (pIII). These antibody fragments (Fab) are fusion proteins capable of binding epitopes(3). These fragments expressed on the surface of this filamentous bacteriophage can be used as reagents for detecting antigens, tumor cells, virus, and toxins. Numerous applications have been reported on the use of Fab fragments(4), including in immunotherapy, delivery of molecules to kill specific cells, neutralization of HIV-2, vaccine development and the activation of T-cells around tumor cells(5-7).

Dextrans are synthesized enzymatically from sucrose by dextransucrases, glucansucrases, or glucosyltransferases produced by Leuconostoc or Streptococcii bacteria. They are linked with α-1,6 linked bonds and contains variable amounts of α-1,2 α-1,3, or α-1,4 linked side-chains(8,9). Generally, they are poorly immunogenic, requiring protein conjugation to elicit antibody responses(10). Commercially they are used as blood plasma extenders, in chromatographic media and as medical conjugates for treatment of iron deficiency(9). Their presence in cane sugar processing causes serious economic losses(11). If the dextran levels are high it may not clarify. Dextran in syrups cause production losses due

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to increased viscosity, lowered crystallization rate and changes in sugar shape, increasing losses on separation. Because dextran in sugar is not removed by refining, financial penalties are imposed on the seller of raw sugar for sugar containing dextran above 250 ppm(11). The proper management of a sugar cane processing facility would benefit from targeted exclusion of loads of stale cane from the process. Dextrans are monitored in the sugar processing industry by either the Roberts copper method(11), the alcohol haze test(12), the ASI II method(13), the Optical Activity Ltd. DASA method(14) or the Midland SucroTest™(15). Each method suffers from some fault which precludes its use for routine screening of dextran in loads of sugar cane. Previous study showed the use of antibody displayed phage for the detection of dextran using a dipstick assay and transmission electron micrograph(16). In the course of research on developing a simple method for dextran analysis we found a simple method for selecting dextran binding phages from phage library. This report described a method for isolation of dextran binding phage.

Materials and Methods

Organism and Maintenance

E. coli TG1Tr (K12, D(*lac-pro*), *sup*E, *thi, hsd*D5/ F'*tra*D36, *pro*A+B+, *lacI*q, *lac*ZDM15) was acquired from the Cambridge Center for Protein Engineering, England. This strain was maintained on M9 minimal media agar plates, at 4℃(12). Colonies were grown at 35℃ for 24 hr in a 7-L fermenter (KoBioTech Co., Incheon, Korea) containing 5 L of TY medium (16g Trytone, 10g Yeast extract, and 5g NaCl/L) and incubated overnight at 37℃ before infection with phage.

Phage Infection

Log phase *E. coli* TG1Tr was exposed to phages at 37℃ for 30 min. Cells were removed by centrifugation at 3300 × g for 20 min, resuspended in TY media and incubated overnight at 37℃. The infected cells were centrifuged at 3300 × g for 20 min and stored in a TY-15% glycerol at -70℃, for use as a phage source. Supernatants of broth culture were concentrated as described by Medical Research Council, England(17).

Selection of Phage that Display Ligand for Dextran by Sephadex™Column

Phage library solutions (100 μL of 10^8 titration unit, t.u./mL) were applied to columns containing 10 mL of Sephadex-50G (Phamacia Fine Chemical, Ltd., Uppsala, Sweden). Each column was eluted with 100 mL of PBST (PBS + 0.05% Tween 20), followed by 100 mL of phosphate-buffered saline (PBS). Triethylamine (1 mL of 100 mM) was then added to the column to elute those phages bound to the sephadex beads, then 1 M Tris-HCl (pH 7.4, 0.5 mL) was added to neutralize the triethylamine in the eluted samples. *E. coli* TG1Tr, in log phase, was infected with phage eluted from these columns for phage propagation.

Immunonephelometric Assay

A dextran solution (600 μL, T2000) was diluted 1 to 1 with distilled water and the suspended solids were removed either by centrifugation at 12,000 rpm for 10 min, or by filtration through a membrane filter (0.45 μm pore size, Gelman Coporation, Ann Arbor, MI, U.S.A.). Measurements of dextran concentrations in samples were performed according to a modification of the procedure described for the MCA-SucroTest™(15,18), where the initial reading (No) of 1200 μL antibody solution (600 μL of phage antibody in 600 μL of PBS buffer) was recorded, then 12 μL of dextran solution was added to the 1200 μL of phage antibody solution. After mixing, the turbidity (N) was read for 60 min and the differential (Δ N) was measured. The differential (Δ N) obtained by subtracting the No value from the reading (N) obtained was a measure of the dextran in the solution.

Paper-Dip Stick Assay

Polyvinylidene difluoride membranes (PVDF, 0.45 μm pore size, Gelman Coporation, Ann Arbor, MI, U.S.A.) were used to make paper strips (2 cm × 0.5 cm)(19). In order to block nonspecific phage antibody binding sites on the strips, they were dipped into either 10% MPBS (PBS + 10% skim milk) or 10% BSA (Bovine serum albumin) and dried. The strips were dipped into sugar solutions, containing between 10 and 1000 ppm of dextran (T2000, 2×10^6) concentration and dried at room temperature. Then 5 μL of a predetermined concentration of phage was applied to the paper, incubated for 1 min, and then washed once by dipping in 1% PBST (PBS + 1% Tween 20). After drying at room temperature, the strips were incubated with 1μL of a dilution (1: 10000) of HRP-anti-M13 in 5% MPBST (PBS + 1% Tween 20 + 5% skim milk) for 5 sec and then washed three times by dipping into 1% PBST. The paper was developed with 0.5 mL of TM Blue™ substrate, which produced a blue colored spot if dextran was present. The intensity of the spots was

determined by scanning densitometry using NucleoTech's (NucleoTech LLC., San Mateo, CA, U.S.A.) NucleoVision with GelExpert software. The normalized intensity was calculated as scanned intensity divided by area of the blot.

Confirmation of Recombinant Monoclonal Phage and DNA Sequencing

A polymerase chain reaction and β-galactosidase assay were used to determine the presence of Fab inserts in the phages obtained from each round of selection(20). Template DNA from phage infected bacterial stocks was obtained with a Wizard Plus Mini-preps DNA purification system (Promega, Madison, WI, U.S.A.). Fab DNA amplification was carried out at 94℃, with 5min for pre-soaking, followed by 30 cycles at 94℃, 1 min for denaturation, 60℃, 1 min for annealing, 72℃, 1.5 min for extension and finished by incubation at 72℃ for 5 min with 50 mL PCR reaction mixture containing the primer Fdpcrback-1 (GCGATGGTTGTTGTCATT) and the primer Fdseq1 (CCTCATACAGAAAATTC), 5 mM of dNTP, 25 mM of $MgCl_2$, *Taq* polymerase and *Taq* buffer. The PCR products were separated by electrophoresis at 70 volts for 60 min on 1% agarose gels using Tris-acetate/ethylenediamine tetraacetic acid electrophoresis (TAE) buffer. The PCR products of the κ light chain antibody were amplified from phage (SD-3) by *Taq* polymerase using C κ.lib.seq primer (5'-CAACTGCTCATCAGATGGCG-3') and LMB3 primer (5'-CAGGAAACAGCTATGAC-3') for kappa chain. These PCR products were combined in a cloning vector (pCR 2.1 TOPO®, Invitrogen, Carlsbad, CA, U.S.A.) for ligation and then used to transform *E.coli*. Plasmid DNA was prepared by the method of Sambrook *et al.* (1989). The PCR products of heavy chain antibody were amplified by *Pfu* polymerase using primers, CH1.lib.seq primer (5'-GGTGCTCTTGGAGGAGGGTGC-3') and pelBback primer (5'GAAATACCTATTGCCTACGG-3'). DNA sequencing analysis of κ light chains and heavy chain were performed using a API PRISM 377 DNA sequencer (PerkinElmer) with the following primers, T7 promoter primer (Invitrogen) for kappa chain and CH1.lib.seq for heavy chain (Division of Biotechnology and Molecular Medicine, School of Veterinary Medicine, Louisiana State University at Baton Rouge, LA, U.S.A.).

Statistical Analysis

All experiments were performed in triplicate and the data were statistically analyzed by the SPSS program version 12. The comparison of means was done using Duncan's multiple range tests at a level of 0.05.

Results and Discussion

Phage Enrichment Using Sephadex™Columns

Sephadex, which is composed of beads of cross-linked dextran, provide an alternate to biopanning for dextran binding phages using immunotubes. Three sequential screens using Sephadex columns (SC) enriched for dextran binding phages. Phage collection from the third iteration, SC-3R, produced a 5.4 nephelos turbidity unit (NTU) difference (ΔN) in a nephelometric assay after 60 min, 2.2 fold higher than SC-1R, the phage preparation from the first round selection. Because turbidity formation by a commercial anti-dextran monoclonal antibody is concentration dependent, where the reaction goes to completion after 3 min due to the high concentration of antibody(15). The reaction rate of phage antibody was slow in comparison, probably because of the low concentrations of phage used (4.4×10^7 transducing unit, t.u./mL). SC-1R and SC-3R produced 12 and 27 fold higher NTU, respectively, than control, Fab 2lox library(Fig. 1). The columns were an improvement over biopanning as they not only provided antigen (unmodified dextran) but concurrently removes salts and small impurities from phage preparations. The phage preparation, (SC-1R and SC-3R) initially selected by this method produced higher nephelometric turbidity with each iteration.

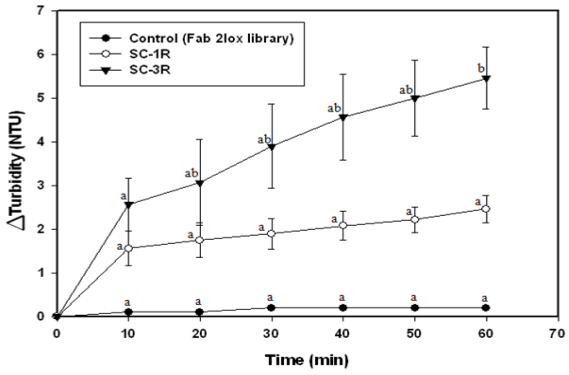

Fig. 1. Immunonephelometric assay of phages (SC) selected using Sephadex column.

Fab 2lox library was used as control for zero round selection. SC-1st and SC-3rd round indicates the phages obtained after each round of Sephadex™ column selection. Immunonephelometric assay measures turbidity using scattered light of the lattice complex formed by dextran and phages using an MCA-SucroTest™. ΔTurbidity(ΔN) was calculated by subtracting the initial reading (No) from the reading (N) obtained to measure the dextran in the solution. The error bars represent standard deviation of triplicate experiments and those with different alphabet letters are significantly different at $p<0.05$.

Effects of Dextran Size and Dextran Concentration

The effects of dextran concentration, and size were determined using phage (SC-3R, 4.4 $\times 10^7$ t.u./mL) that display antibody specific for dextran. The highest normalized intensity (N.I.) was found with 500 ppm of dextran (T2000), which showed 89±6 N.I.. The normal intensities did not increase with concentration above 500 ppm of dextran(Fig. 2). Saturation of antigen on the polyvinylidene difluoride membranes (PVDF) limits the upper range of the assay. The detection of dextran was linear with the log of the molecular size and the detectability of high molecular weight (T2000) was about 2.5 times higher than for low molecular weight (T10) because high molecular size dextran has more α-1,6 linked bonds that can react with a paratope, antigen binding sites on antibodies, than low molecular size dextran. The normal intensities of a 500 ppm test dextran solution of T10 (10^4), T40 (4×10^4), and T2000 (2×10^6) were 30±6, 40±6, and 72±10 N.I., respectively(Fig. 3).

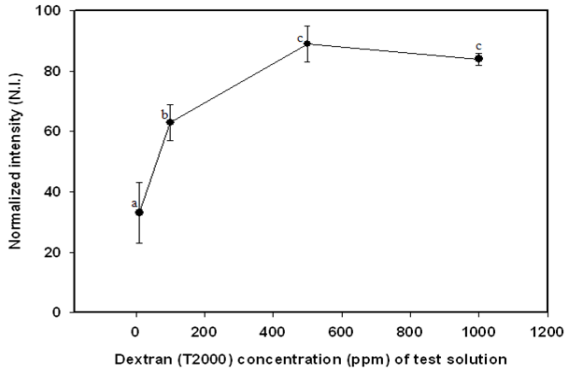

Fig. 2. The effect of dextran (T2000) concentration on detection using a paper-dip assay with phage (SC-3R).

Dextran (T2000, 5000 ppm) was diluted with PBS to make 10, 100, 500, and 1000 ppm and dextran solution (5 mL/paper) was applied to each paper using phage (SC-3R, 4.4 x 10^7 t.u./mL). Horseradish peroxidase anti-M13 antibody conjugated and TMBlue™ substrate produced blue color. The blue color of a paper-dip assay was scanned by NucleoTech's NucleoVision scanning densitometry system with GelExpert software. The error bars represent standard deviation of triplicate experiments and those with different alphabet letters are significantly different at $p<0.05$.

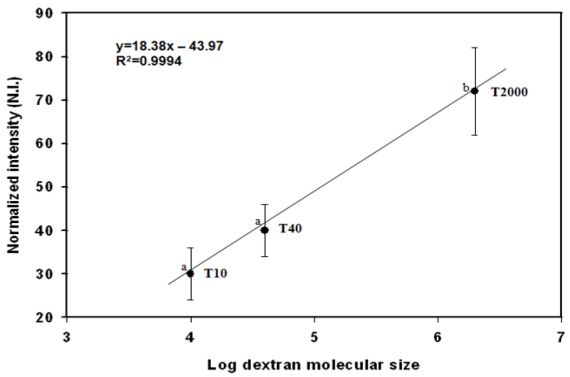

Fig. 3. The effect of dextran size on detection using a paper-dip assay.

Phage (SC-3R, 4.4 x 10^7 t.u./mL) was tested to determine the effects of dextran size. Horseradish peroxidase anti-M13 antibody conjugated and TMBlue™ substrate produced blue color. The blue color of a paper-dip assay was scanned by NucleoTech's NucleoVision scanning densitometry system with GelExpert software. The error bars represent standard deviation of triplicate experiments and those with different alphabet letters are significantly different at $p<0.05$. R^2. The coefficient of determination represents of how well the regression model predict the dependent variables from the independent variables.

DNA Sequencing

The phage clones were tested for the presence of insert DNA within the pIII. *E.coli* TG1 with an Fab insert DNA within the pIII are visually colorless on LB (Luria-Bertani) plates toped with agar containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 20 mg/mL, Sigma, St. Louis, MO, U.S.A.) and IPTG (Isopropylthio-β-D-galactoside, 200 mg/mL, Sigma, St. Louis, MO, U.S.A.), whereas, *E.coli* TG1 with out the insert produces blue colonies within 12 to 20 hr. Template DNA from phage (SD-3) infected bacteria after amplification showed a single band of approximately 1.6 kb in 1 % agarose gels (data not shown). The PCR products of light chains (κ) and the heavy chain from phage (SC-3R) were approximately 550 bp and 600 bp (Fig. 4). The phage displayed V_H-Vκas Fab inserts. Light chain and heavy chain were aligned to check homology with human DNA sequences by BLAST search. Kappa chain showed a partial homology (Expectation, E, value, 7e-08) with Homo sapiens immunoglobulin kappa locus, proximal V-cluster and J-C cluster (IGK-proximal) on chromosome 2; and the heavy chain with Homo sapiens cDNA FLJ40046 fis, clone SYNOV2001300, immunoglobulin heavy chain (E= 8e-08).

Polymerase chain reaction and β-galactosidase assay is essential in selection of the desired phages, confirming the

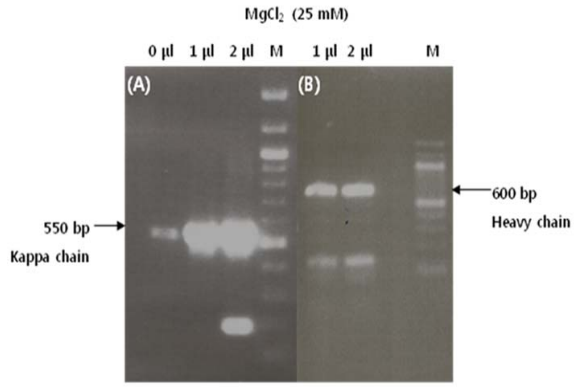

Fig. 4. PCR products of light (κ) and heavy chains in phage antibody.

(A) Kappa chain PCR products and (B) heavy chain PCR products of SC-3R. M, 100 bp DNA ladder size marker. DNA amplification was carried out at 94℃, with 5 min for pre-soaking, followed by 30 cycles of 94℃, 1 min for denaturation, 60℃, 1 min for annealing, 72℃, 1.5 min for extension and finished by incubation at 72℃ for 5 min in 50 mL PCR reaction mixture using primers, 5mM of dNTP, 25mM of $MgCl_2$ (0 to 2 μL), *Taq* polymerase and *Taq* buffer.

presence of Fab inserts after each round of selection. Single colonies, obtained after three rounds of selection, were screened for the dextran binding using immunoblotting after column chromatography. This screening indicated that phages the propagated from each round of selection were mixtures of phages with varying dextran binding affinities. Each round of selection increased the enrichment of dextran binding phage maximizing the possibilities of selecting for high dextran binding phages. The use of Sephadex column had the following advantages over traditional biopanning: ease of use, the antigen binding procedure to plastic surfaces of biopanning tubes could be omitted, effective performance of enrichment and concurrent purification of phage.

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