The production of a dextran binding antibody by phage display library and its applications to sugar processing

Duwoon Kim

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THE PRODUCTION OF A DEXTRAN BINDING ANTIBODY BY PHAGE DISPLAY LIBRARY AND ITS APPLICATION TO SUGAR PROCESSING

A Dissertation

Submitted to The Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by

Duwoon Kim
B.Sc., Chonnam National University, 1997
M.Sc., Louisiana State University, 1999
May 2004
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<table>
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<tr>
<th>Abbreviation</th>
<th>Term</th>
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<tbody>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen binding fragments</td>
</tr>
<tr>
<td>MPBS</td>
<td>Skim milk phosphate buffered saline</td>
</tr>
<tr>
<td>MPBST</td>
<td>Skim milk phosphate buffered saline with tween 20</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>SBAE</td>
<td>Sephadex bead agarose electrophoresis</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity-determining-region</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonate)</td>
</tr>
<tr>
<td>NTU</td>
<td>Nephelos turbidity unit</td>
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ABSTRACT

A phage collection (M1114), which is a mixture of two collections, M710-3R and Silica 426 each derived from a human synthetic phage antibody library (Fab 2lox), was used further enriched for dextran binding using enzyme-linked immunosorbent assays (ELISA) screening. The effects of dextran concentration on phage binding affinity were tested using indirect sandwich ELISA on phage collections, M1114-m74 and AE-M1114-m74-2R. Most of the phage bound to dextran (T2000) coated on a sandwich ELISA. The combination of ELISA screening and a sephadex columns enriched dextran binding 7 fold over enrichment by a single ELISA screen. Phage collection (M1114-m74) produced by combination screening showed the greatest binding on ELISA. The color intensity produced by phage collection (AE-M1114-m74-2R) obtained after the 2nd round of selection was 3.5 fold higher than that of phage collections, AE-M1114-m74-1R after the 1st round. Dextran binding by phage collection (AE-M1114-m74-2R) was illustrated using image analysis of transmission electron micrographs. Sephadex bead agarose electrophoresis (SBAE) screening produced phage collections (AE-M1114-m74-1R and 2R) which were used in a paper-dip assay. A dip stick assay using a protein blocked paper with adsorbed high molecular size dextran (T10,000, 10^7) produced the most color (59 ±5) using anti-dextran phage enzyme linked assays. Low molecular size dextran (T40, 4x10^4) produced significantly lower color (15 ±1). Phage collection (AE-M1114-m74-2R) was tested for specificity against dextran (T2000), corn starch, sucrose, dextrose, and chitin. Dextran produced up to 18 fold the normalized intensity of the other carbohydrates.
The presence of Fab inserts in the phage collections was confirmed using PCR, and the presence of the same insert in the host *E.coli* was checked using a β-galactosidase linked assay. DNA sequencing of phage collection (AE-M1114-m74-2R) confirmed that human origin antibody was present. The PCR products of λ, κ light chains and heavy chain from phage collection (AE-M1114-m74-2R) were approximately 420 bp, 550 bp and 600 bp.

This research used various selection methods to isolate anti-dextran phages from a library. These were used to develop a paper-dip stick method for dextran detection used for routine screening of sugar juices.
INTRODUCTION

Display technology can be used to develop biological reagents with affinity for specific antigens. Display systems have been studied that use surfaces of phage, yeast, flagella, Bacillus spores, and Staphylococcal cell walls. Yeast cells expressing cell receptors on their surfaces can function as antigens, producing T cell activation (Cho, et al., 1998). Flagella can express small fragments of foreign peptides, inserted in a variable domain of the E.coli or Salmonella FlicC, without harming flagella function. Due to strong immune reactions and the adjuvant effect of flagella, a flagella peptide display was used to produce vaccines against cholera toxin, hepatitis B, influenza A, and HIV-1. Peptides fused to FliC of E.coli were also used for production of random peptide libraries and antibodies against lysozyme. Bacillus subtilis spore coat protein gene, cotB, was modified so as to express foreign proteins, such as tetanus toxin (C fragment) on the spore surface (Isticato, et al., 2001). Gram positive bacteria such as Staphylococcus xylosus and Staphylococcus carnosus have been shown to express in a surface display a mouse immunoglobulin G1(κ) anti-human IgE single chain Fv antibody fragment (Gunneriusson, et al., 1996).

A phage display antibody library contains all possible antibody fragments as inserts in a viral coat protein. Use of a library simplifies the process for production of antibody binding proteins (Harrison et al., 1996 and Clackson et al., 1991). Fd phage is a filamentous phage that can display Fab (antigen binding fragments) as fusion proteins with pIII, a minor coat protein of the phage. Phages containing the fusion protein with antibody fragments (Fab) have the ability to bind epitopes (Harrison et al., 1996).
Antibody fragments expressed on the surface of the filamentous bacteriophage, fd, can be used as a reagent to detect antigens, tumor cells, virus, and toxins. Numerous reported applications use Fab fragments (Itoh, et al., 2001), including immunotherapy, delivery of molecules to kill specific cells, neutralization of HIV-2, vaccine development and activation of T-cells around tumor cells (Bjorling, et al., 1999, Chames, et al., 2000 and Nilsson, et al., 2000).

Dextran are high molecular weight poly glucans with α-1,6 linked main chains and varying degrees of α-1,2 α-1,3, or α-1,4 linked branch chains. It is synthesized enzymatically from sucrose by dextran transferases, glucansucrases, or glucosyltransferases produced by Leuconostoc or Streptococcii bacteria (Newman, et al., 1985 and Robyt, 1986). These bacteria growing in sugar juices produce dextran. High concentrations of dextran on solids (> 1000 ppm) can produce severe financial loss to the sugar industry (Clarke and Godshall, 1988). Several methods for dextran analysis are currently available to this industry. They are the Roberts copper method (Clarke and Godshall, 1988), the alcohol haze test (ICUMSA, 1994), the ASI II method (Sarkar and Day, 1986), the Optical Activity Ltd. DASA method (Singleton, 2001) and the Midland SucroTest™ (Day, et al., 2002). Midland SucroTest™ is an immunological method that uses a dextran specific monoclonal antibody to produce an increase in turbidity. The assay method is rapid, utilizes a minimum of equipment and highly specific to dextran but the cost per analysis is considered to be high for bulk screening of agricultural products. Surface display technology, can extend the analytical options for dextran analysis, in order to provide a low cost, dextran specific analytical method that can be used for routine screening of sugar cane.
1. LITERATURE REVIEW*

1.1. Overview of Phage Display Antibody Technology

1.1.1. Filamentous Phages. Filamentous phages, including M13, fd, and f1, are composed of 6500 bases of circular single-stranded DNA covered by a single layer containing 2,700 molecules of a single protein (VIII). In addition, four gene products, IV and VII and III and VI, are located at the distal end of each phage. Filamentous phages are a convenient vehicle for expressing proteins from cloned DNA, because sequences can be inserted into sites within gene III, which codes for protein (g3p), without disrupting functionality of the phage. Between 100-200 phages are discharged from each infected *E.coli* cell, but infection is not lethal to the host, it just slows growth (Breitling and Dubel, 1999). The infection cycle of filamentous phages are shown in Figure 1. The initial infection is mediated by interactions between the F-pili of *E.coli* carrying an F episome and the gene 3 protein (g3p). The N-terminal domain of g3p binds to TolA proteins in the periplasmic membrane. TolA proteins bring the phages close to inner membrane where the phage DNA is released to the cytoplasm (Breitling and Dubel, 1999 and Karlsson, *et al.*, 2003).

1.1.2. Phage DNA Replication. After the single-stranded phage DNA (plus strand) enters the host cell, synthesis of the complementary minus strand begins from an RNA primer, producing a double-stranded replicative form (RF) DNA (Fig. 2). The RNA primer is made at a specific site on the plus strand DNA by host DnaG protein or RNA polymerase and is removed by host DNA polymerase I. The product of gene II, an endonuclease, nicks the plus strand of RF, allowing synthesis of more RFs via rolling-circle replication.

* Portions of this chapter are reprinted by permission of Food Science and Biotechnology
Figure 1. The infection cycle of filamentous phage. The infection of filamentous phage is initiated by interaction between F-pilus and gene 3 protein (g3p) in step I, TolA proteins bring the phages close to the inner membrane where the phage DNA is released into the cytoplasm in step II, DNA is replicated by rolling circle replication in step III, phages are packed with coat proteins in step IV, and released in step V. Source: Adapted from Breitling and Dubel (1999) and Karlsson, et al. (2003). OM (IM), outer (inner) membrane.

\[\text{pIII; pVIII; pVI; pVII+ pIV; pV.}\]
Figure 2. DNA replication cycle of filamentous phage.
A double-stranded replicative form (RF) is synthesized from a single-stranded DNA of phage infecting the host cell by host enzymes (DnaG protein or RNA polymerase). Synthesis of RFs via rolling-circle replication continues after nicking the plus strand. After accumulation of RF molecules, the phage coat proteins coat the DNA at unique packaging sites before it leaks out of the cell. Source: Adapted from Sambrook, et al. (1989a).
The free 3’ end of each plus strand serves as a primer for a new plus strand, produced by the DNA polymerase. As each new plus strand is synthesized, the old plus strand peels off and is used as a template to synthesize another strand, forming another RF. After a few RF molecules accumulate, the plus strands displaced by rolling-circle replication are no longer used as templates. The gene IV product binds to plus strands preventing them from being further used as templates for RF synthesis, and helps package them into phage heads. The phage coat proteins bind and coat the DNA at unique packaging sites. Finally the new phage particles leak out of the cell (Sambrook, et al., 1989a).

1.1.3. Construction of a Phage Display Antibody Library. The technology to produce a phage display library is based on the molecular genetic work of McCafferty (McCafferty, et al., 1996). Antibody heavy chains are composed of about 50 V\(_H\), D and J\(_H\) segments with three hypervariable loops (H1, H2, and H3). Light chains are composed of about 30 V lambda and about 30 V kappa and J lambda and J kappa segments with three hypervariable loops (L1, L2, and L3). Total RNA extracts of either human lymphocytes or spleen cells from immunized mice are used in preparing a phage antibody library. RNA is isolated by phenol and chloroform extraction followed by ethanol precipitation. An oligo (dT) column is used to isolate pure mRNA (McCafferty, et al., 1996 and Davis, et al., 1994). cDNA strands are synthesized from this mRNA using random hexamer primers, dNTP, reverse transcriptase, and RNasin (a ribonuclease inhibitor) in a buffer (KCl, Tris-HCl, and MgCl). Expressed Variable genes (V\(_H\) and V\(_L\)) of cDNA or genomic DNA are amplified using polymerase chain reaction with specific primer sets encoding variable gene or leader sequences and J genes to create gene collections of variable heavy and light chains (McCafferty, et al., 1996). Thirty one
specific primers are required for synthesis of an antibody library from human blood. It requires six VHBACK and four JHFOR primers for heavy chain genes and six VKBACK and five JKFOR primers for the kappa light chain genes, and seven JλBACK and three JλFOR primers for the lambda light chain genes (McCafferty, et al., 1996). After amplification of the cDNA, the various heavy and light genes which are produced by the 31 primers, make a library. The variable heavy chains and variable light chains are amplified using primers with specific restriction sites (ApaI, AscI, SfiI, NotI) and then digested with ApaI, AscI, SfiI, and NotI and cloned to vectors (Waterhouse, et al., 1993). There are several cloning methods available for creating gene collections of heavy and light antibody including using phagemid vectors, phage vectors, by in vitro recombination or combinatorial infection and in vivo recombination (Winter, et al., 1994). Phage vectors contain all the genes required for infection, replication, assembly, a tetracycline marker and protein III for phage production. A phagemid vector consists of origin sites for plasmid and phage, an ampicillin marker and needs a helper phage for replication and packaging of the phagemid (Harrison, et al., 1996).

1.1.4. Producing a Large Antibody Library. Combinatorial infection and in vivo recombination is used to construct large antigen binding fragment (Fab) repertoires in gene libraries (Griffiths, et al., 1994). The lox-Cre system (Davies, et al., 1995 and Griffiths, et al., 1994) is used to produce variable genes by combinatorial infection between the fd phage vector, fdDOG-2lox and the plasmid vector, pUC19-2lox. Recombination occurs between the heavy and light chains from the two vectors (Waterhouse, et al., 1993 and Winter, et al., 1994). The Cre recombinase of bacteriophage P1 mediates recombination of the loxP site, which consists of two inverted
repeats and 8 bp spacers and is flanked by heavy and light genes. DNA sequences from
\textit{loxP 511} (mutant loxP) and \textit{loxP wt} (wild type lox P) are present in the 2lox phage vector
DNA sequence (Fig. 3) with a phage fd gene III leader peptide sequence (Lg3), a pelB
leader peptide sequence (LpelB), and a ribosomal binding site (r.b.s.) (Davies, \textit{et al.},

The fd phage (fd DOG-2lox vector) is used as a loxP acceptor, encoding the light
chains, and pUC19-2lox as a loxP donor, encoding the heavy chains (Fig. 4). The
variable heavy chains and variable light chains are amplified using primers with specific
restriction sites from antibody cDNA by polymerase chain reaction, and then variable
light chain DNA are cloned to an fdDOG-2lox vector (fd phage) and heavy chain DNA to
a pUC19-2lox. \textit{E.coli} TG1 infected with fd phage carrying a light chain gene library is
incubated for propagation of the fd phage. Separately, \textit{E.coli} TG1 is transformed by the
pUC19-2lox carrying the heavy chain gene library, and the culture containing pUC19-
2lox is incubated to produce a log phase culture. The log phase culture is infected with
fdDOG-2lox, containing a variable kappa or lambda gene library and then incubated to
produce the combination of a heavy and a light gene library. Two loxP sites flanked by
the variable heavy chain link the heavy and light chains during combinatorial infection in
host cells. This culture is infected with phage P1, to use its lox-Cre system for \textit{in vivo}
recombination. Cre recombinase combines a light and a heavy chain gene into an fd
phage vector that encodes Fab fragments expressed on the surfaces of fd phages. From
the resulting recombinants, the fd phage repertoire reaches to $4.1 \times 10^{13}$ transducing unit
(t.u.) (Breitling and Dubel, 1999 and Griffiths, \textit{et al.}, 1994).
GCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTGACTCCC
TGCAAGCCTCACTGCGACCAGATATAATCGGTTATGCGTGGGCAGATGGTTGTC
Fd-tet-Dog1
ATTGTACGCGCTACTATCGGTATCAAGCTTTATAAGAAATTCACCTCGAAAAG
CAAGCTGATAAACCCGATACAATTTAAAGGCTCTCTTTTGGAGCCTTTTTTTTTGG
AGATTTTCAACGTGAAAAATTATATTATTCGCAATTTCTTTTACTTTATTTCTTCT
ATTCTCACAGTGCACAGGTCCCtataagggccgcaagacctcttaatatagctgataagtaacttaattataacttcgtata
511 r.b.s. LpelB
tactcgccaggaggccttgcccttcgcaCAACTGCGAGCGTGATCAAAAGCAGGCGCCGCGGcc
511
TatatataacctctgtaatatgtgctataaaggatatgtaggtctggtcggcgaaGAAACTTGTTGAAAGTTGTTTTTAG
CAAAACCTCATACAGAAATTCATTTACTAAACGCTGAAAAGACGACAAAAAC
TTTAGATCGTTACGCTAACTATGAG

Lg3: phage fd gene III leader peptide sequence
LpelB: pelB leader peptide sequence
r.b.s.: ribosomal-binding sites
loxP wt: wild-type loxP
loxP 511: mutant loxP
VL : Light chain gene
H: Heavy chain gene
Figure 4. Combinatorial infection and \textit{in vivo} recombination of variable gene repertories. The variable heavy chains and variable light chains are amplified by PCR using primers with restriction sites from cDNA (I) and then variable light chain DNA are cloned to fdDOG-2lox vectors (fd phage) and heavy chain DNA, pUC19-2lox (II). \textit{E.coli} TG1 infected with fd phage carrying light chain gene library is incubated for propagation of the fd phage (III-1) and \textit{E.coli} TG1 transformed by pUC19-2lox carrying heavy chain gene library is incubated to produce a log phase culture (III-2). The log phase culture is infected by fdDOG-2lox containing a variable kappa or lambda gene library and then incubated for the combination of a heavy and a light gene library (IV). Cre recombinase combines a light and a heavy chain gene into an fd phage vector (V). H: Heavy chain and L: Light chain. Circles: recombination signals. Source: Adapted from Winter, \textit{et al.}(1994) and Breiling and Dubel (1999).
1.1.5. Phage Display Antibody Expression Formats. Immunoglobulin G (IgG) consists of two light chains (V_L and C_L) and two heavy chains (V_H, C_H), with complementarity-determining-regions (CDRs) in the variable domains of the light and heavy chains with disulfide bridges connecting the light and heavy chains (Fig. 5). Functionally, IgG consists of Fab fragments where antigen binds (V_H, V_L, C_H1, and C_L) and Fc fragments for complementation activation (C_H2) and macrophage binding (C_H3) (Breitling and Dubel, 1999). The display antibody formats are antigen binding fragments (Fab), single chained fragment variable (scFv), Fragment variable (Fv) and two linked scFv (diabody) (Fig. 6). To form Fab, light and heavy chains are batched cloned into a phage genome as a fusion to the gene encoding pIII. Both chains fuse in the periplasmic space, producing Fab consisting of V_H, V_L, C_H1, and C_L on the phage surface (Hoogenboom, _et al._, 1998). An Fab was chosen by Chames, _et al._ (2000) as an alternative to scFv because it maintains a more stable form after purification and permits the use of C_H1 as a tag for detection and purification of Fab. scFv has a peptide (15-20 residues) that links the variable domains of the heavy (V_H) and light chains (V_L). Linker DNA, which has sequences common to the C terminus of V_H and the N terminus of the V_L genes, links the V_H and V_L genes. These linked variable genes that contain restriction sites are amplified by primers and then ligated into the phagemid vector. This produces 4 types of libraries, VHμ-Vκ, VHμ-Vλ, VHγ-Vκ, and VHγ-Vλ (Marks, _et al._, 1991). The smallest format is an Fv fragment consisting of only a variable heavy chain. Minibodies are fused formats with a structure stabilized by linking a C_H3 and either an Fab or scFv fragment. They are used for diagnostic assays (Chames and Baty, 2000 and Nilsson, _et al._, 2000).

Diabody, triabody and tetrabody are created by linking scFV with short linkers.
Figure 5. A schematic structure of an immunoglobulin G. CDRs, complementary-determining regions; $V_H$, variable region of the heavy chain; $V_L$, variable region of the light chain; $C_L$, $C_H1$, $C_H2$, and $C_H3$, constant regions; Brown bars, disulfide bonds.
Source: Adapted from Breitling and Dubel (1999).
**Figure 6. Antibody expression formats.** Immunoglobulin G (IgG) consists of two light chains (V_L and C_L) and two heavy chains (V_H, C_H), with complementarity-determining-regions (\(\otimes\)) in the variable domains of light and heavy chains and disulfide bridges (blue lines) that connect the light and heavy chains. Fab consists of V_H, V_L, C_H1, and C_L. scFv has a peptide (red line) that links the variable domains of the heavy (V_H) and light chains (V_L). Fv fragment consists of a variable heavy chain. Minibodies are fused formats with a structure stabilized by linking a C_H3 and either an Fab or scFv fragment. Diabody, triabody and tetrabody are created by linking scFV using short linkers (black lines and dots).

Source: Adapted from Chames and Baty (2000).
There can be homobivalent or bispecific scFv fragments in a diabody. A diabody composed of two identical fragments (scFv) can show improved binding affinity (Chames and Baty, 2000).

1.1.6. Phage Display Antibody Selection. The process for selecting and screening for phage display antibodies is summarized in Figure 7. Isolation of a desired phage from a phage antibody library (naïve phage library, an immunized phage library or synthetic phage library) may require a combination of different selection methods, which may include biopanning, biotinylation, and affinity columns. In biopanning, the phage that displays the relevant ligand for a specific antigen will be retained in an immunotube, while non-adherent phage will be washed away. After vigorous washing, bound phage can be eluted by acid denaturation, reinjected into the host bacteria and re-grown for further enrichment. ELISA screens are conducted on populations of phage produced at each round of selection. The binding ability of biotinylated antigens and streptavidine on magnetic beads can be used in solution, which skips the antigen coating step to a solid surface, and the antibodies bound to biotinylated antigen are selected by streptavidine-coated magnetic beads. The binding affinity of between antigens and beads of Sepharose or Sephadex can also be used to select and enrich for a desired antibody (Hoogenboom, et al., 1998 and Harrison, et al., 1996).

1.1.7. Applications. The expression of antibody on the surface of a filamentous bacteriophage, fd, provides a rapid method to produce reagents for detection of various antigens, such as tumor cells, viruses, toxins and carbohydrates. These phages may be used for both analytical and clinical applications. For example, a phage cloned with anti-CD98 gene displayed as an Fab fragment bound to a cell surface containing a CD98
Immunized or non immunized donors (Human, Mouse, Rabbit, Chicken, etc)

Synthesis of various antibody genes by PCR, combinatorial infection and *in vivo* recombination

Immunized phage library

Naïve phage library

Synthetic phage library

Biopanning

Immunotubes

Affinity chromatography, etc.

Screening of binding affinity

ELISA

Paper dip stick

Immunonephelometer

Preparation of various antibody formats

Phage antibody

Antigen binding fragment (Fab)

Single chained fragment variable (scFv)

**Figure 7. Summary of phage antibody selection and screening.**

Selection of a desired phage antibody from a phage antibody library (naïve phage library, an immunized phage library or synthetic phage library) requires multi-biopanning steps to enrich for a desired phage antibody. The binding to target molecules is screened by immunological methods.

Source: Adapted from Harrison, *et al.* (1996) and Siegel (2002).
oncoprotein and produced growth inhibition of tumor cells, indicating a potential application for immunotherapty (Itoh, et al., 2001). Phage antibody against tumor targeting peptides made it possible to locate tumor cells. Phage antibody (scFV or Fab) fragments fused with carrier molecules, such as toxins, liposomes, radio-labeled markers or cytokines (IL-2), have been used as vehicles to deliver and capture specific targets, or kill specific cells, or activate T-cells around tumor cells (Chames, et al., 2000 and Nilsson, et al., 2000). Glycoprotein 125 bound human Fab fragments were isolated using phage display technology to neutralize HIV-2 (Bjorling, et al., 1999). Binding of human display Fab fragments neutralizes virus proteins of rotavirus, measles virus and puumala hantavirus (Itoh, et al., 1999, Nicacio, et al, 2000 and Nicacio, et al., 2002) making possible their use in vaccines. Mycotoxins, including zearalenone and fumonisin B₁ from the genus Fusarium, and microcystins of Cyanobacteria, have been studied using phage display antibodies. Mycotoxin detection technology has now changed to include phage display technology because of cost (McElhiney, et al., 2000, Yuan, et al., 1997 and Zhou, et al., 1996).

1.2. **Carbohydrate Binding Antibodies**

1.2.1. **Phage Display Antibodies with Carbohydrate Binding Affinity.** Various phage display technologies have been developed for selection of antibodies with carbohydrate binding affinity because it provides a technology to produce antibodies against poorly immunogenic antigens. Hydrogen bonds and electrostatic properties are considered to be the primary mode of binding between carbohydrates and proteins, because hydrogen bonds have higher directional property than van der Waals forces and electrostatic properties increase binding affinity for polar substrates (Quiocio, 1986). Polar planar
side chains such as Asn, Asp, Glu, Gln, Arg, and His form hydrogen bonds in sugar binding sites (Quiocio, 1989).

Variable domains with Fab, scFv or various ligand proteins can be fused with capsid proteins for display in a phage (Yamamoto, et al., 1999). Mouse origin, Fab-phage antibody from a phage display library was selected against rhamnogalacturonan by serial panning enrichment (Williams, et al., 1996). Enhanced binding to Samonella serotype B O-polysaccharide was produced by a single chain variable fragment (scFv) after site-directed PCR mutagenesis in the heavy chain changed Ile into Thr (Deng, et al., 1994). An scFv against cancer cell related-carbohydrate antigens, sialyl Lewisx and Lewisx has been used for cancer identification. This library was prepared from human blood donated from patients (Mao, et al., 1999). Galectin-3 is one of β-galactoside-binding lectins with a specific binding affinity for galactose and lactose. T7 phage are used for cloning vector expressing proteins from cloned DNA, where their sequences can be fused with gene X encoding for capsid proteins. T7 phage displaying capsid proteins fused with galectin-3 showed a binding affinity for galactose and lactose (Yamamoto, et al., 1999 and Yang, et al., 1998).

1.2.2. Dextran Binding Antibodies. Some dextrans are reported to be antigenic in man. There are reports of highly branched dextrans causing skin reactions and formation of an antidextran to native dextran (B742 and B512) produced by Leuconostoc mesenteroides (Kabat, et al., 1953 and Newman, et al., 1985), although linear dextran is normally considered to be non-immunogenic. There are also reports of dextran binding antibodies being found in human, mouse and rabbit antisera (Kabat, et al., 1953, Sharon, et al., 1981 and Outschoorn, et al., 1974). Kabat, et al. (1953) reported that humans injected with 1
mg of native or clinical dextran produced precipitins and had cutaneous sensitivity to
dextran. Sharon, et al. (1981) obtained antibodies (IgM and IgA) to dextran B512 from
hybridomas of BALB/c and C57BL/6 origin. Rabbit antisera with dextran binding
affinity were formed after immunization with isomaltohexaonic acid coupled to bovine
degrees of precipitation of BALB/c mouse antibody (IgA), W3129 and QUPC 52 with
dextran. QUPC 52 precipitated with both branched and linear dextran but W3129
precipitated with only branched dextran. A quantitative precipitin assay was used to
determine the type of the antibody binding site to dextran, and mouse monoclonal
antibodies were classified by the type of site (Table 1): “Groove-type” is defined as a
type that binds to unbranched or internal linear or non-terminal sections of α (1-6)-linked
dextran and was produced by “soluble dextran”. “Cavity-type”, bound branched or
terminal non-reducing ends of dextran was produced by “isomaltosyl oligosaccharide-
Dextran B512 is a T cell independent antigen consisting of α (1-6)-linked glucan and is
“groove-type” for antibody binding sites (Tanaka, et al., 1994).

1.3. Dextran Analysis

1.3.1. Dextran. Dextran are composed of chains of D-glucan (1 to 20 x10^6) with α-1,6
as the main chain linkage and variable numbers of α-1,2 α-1,3, or α-1,4 branched chain
linkages. Dextran is synthesized from sucrose by dextranucrases, glucansucrases, and
glucosyltransferases, produced by Leuconostoc or Streptococcii (Newman, et al., 1985
and Robyt, 1986). Table 2 shows the different linkage structures found in dextran.

Dextrans of low molecular size (70,000 ± 25,000) are used as blood volume extenders
Table 1. Immunochemical types of mouse anti-dextran monoclonal antibody

<table>
<thead>
<tr>
<th>Antibody</th>
<th>16.4.12E</th>
<th>28.4.10A</th>
<th>35.8.2H</th>
<th>36.1.2D</th>
<th>W3129</th>
<th>19.1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>C57BL/6</td>
<td>BALB/c</td>
<td>BALB/c</td>
<td>BALB/c</td>
<td>BALB/c</td>
<td>BALB/c</td>
</tr>
<tr>
<td>Immunogen</td>
<td>IM6-KLH</td>
<td>IM6-BSA</td>
<td>IM3-BSA</td>
<td>IM3-BSA</td>
<td>Unknown</td>
<td>B512</td>
</tr>
<tr>
<td>Isotype</td>
<td>IgA</td>
<td>IgM</td>
<td>IgG1</td>
<td>IgM</td>
<td>IgA</td>
<td>IgM</td>
</tr>
<tr>
<td>Type of site</td>
<td>Cavity</td>
<td>Groove</td>
<td>Groove</td>
<td>Cavity</td>
<td>Cavity</td>
<td>Groove</td>
</tr>
</tbody>
</table>

KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; IM3, isomatotriose; IM6, isomatohexaose; B512, dextran B512.
Source: Adapted from Matsuda, et al. (1989).
Table 2. Linkages in different dextrans

<table>
<thead>
<tr>
<th>Dextran</th>
<th>Solubility</th>
<th>Linkages, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α 1-6</td>
</tr>
<tr>
<td>L.m.B512F</td>
<td>Soluble</td>
<td>95</td>
</tr>
<tr>
<td>L.m. B742</td>
<td>Soluble</td>
<td>50</td>
</tr>
<tr>
<td>L.m. B742</td>
<td>Less soluble</td>
<td>87</td>
</tr>
<tr>
<td>L.m. B1299</td>
<td>Soluble</td>
<td>65</td>
</tr>
<tr>
<td>L.m. B1299</td>
<td>Less soluble</td>
<td>66</td>
</tr>
<tr>
<td>L.m. B1355</td>
<td>Soluble</td>
<td>54</td>
</tr>
<tr>
<td>L.m. B1355</td>
<td>Less soluble</td>
<td>95</td>
</tr>
<tr>
<td>S.m. 6715</td>
<td>Soluble</td>
<td>64</td>
</tr>
<tr>
<td>S.m. 6715</td>
<td>Insoluble</td>
<td>4</td>
</tr>
</tbody>
</table>

¹L.m., *Leuconostoc mesenteroides*; ²S.m., *Streptococcus mutans*; ³Br, Branch linkage. Source: Adapted from Robyt (1986).
and in blood substitutes (Kim, 1992 and Robyt, 1986). Dextran derivatives, such as
cross-linked dextran (Sephadex™, Phamacia Fine Chemical, Ltd., Uppsala, Sweden),
dextran sulfate, mercaptodextran, and iron-dextran have been used in affinity
chromatography, as anticoagulants, in removal of heavy metals and in treatment of iron
deficiency anemia (Kim, 1992). Dextran (D-glucan) from sucrose produced by \textit{S. mutans}
is part of dental plaque, providing attachment and protection for \textit{S. mutans} in the oral
environment. The fructose produced during dextran formation normally is metabolized to
D-lactic acid and the localized acidity causes tooth lesions (Robyt, 1986).

1.3.2. Quantitative Dextran Analysis in the Sugar Industry. Several different methods
are in use for the determination of dextran in the sugar industry. They are Roberts copper
method, ASI II method, the Haze method, Midland SucroTest™ and the Optical Activity
Ltd. DASA method. Roberts copper method determines dextran after polysaccharide
precipitation from sugar solutions by 80% ethanol, and quantitates colorimetrically using
by phenol-H₂SO₄, after a second precipitation with alkaline copper (II) reagent (Clarke
and Godshall, 1988). The ASI II method, after removal starch by amylase, treats alcohol-
precipitated samples with dextranase and \(\alpha\)-glucosidase. This results in the conversion of
dextran to glucose. The glucose produced is then quantitated by the Nelson-Somogyi
arsenomolybdate method (Sarkar and Day, 1986). In the alcohol Haze method, dextran is
quantified by determining turbidity at 720 nm, of a 50% alcohol precipitate, after removal
of starch and proteins by amylase and trichloroacetic acid treatment (Day \textit{et al.}, 2002).
These methods, except ASI II, are not specific to dextran and may cross react with other
polysaccharides that may be present. Two newer methods, the Midland SucroTest™ and
the Optical Activity Ltd. DASA methods are reported to determine only dextran. The
DASA method uses a near infrared spectrometer to detect the change in optical rotation in a sample prior to, and after dextranase treatment. The dextran concentration is calculated and displayed as a change in optical rotation in an OA Ltd. SacchAAr 880 polarimeter (Singleton, 2001). The DASA method was reported to give erroneously high levels of dextran, due to either partial dextran hydrolysis or poor filtration (Singleton, 2001). Midland SucroTest™ is an immunological method that uses a monoclonal antibody to produce a linear increase in turbidity in response to dextran. The dextran concentration is determined from the change in turbidity after addition of the sample into a buffer solution containing a monoclonal antibody and comparing the difference to a standard. Midland SucroTest™ showed consistent results and a high correlation with the Haze test with a R² of 0.8722 (Day et al., 2002). This assay method is a rapid test that utilizes a minimum of equipment, and is highly specific to dextran, but the cost per analysis is considered to be too high for extensive use.

1.4. Raw Cane Sugar Processing and Dextran Effects.

Louisiana and Florida produce most of the sugarcane in the US (Table 3). The impact of the sugar industry on the economy of Louisiana is over $21 billion, it provided 372,000 jobs in 2000 (LMC International Ltd, 2002). The flow diagram for raw cane sugar production is shown in Figure 8. The first step in processing is the shredding of the delivered sugar cane by knives and/or shredders. The shredded cane is then milled to extract juice. This step produces juice and bagasse. The bagasse is used for fuel. The second step is purification of the juice. Milk of lime is added to adjust the pH of the extracted the juice to neutrality, followed by flash heating to denature protein, fats, waxes and gums. Soluble phosphates such as superphosphate, dicalcium phosphate as well as
<table>
<thead>
<tr>
<th>State</th>
<th>Area harvested (,000 acres)</th>
<th>Yield (tons/acre)</th>
<th>Production (,000 tons)</th>
<th>Price($) (per ton)</th>
<th>Value of cane (million)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Louisiana</td>
<td>500</td>
<td>27.6</td>
<td>13,811</td>
<td>20.7</td>
<td>285.3</td>
</tr>
<tr>
<td>Florida</td>
<td>445</td>
<td>36.8</td>
<td>16,354</td>
<td>24.5</td>
<td>401.2</td>
</tr>
<tr>
<td>Texas</td>
<td>46.3</td>
<td>38.1</td>
<td>1,765</td>
<td>23.5</td>
<td>41.6</td>
</tr>
<tr>
<td>Hawaii</td>
<td>34.4</td>
<td>68.7</td>
<td>2,364</td>
<td>27.1</td>
<td>64</td>
</tr>
<tr>
<td>Total US</td>
<td>1,025.7</td>
<td>33.4</td>
<td>34,294</td>
<td>23.1</td>
<td>792.0</td>
</tr>
</tbody>
</table>

Figure 8. A flow diagram of raw cane sugar production.
Source: Adapted from Chung (2000).
polymer flocculants may be used to aid the clarification of the juice. If conditions warrant, dextranase may be added to the juice to reduce the dextran concentrations. Muds are removed by defecation and concentrated on rotary drum vacuum filters. The clarified juice is concentrated by vacuum evaporation. The clarified juice, which contains about 85% water, is concentrated to a 65% solids, 35% water syrup. The final stage is crystallization. The syrup is evaporated in vacuum pans to super saturation and mixed with seed crystals to form a massecuite, a mixture of crystals and mother liquor. The crystals are allowed to grow to commercial size (1.05 mm) by a balancing syrup feed and water evaporation. The raw sugar is then separated by centrifugation into the mother liquor and sugar. The crystallization process is repeated three times for a given syrup. The sugar from the first and second crystallizations are commercial grade. The third crystallization is the source of seed grain (Chen and Chou, 1993).

Dextran producing bacteria are found in high sucrose areas, including the soil in sugarcane fields, in sugar juices and on exposed equipment in cane and beet sugar mills and sugar refineries (Robyt, 1986 and Clarke and Godshall, 1988). The concentration of dextran found in sugar mill process streams can be greater than 5000 ppm on volume and 750 ppm or higher in sugar. Dextran in process streams can cause production loss by increasing viscosity, lowering crystallization rate and changing sugar shape. This has resulted in dextran in sugar of > 250 ppm bringing direct financial penalties to the producer from the refiner (Clarke and Godshall, 1988). Problems in sugar processing associated with the presence of dextran include high viscosity, poor filtration, low evaporation rates, poor crystallization, and slow mud settling. The presence of dextran in juices is normally indicative of stale cane. Juice from stale cane requires more lime to
control pH and longer heating treatment in the clarification step (Chung, 2000). High dextran concentrations also inhibit crystal growth, forming elongated (needles) crystal and lowering the quality of the sugar. An hour delay in processing delivered cane can result in as much as a 6.5% reduction in sucrose recovery (Chung, 2000). The presence of dextran above 250 ppm on solids in raw sugar brings forth penalties from the sugar refiner (Chung, 2000 and Day, 1992). Effects of dextran in juice are shown in Table 4 (Chung, 2000). Management of dextran problems requires early detection in order to control quality. There is a need for a rapid, simple, low cost method for dextran analysis in order to measure dextran in sugarcane as it is delivered to the factory.

1.5. Dip-Stick Assay Systems

Dip stick assays have the advantages of simplicity, rapidity and low cost. Most dip-stick assays are based on “dot-immunobinding”, which is a basic research tool for separation of identification of ligands. Clinical diagnostic applications of dip stick tests have included detection of adenovirus, Chlamydia, influenza A, and B, and Mycoplasma (Towbin and Gordon, 1984). Willates et al. (1998) showed the specific binding of (1-5)-α-L-arabinan by a monoclonal antibody and Sanden et al. (1993) tested a monoclonal antibody specific to a lipooligosaccharide of Bordetella pertussis in a dot assay. scFv phage antibody fused with alkaline phosphatase was used to develop color on a nitrocellulose membrane for the detection of maltose-binding proteins or glutathione S-transferase (Nakayama, et al., 2001). Park et al. (1992) developed the Ciguatect™ test kit to detect ciguatera toxins in fish, using solid phase immunobeads and membrane dip-sticks. A dip-stick assay can use any of a range of papers, such as nitrocellulose, cellulose acetate or polyvinylidene difluoride as supports for antigens. Solid supports currently used
Table 4. Effects of dextran in juice

<table>
<thead>
<tr>
<th>Dextran in juice (ppm/Brix)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>250</td>
<td>1-point loss in molasses purity*</td>
</tr>
<tr>
<td>500</td>
<td>2-point loss in molasses purity</td>
</tr>
<tr>
<td>750</td>
<td>Production of 250 MAU sugar</td>
</tr>
<tr>
<td>1000</td>
<td>Detectable crystal elongation, 5-point loss in molasses purity</td>
</tr>
<tr>
<td>1500</td>
<td>Significant operational problems</td>
</tr>
<tr>
<td>3000</td>
<td>Severe problems</td>
</tr>
</tbody>
</table>

*Purity means % sucrose on solids
Source: Adapted from Chung (2000) and Day (1986).
for immunoassays are listed in Table 5. Blocking buffers such as skim milk, gelatin or bovine serum albumin may be used to reduce non-specific binding. The primary antibody that is bound to the specific antigen is detected by a secondary antibody conjugated with a peroxidase or other color developing system. The color is then developed, in the case of peroxidase by the addition of ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) or a 4-chloro-1-naphthol peroxidase substrate (Dewey, et al., 1989, Hawkes, et al., 1982, Lavanchy, et al., 1990 and Willats, et al., 1998). Enzyme-substrate based color development is one dip-stick assay format. A paper is dipped into a PBS buffer solution containing an antigen and then incubated first with the antibody against the antigen, and then washed with a washing buffer containing a detergent to remove unbound antibody. The presence of the antibody is detected by application of a second antibody conjugated to an enzyme and color is developed by addition of substrate (Hawkes, et al., 1982).

The presence of proteins on PVDF membranes can be detected by dyes such as Amido black, Coomassie Brilliant Blue R-250, colloidal gold and Ponceau S (Harlow and Lane, 1988). Florescent dye labeling kits can be used to detect antigens using labeled antibodies. Commercial protein labeling kits (FluoReporter® protein kits) are available to label proteins such as Fab or monoclonal antibodies with fluorophore and biotin for staining (Haugland, 1996). Ninhydrin has been used in a histochemical test for keratin and in chromatography for staining amino acids (Holmes, 1968). Ninhydrin is a dye that reacts with α-amino groups producing a purple color (Holmes, 1968).

Our approach was to develop a rapid, paper-dip stick assay for dextran detection in sugar juices using the dextran binding affinity of a phage display antibody.
Table 5. Solid supports of immunoassays

<table>
<thead>
<tr>
<th>Support</th>
<th>Form</th>
<th>Binding methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrocellulose</td>
<td>Membranes, Microtiter wells</td>
<td>Noncovalent</td>
</tr>
<tr>
<td>Polyvinylchloride</td>
<td>Microtiter plates, Sheets</td>
<td>Noncovalent</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>Beads, Microtiter plates</td>
<td>Noncovalent</td>
</tr>
<tr>
<td>Diazotized paper</td>
<td>Sheets</td>
<td>Covalent by free amino acids</td>
</tr>
<tr>
<td>Activated beads</td>
<td>Beads</td>
<td>Covalent by free amino acids</td>
</tr>
<tr>
<td>Protein A beads</td>
<td>Beads</td>
<td>Noncovalent</td>
</tr>
</tbody>
</table>

Source: Adapted from Harlow and Lane (1988).
2. MATERIALS AND METHODS

2.1. Human Synthetic Phage Antibody Library

A human synthetic phage-antibody library (Human Synthetic Fab 2lox Library) was acquired from the Cambridge Center for Protein Engineering, Cambridge, England. This library contains a large synthetic repertoire, $1.2 \times 10^{12}$, of Fab fragments. Variable regions of heavy and light chains of Fab fragments are expressed with the coat protein (pIII) in the fd filamentous phage.

2.2. Organism and Maintenance

*E. coli* TG1Tr (K12, D(lac-pro), supE, thi, hsdD5/F'traD36, proA+B+, lacIq, lacZDM15) was acquired from the Cambridge Center for Protein Engineering, Cambridge, England. This strain was maintained on M9 minimal media agar plates, at 4°C [100 ml of 10X salts (0.22M Na$_2$HPO$_4$7H$_2$O, 0.22M KH$_2$PO$_4$, 0.09M NaCl, and 5.35M NH$_4$Cl /liter dH$_2$O), 10 ml 20% glucose (filter sterilized), 1ml of 0.1M CaCl$_2$, and 1ml of 1M MgCl$_2$, 50µl thiamine (100µg/ml), 15g agar in 900 ml dH$_2$O at pH 7.0], and transferred monthly (Sambrook, *et al.*, 1989b). *E. coli* TG1Tr colonies were transferred from M9 minimal agar plates to 5 ml of a TY broth medium (16g tryptone, 10g yeast extract, 5g NaCl/liter and pH 7.4) and incubated overnight at 37°C. To produce working cultures, the organism was transferred to TY broth and grown at 37°C for 4 hours ($A_{660}$ 0.4) prior to infection with phage.

2.3. Purification of Phage Stock

30 µl of log phase *E. coli* TG1Tr ($10^6$ cfu/ml) was exposed to the 10 µl of phage library at 37°C for 30 min. Cells were removed by centrifugation at 3300g for 20 min, resuspended in TY media and incubated overnight at 37°C. The infected cells were
centrifuged at 3300g for 20 min and stored in a TY-15% glycerol at -70°C, for use as a phage source. PEG/NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) was mixed in a 1:5 v/v ratio with supernatants of broth culture, obtained after centrifugation of infected cultures. The mixture was incubated for 1 hr at 4°C to allow for separation of phages. Then the PEG/NaCl supernate mixtures were centrifuged at 10,800g, for 30 min, and the pellets resuspended in phosphate-buffered saline (PBS) (0.13M NaCl, 0.02M Na₂HPO₄ anhydrous, 0.01M NaH₂PO₄·2H₂O per 1 liter, pH 7.2). After recentrifugation, at 3,300 g for 10 min, cell free phage solutions were transferred to new tubes and stored in 15% glycerol in PBS at -70°C.

2.4. Titering Phages

Filamentous phages (Fd, M13, F1) are non-lytic and do not form clear plaques. Transducing units (t.u.) of phages were obtained by counting phage infected E.coli TG1Tr colonies growing on TYE (15g agar, 8g NaCl, 10g tryptone, 5g yeast extract/liter) +12.5 µg/ml tetracycline (tet) plates. Phages solutions were titered by diluting with TY and incubating with log-phase E.coli TG1Tr at 37 °C for 30 min and then plating on TYE+tet plates (Zacher, et al., 1980 and Medical Research Council, 1995).

2.5. Selection of Phage Display Antibody

Phages with dextran binding affinity were selected using five different screening methods; thin layer chromatography (TLC), Sephadex columns, enzyme-linked immunosorbent assays (ELISA), a combination of ELISA and Sephadex column and Sephadex bead agarose electrophoresis (SBAE). The dextran binding of phages enriched by each selection was determined by an immunonephelometric assay and confirmed using paper chromatography and indirect sandwich ELISA.
2.5.1. Selection by Thin Layer Chromatography. Dextran (T2000, 2µl volumes of 1000 ppm) was streaked in two discrete bands, on silica plates (Uniplate™ Silica gel GF, 10x20cm, Analtech., Inc.). After drying at room temperature, each silica plate was spotted with 5 µl of phage library solution (10^8 t.u./ml), below the lower dextran band, and then developed for 9 min using PBST (PBS + 0.2% Tween 20, Amresco, Solon, OH). Each dextran containing band on the silica plate was removed by scraping. The removed portion of silica gel was suspended in 600 µl of PBS to desorb non-attached phage. This supernatant, which contains unbound phage, was discarded. Phage adsorbed to 15 mg of silica gel was then desorbed by washing the gel with 500 µl of 100 mM triethylamine (TEA, pH 10.5) for 3 min, followed by the addition of 250 µl of 1 M Tris-HCl to return the pH to 7.4. Then 200 µl of TEA/Tris-HCl containing phage was used to infect 20µl of E.coli TG1 (10^6 cfu/ml), which was then plated on TYE with tetracycline. The plates were harvested and the phages produced by these cells were purified from cell pellets using PEG/NaCl as described above.

2.5.2. Selection by Sephadex Column. Sephadex columns were used to select clones of interest based on the following procedure (Medical Research Council, 1995). Phage library solution (100 µl of 10^8 t.u./ml) was applied to a column containing 10 ml of Sephadex-50G (Phamacia Fine Chemical, Ltd., Uppsala, Sweden). This column was eluted with 100ml of PBST (PBS + 0.05% Tween 20) followed by 100 ml of PBS. 1 ml of triethylamine (100 mM) was added to the column to elute bound phages and then 1 M Tris-HCl (pH 7.4, 0.5 ml) was used to neutralize the triethylamine in the eluted samples. 30 µl of E.coli TG1-Tr, in log phase, was infected with phage eluted from these columns. After centrifugation of the infected cultures, at 3,300 g for 10 min, the bacterial cell pellet
was resuspended in TY and plated on TYE with tetracycline (12.5µg/ml). Following overnight incubation at 37°C, the colonies on each plate were harvested by washing with 3 ml of TY. They were then incubated for enrichment of phage in 1 liter volumes of TY with tetracycline (12.5µg/ml). Phage was purified from enrichment cell pellets by addition of PEG/NaCl, using the method described for purification of phage stock.

2.5.3. Selection by ELISA Screening. ELISA plate wells were coated with 200 µl of anti-dextran antibody solution (1 vial antibody/10ml PBS, Midland M9010 MCA antibody, mouse origin, Midland Research Laboratories, Inc., Lenexa, KS) and incubated overnight at 4°C. They were then blocked with 200 µl of 1% BSA per well for 1h. After a single washing of the plates with 200 µl of PBS, a suspension of 100 µl dextran (T2000, 10,000 ppm) was added to each well for 2 hours and then washed once with 200 µl of PBS. This was followed by addition of 100 µl of phage (10^8 t.u./ml). The ELISA plate wells were washed 40 x 200 µl of 0.01M sodium phosphate (pH 7.3), a low ionic strength washing buffer (Maruyama et al., 1994). Then 100 µl of *E.coli* TG1Tr (A_660_ 0.2) was added to each washed well, and incubated for 30 min at 37°C to allow infection. The infected cell pellets were resuspended in 100 µl of TY+ tetracycline, and plated onto TYE+tetracycline and incubated overnight at 37°C. Tetracycline resistant colonies were randomly chosen from the plates and each was inoculated into 600 µl of TY+tetracycline and incubated overnight at 37°C. Phage supernatants from TY+tetracycline cultures were used to test binding of phage clones to dextran.
2.5.4. **Selection by Sephadex Bead-Agarose Electrophoresis (SBAE).** Phages (10 µl of 10^7 t.u./ml), m74 were selected by the methods previously described and then was mixed with 100 µl of Sephadex-50G (SD) beads, for 3 hours at room temperature. Agarose gels (0.3%) were prepared and 50 µl of treated SD beads were loaded in each well positioned in the center of the gels. After electrophoresis at 70 volts for 30 min, the SD beads were collected from the wells, washed three times with 100 µl each of distilled H₂O. Phage adsorbed to SD beads were desorbed by washing the gel with 500 µl of 100 mM triethylamine (TEA, pH 10.5) for 3 min, followed by the addition of 250 µl of 1 M Tris-HCl to return the pH to 7.4 and then the phage (30 µl) elutate was incubated for 30 min at 37°C with *E. coli* (30 µl, A_{660} of 0.4) and plated on TYE+tet, which was incubated overnight at 37°C. The plates were harvested and the phages produced by these cells were purified from cell pellets using the PEG/NaCl as previously described. These phages were used for DNA sequencing analysis and characterization of dextran binding using an indirect sandwich ELISA and a paper-dip assay.

2.6. **Confirmation of Presence of an Fab Inserts in Phage**

2.6.1. **Confirmation of an Fab Insert in a Phage Vector by β-Galactosidase Assay.**

*E. coli* TG1Tr was inoculated into 1ml of LB (Luria-Bertani medium, 10g bacto-tryptone™, 5g bacto-yeast extract™, 0.17M NaCl per liter, pH 7.0) and incubated at 37°C for 4 hours. Tubes containing 3ml of top agar (10g bacto-tryptone™, 5g bacto-yeast extract™, 0.09M NaCl, 8g agar per liter at pH 7.0) were prepared and held at 55°C. Ten fold serial dilutions in LB of 10 µl phage stock containing supernatants were dispensed to the top agar tubes, *E. coli* TG1 (50 µl) was added into each tube and mixed well. Then 40 µl of a solution of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 20mg/ml, Sigma,
St. Louis, MO) in DMF (dimethylformamide, Sigma) and 4 µl of a solution of IPTG (Isopropylthio-β-D-galactoside, 200 mg/ml, Sigma, St. Louis, MO) were added to the top agar tubes and the tubes dispensed to LB agar plates. These were left at room temperature for 5 min to solidify, and then incubated overnight at 37°C. Samples with an insert DNA within the pIII were visually colorless, whereas, samples without the insert produced blue colonies after 12 to 20 h.

2.6.2. Confirmation of an Fab Insert in Phage Vector by PCR. Template DNA was obtained from phage infected bacterial stocks using a Wizard Plus Mini-preps DNA purification system (Promega Corporation, Madison, WI) using the manufacturers recommended procedures. Fab DNA amplification was conducted at 94°C, with 5min for pre-soaking, followed by 30 cycles of 94°C, 1 min for denaturation, 60 °C, 1 min for annealing, 72°C, 1.5 min for extension and finished by incubation at 72 °C for 5min in 50 µl of PCR reaction mixture. This PCR mixture consisted of two primers, ten pmole/µl of Fdperback-1 (GCGATGGTTGTTGTCATT, 18mer, Tm 56°C, GC 56%) and ten pmole/µl of Fdseq1Forward (CCTCATACAGAAAATTC, 17mer, Tm 50.3°C, GC 35.2%), mini-prepped DNA, 5mM of dNTP (GibcoBRL/Life Technologies, Grand Island, NY), 25mM MgCl (GibcoBRL/Life Technologies, Grand Island, NY), Pfu polymerase (2.5 U/µl, Stratagene, La Jolla, CA) and 10x Cloned Pfu DNA polymerase reaction buffer (Stratagene, La Jolla, CA) or Taq polymerase (5U/µl, Applied Biosystems, Foster City, CA) and 10x Taq PCR buffer (Applied Biosystems, Foster City, CA). The PCR products were separated by electrophoresis at 70 volts for 60 min on 0.8 % agarose gels using Tris-acetate/ethylenediamine tetraacetic acid electrophoresis (TAE)
buffer (pH 7.2, 0.04 M Tris base, 0.02 M sodium acetate•3H₂O, 0.001 M Na₂EDTA•2H₂O per liter).

2.7. Determination of Binding Affinity using ELISA

2.7.1. Indirect Sandwich ELISA. ELISA plate wells (Micro test III flexible assay plate, Falcon, Becton Dickinson, Franklin Lakes, NJ) were coated with 200 µl per well of anti-dextran polyclonal antibody (Midland M9010 MCA antibody, Midland Research Laboratories, Inc., Lenexa, KS) and incubated overnight at 4°C. Then the ELISA plates were washed once with PBS and then dextran (T2000, 500 ppm, 200 µl/well) was added to the ELISA plates. MPBST (PBS+0.05% Tween+2% skim milk, 100 µl) was added to control wells. The plates were incubated for 2 hr at 37 °C. After one wash with 200 µl of PBST (PBS + 0.05% Tween 20), these plates were blocked using 200µl of MPBST per well for 2 hr at 37°C and then washed twice with PBST. Phage samples (50 µl/well) and 2% MPBST (50µl/well) were added each well and incubated for 2 hr at room temperature. After washing three times with PBST, 100 µl of a dilution (1: 10,000) of horseradish peroxidase anti-M13 antibody conjugated (HRP-anti-M13, Amersham Pharmacia Biotech Inc., Piscataway, NJ) in 2% MPBST was added to each wells and incubated for 90 min at room temperature, and then the wells were washed three times with 200 µl of PBST. Then 100 µl of a substrate solution containing 500 µg/ml 2,2’-azino-bis 3-ethylbenthiazoline-6-sulfonic acid (ABTS, Sigma, St. Louis, MO), 4 µl of 30% hydrogen peroxide in 12 ml of 0.1 M citrate buffer (pH 3.8) was added to each plate wells, incubated for 10 min at room temperature, and ELISA plates were read using a SPECTRAmax Plus microtiter plate reader (Molecular Devices Coporation, Sunnyvale, CA) at A₄₃₀ (Chung et al, 1990).
2.7.2. Optimization of ELISA. The optimization of ELISA was performed according to the methods of Chung et al. (1990). After coating wells overnight at 4°C with 200 µl of anti-dextran polyclonal antibody, plates were washed once with 200 µl of PBS. Dextran (T2000), 1.4, 6.3, 25, 100, or 200 µg per well was added to ELISA plates for 2 hours. The plate wells were washed with 200 µl of PBST, and then blocked with 200µl per well of 2% MPBST for 2 hr at 37 °C. After the wells were washed twice with PBST, phage (10 µl of 10⁵ t.u./ml) was added to each plate well and then they were filled with 90 µl of 2% MPBST and incubated for 90 min at room temperature. The plate wells were washed twice with 200 µl of PBST and color was developed by the procedure described previously.

2.8. Determination of Dextran Binding Using Paper Chromatography

Paper chromatography was used to determine dextran binding of phages using HRP-anti-M13 and 3,3’,5,5’-tetramethylbenzidine (TM) Blue™ substrate (Intergen company, St. Milford, MA) for blue color development. Phages (5 µl of 4.4 x 10⁷ t.u./ml) were mixed with 5µl of a 1000 ppm solution of T2000 at room temperature for 10 min and 1 µl of the mixture of phages and dextran was spotted on cellulose acetate paper. The paper was chromatographed with a solvent of 5% skim milk and 0.3% Tween 20 for 15 min. After drying, the paper was treated by spotting 0.5 µl of HRP-anti-M13 on the origin. The paper was replaced into a chamber containing a fresh solvent for 15 min and dried at room temperature. The paper was then dipped into a solution containing TM Blue™ substrate for development of color, which was scanned by NucleoTech's (San Mateo, CA) NucleoVision scanning densitometry system with GelExpert software. The normalized intensity was calculated as scanned intensity divided by area of the blot.
2.9. Determination of Dextran by 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 Corporation, Ann Arbor, MI.). Measurements of dextran concentrations in samples were performed according to a modification of the procedure described for the MCA-SucroTest™, 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 (ΔN) was measured. The differential (ΔN) obtained by subtracting the No value from the reading (N) obtained was a measure of the dextran in the solution. The concentration of dextran was calculated by comparison with a standard run at the same time.

2.10. Paper-Dip Stick Assay

Polyvinylidene difluoride membranes (PVDF, 0.45 µm pore size, Gelman Corporation, Ann Arbor, MI.) were used to make paper strips (2cm x 0.5cm). 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 dextran 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 (San Mateo, CA) NucleoVision with GelExpert software. The
normalized intensity was calculated as scanned intensity divided by area of the blot. A
diagram of the dip assay step is shown in Figure 9.

2.11. Transmission Electron Microscopy (TEM)

The binding of phage to dextran was visualized by TEM by modification of the
method of Derrick and Bransky (1976). Copper TEM specimen grids coated with
collodion and carbon were placed on for 5 min drops (10 µl) of anti-dextran antibody
from mouse origin, diluted 1:500 with sterile distilled water. Excess anti-dextran
antibody was washed of the grids by touching each to 10 µl drops of distilled water,
twice. The copper TEM specimen grids were then placed for 1 min on drops (10 µl) of
dextran (T10, 500 ppm) dissolved in filtered water, air dried for 5 minutes, blotted with
filter paper to drain excess solution, then touched to a 20 µl drop of test or control phage
(Fab 2lox library) solution (10^5 t.u/ml) and rinsed once with filtered water. The grids
were exposed to 50 µl of 2% uranyl acetate negative stain, wicked off and then imaged
with a JEOL 100CX TEM operating at 80KV (The Socolofsky Microscopy Center,
Louisiana State University at Baton Rouge, LA). Test or control phage (Fab 2lox library)
were quantitated by counting phages per µm² TEM images obtained from forty eight
fields on 8 grids. Control phage (Fab 2lox library) was used to calculate the degree of the
non-specific binding on the copper TEM grids coated with dextran.
1. Preparation of paper strips using PVDF (2cm x 0.5cm)

2. Blocking of paper strips with 10% MPBS or 10% BSA

3. Dipping into sugar solutions and dried at room temperature

4. Applying 5 µl of phage for 1 min and washing with 1% PBST

5. Applying 1µl of HRP-anti-M13 and washing three times with 1% PBST

6. Adding 0.5 ml of TM Blue™ substrate and determining color intensity

Figure 9. A diagram of a paper-dip stick assay.
2.12. Sequencing of Fab Genes

The PCR products of the κ or λ light chain antibody were amplified from phage (AE-M1114-m74-2R) by *Taq* polymerase using Ck.lib.seq (Light constant kappa chain library sequence) primer and LMB3 (Laminin β-3) primer for kappa chain and C\(\lambda\).lib.seq (Light constant lambda chain library sequence) primer and LMB3 primer for lambda chain. These PCR products were combined in a cloning vector (pCR 2.1 TOPO®, Invitrogen, Carlsbad, CA) for ligation and then used to transform *E.coli*. A map of the cloning vector, pCR 2.1 TOPO®, is shown in Figure 10. Plasmid DNA was prepared by the method of Sambrook *et al.* (1989c). The PCR products of heavy chain antibody were amplified by *Pfu* polymerase using primers, CH1.lib.seq (Heavy constant chain library sequence) primer and pelBback (Leader peptide of the pectate lyase backward) primer. DNA sequencing analysis of κ, λ light chains and heavy chain were performed using a API PRISM 377 DNA sequencer (PerkinElmer, Wellesley, MA) with the following primers, T7 promoter primer (Invitrogen, Carlsbad, CA) for kappa chain and lambda 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). The primer sequences for PCR and DNA sequencing are shown in Table 6.

2.13. Statistical analysis

Means and standard deviation were used for data comparison. \(R^2\), the coefficient of variation was used to describe fit of the regressions.
Table 6. Primer sequences for PCR and DNA sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cκ.lib.seq</td>
<td>5’CAACTGCTCATCAGATGGCG</td>
</tr>
<tr>
<td>LMB3</td>
<td>5’CAGGAAACAGCTATGAC</td>
</tr>
<tr>
<td>Cλ.lib.seq</td>
<td>5’GTGGCCTTTGTTGGCTTGAAGC</td>
</tr>
<tr>
<td>CH1.lib.seq</td>
<td>5’GGTGCTCTGGAGGAGGGGTGC</td>
</tr>
<tr>
<td>pelBback</td>
<td>5’GAAATACCTATTGCCTACGG</td>
</tr>
<tr>
<td>T7 promoter primer</td>
<td>5’TAATACGACTCACTATAGGG</td>
</tr>
</tbody>
</table>
Figure 10. A schematic map of pCR® 2.1-TOPO® cloning vector.
Source: Invitrogen catalog (2002).
3. RESULTS

3.1. Pathway for Selection of Dextran Binding Phages and Phage Histories

The pathways for selecting dextran binding phages are summarized in Figure 11. Dextran binding phages were selected from an Fab 2lox phage library using combinations of five methods: thin layer chromatography (TLC), sephadex column, ELISA screening, sephadex column followed by ELISA (or combination of ELISA and sephadex column) and Sephadex bead-agarose electrophoresis (SBAE). Phages (Silica 426) were selected using TLC; phages (M710-1R and M710-3R) were selected from sephadex columns and phages (SD-1, 2, 3, 4, 5 and 6) were randomly chosen from plates of phages M710-3R. Phages, M1114 (a mixture of phages, Silica 426 and M710-3R) were screened for dextran binding using ELISA. Phage (M1114-89) was randomly chosen from those selected by ELISA and was further screened using a sephadex column, producing a high dextran affinity phage collection, M1114-m74. Phage (M1114-m74) then panned using sephadex bead-agarose electrophoresis, from which phages (AE-M1114-m74-1R and AE-M1114-m74-2R) were selected. Phage (AE-M1114-m74-2R) was the organism of choice for use in a paper dip stick assay.

3.2. Selection of Dextran Binding Phages by Thin Layer Chromatography

As dextran binding phages bind to dextran adsorbed on the silica, thin layer chromatography was used to select dextran binding phages from the phage display library. A band of dextran on the TLC plates prevented those phages which bind dextran from migrating up the plate. Phages that were found absorbed on the dextran sections (lower and upper) were collected and their dextran binding ability tested using an immunonephelometric assay (Fig.12). The immunonephelometric assay
Figure 11. Summary of selection pathway and history of dextran binding phage selected from human synthetic Fab 2lox library. Five different methods were used to select a dextran binding phage from the Fab 2lox phage library. They were thin layer chromatography (TLC), sephadex column, ELISA screening, a combination of ELISA screening and sephadex column, and sephadex bead-agarose electrophoresis.

, selected phages; , selection methods.
Figure 12. Immunonephelometric assay for determination of the dextran binding affinity of phages recovered from dextran bands on silica plates. Dextran (2 µl volumes of 1000 ppm T2000) was streaked in two discrete bands, an upper and a lower section on silica plates. The bands were 1.5 cm apart. Each silica plate was spotted with 5 µl of phage library solution (10^8 t.u./ml), 1 cm below the lowest dextran band, and developed with PBST for 9 min. The dextran bands were removed by scraping. Phage adsorbed to silica gel was then desorbed by washing the gel with 500 µl of 100 mM triethylamine (TEA, pH 10.5) for 3 min, followed by the addition of 250 µl of 1 M Tris-HCl to return the pH to 7.4. Phages that were found in dextran adsorbed sections were collected and their binding tested using an immunonephelometric assay. The assay measures light scattered by the lattice complex formed by dextran and phages and is based on the MCA-SucroTest®. ΔTurbidity (Δ N) was calculated by subtracting the initial reading (N₀) from the reading (N) obtained with dextran in the phage solution. The error bars represent standard deviation of triplicate experiments. R², the coefficient of determination represents of how well the regression model predict the dependent variables from the independent variables.
measured light scattered by lattice complexes formed between dextran and phages (Whicher, et al., 1983). It is based on the MCA-SucroTest™. The initial reading ($N_0$) of the anti-dextran test solution was recorded, then $12 \mu l$ of solution containing dextran (T2000, 500 ppm) was added. After mixing, the turbidity ($N$) was determined after 60 min and the differential ($\Delta N$) calculated by subtracting the $N_0$ value from the end reading ($N$). Phages (Silica 426) collected from the lower dextran section from TLC plates produced a 21 fold increase in turbidity over phage on upper band. The turbidity increase was linear with time over 60 min.

3.3. Selection of a Dextran Binding Phages by Sephadex Columns

To enrich for dextran binding, three sequential column selections were performed on the same phage preparation. Dextran binding was monitored by immunonephelometric assay using Fab 2lox library as control. M710-1R and M710-3R are the phage preparations isolated from the first and third round selections (Fig. 13). M710-3R produced a 5.4 nephelos turbidity unit (NTU) difference ($\Delta N$) after 60 min, 2.2 fold higher than M710-1R, the phage preparation from the first round of selection. M710-1R and M710-3R, respectively, produced 12 and 27 fold higher turbidities than the control, Fab 2lox library. A rapid turbidity increase was shown for 10 min. After 10 min, non-linear turbidity rate continued to increase for 60 min. The formation of turbidity produced by light scattering will reach to equilibrium and decrease because the size of dextran and antibody complex will increase and precipitate (Lente, 1997). Enrichment of dextran binding phage with sequential column selections was not linear with repeated selection. A rapid improvement in dextran binding was seen with the first selection, with decreasing increments seen with further repetition.
Figure 13. Immunonephelometric assay of phage preparation selected using Sephadex column. Phage library solution (100 µl of $10^8$ t.u./ml) was applied to a column containing 10 ml of Sephadex-50G and phages binding to the column were collected. M710-3R, a phage isolated from the third round of selection showed a 5.4 nephelos turbidity unit (NTU) differences ($\Delta N$) after 60 min, a 27 fold higher value than control, Fab 2lox library and M710-1R, the phage preparation from the first round selection, a 12 fold higher than control in immunonephelometric assay, which measures turbidity using scattered light of the lattice complex formed by dextran and phages using an MCA-SucroTest™. $\Delta$Turbidity ($\Delta N$) was calculated by subtracting the initial reading ($N_o$) from the reading (N) obtained to measure the dextran in the solution. The error bars represent standard deviation of triplicate experiments.
Phages may lose their inserts (Fab) during propagation. Phages without inserts will propagate more rapidly than those with inserts, enriching them in the population, hence causing an overall reduction in dextran binding affinity. Six *E.coli* TG1Tr colonies infected with phage preparation (M710-3R) were chosen from TYE+tet plate grown colonies and propagated. Phages from these randomly selected colonies were separately collected (SD-1, SD-2, SD-3, SD-4, SD-5, and SD-6). After PEG/NaCl concentration, phage preparation of identical concentration was tested for dextran binding (Fig. 14). The presence of phages that bound to dextran on cellulose acetate paper chromatography was determined by treatment with HRP-anti-M13 and TM Blue™ substrate, which produces a blue color. It was quantitated by scanning densitometry. This system compares the color density of interest with one chosen from the background of the paper. Dextran binding phages bound to the dextran deposited area on the cellulose acetate paper separating then from the non-binding phages. The six isolated phages produced different color densities, indicating different levels of dextran binding phage were present. The normalized intensity was calculated as scanned intensity divided by area of the blot. Two-thirds of the phages selected, SD-1 (lane 1), SD-2 (lane2), SD-3 (lane 3) and SD-4 (lane 4) showed higher normalized intensities (N.I.) than the control (-2 N.I.). The control was below the color density of the background paper. The color densities of SD-5, and SD-6 were below 2 (N.I.) and were considered to be non-specific phages.

3.3.1. Characterization of Phage SD-3. A paper-dip assay was developed using SD-3. Protein coated paper (PVDF membranes) was dipped into dextran solution (T2000, 1000 ppm) 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
Figure 14. Determination of dextran binding by phages, SD-1, 2, 3, 4, 5, and 6 on cellulose acetate paper chromatography. Phages (5 µl of 4.4 x 10^7 t.u./ml) were mixed with 5µl of a 1000 ppm solution of T2000 at room temperature for 10 min and 1 µl of the mixture of phages and dextran was spotted on cellulose acetate paper. The paper was chromatographed with a solvent of 5% skim milk and 0.3% Tween 20 in 100 ml for 15 min. After drying, the paper was treated by spotting a 0.5 µl HRP-anti-M13 on the origin. The paper placed into a chamber for 15 min containing a fresh solvent, 5% skim milk and 0.3% Tween 20 in 100 ml. The paper was then dipped into a solution containing TM Blue™ substrate for development of color, which was scanned by NucleoTech's (San Mateo, CA) NucleoVision scanning densitometry system with GelExpert software and density increase was calculated by comparison of color density with the background. (A) In the paper chromatogram: lane 1 is SD-1; lane 2, SD-2, Lane 3, SD-3; lane 4, SD-4; lane 5, SD-5; Lane 6, SD-6; C, control (no phage). (B) Comparison of normalized intensities produced by phages (SD-1, 2, 3, 4, 5, and 6). A color increase of < 2.0 was taken as no increase.
1% PBST (PBS + 1% 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. The intensity of the spots was determined by scanning densitometry using NucleoTech's (San Mateo, CA) NucleoVision with GelExpert software. The normalized intensity was calculated as scanned intensity divided by area of the blot. The effects of the phage concentration, dextran concentration, and dextran molecular size on dextran binding affinity on the dip assay were studied.

To determine the effects of the phage concentration on the amount of dextran bound, two concentrations of SD-3 (4.4 x 10^5 titration unit, t.u./ml and 4.4 x 10^7 t.u./ml) were prepared. The normalized intensities produced by the phage preparations on a paper dip assay were 62±7.7 N.I. and 72±20 N.I., respectively, against 1000 ppm dextran, indicating saturation at phage concentrations above 4.4 x 10^5 t.u./ml. For dextran concentration determinations, a dextran solution (T2000, 5000 ppm) was diluted with PBS to make 10, 100, 500 and 1000 ppm solutions. Test solution (5 ml) was absorbed to each paper strip to give 0.15, 1.5, 7.5 or 15µg of dextran. The detection of dextran was tested using phage SD-3 (4.4 x 10^7 t.u./ml). The highest normalized intensity was found with 500 ppm of dextran (T2000, 7.5 µg), which showed 89±6 N.I. There was no increase in normalized intensity with higher concentration, possibly due to blinding of the substrate or a limitation in the dextran binding capacity of the paper (Fig. 15). The linear region appears to be between 0 – 200 ppm dextran. The normalized intensity in this assay...
Figure 15. The effect of dextran (T2000) concentration on detection using a paper-dip assay with phage (SD-3). (A) 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 to give 0.15, 1.5, 7.5 or 15 µg of dextran. The presence of dextran was tested in the paper dip assay using phage (SD-3, 4.4 x 10^7 t.u./ml). Horse radish peroxidase anti-M13 antibody conjugated and TM Blue™ substrate produced blue color. (B) The blue color of a paper-dip assay was scanned by NucleoTech's (San Mateo, CA) NucleoVision scanning densitometry system with GelExpert software. The error bars represent standard deviation of triplicate experiments (n = 6).
increased as the molecular size of dextran increased. The normal intensities of a 500 ppm test dextran solution, 7.5 µg of dextran on the paper of T10 \((10^4)\), T40 \((4 \times 10^4)\), and T2000 \((2 \times 10^6)\) were 30±6, 40±6, and 72±10 N.I., respectively (Fig. 16). 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).

3.4. Selection by ELISA Screening

A new phage collection (M1114) was prepared by mixing phage collections M710-3R and Silica 426. This was used for selection by ELISA. This collection (M1114) was screened for dextran binding using ELISA plates, coated with anti-dextran polyclonal antibody (Midland M9010 MCA antibody) followed by dextran T2000. Ninety four \(E.coli\) TG1Tr colonies carrying phages were selected from this screening and propagated. Supernatant phages were screened. Their dextran binding was determined by indirect sandwich ELISA. Of ninety four phages selected by ELISA, 78% of them showed no binding, 18% were 2 fold over control \((A_{430} 0.11)\) and 4% were more than 2.5 fold over control (Table 7). The phage producing highest absorbance \((0.36)\) at 430 nm was chosen and labeled M1114-89.

3.5. Selection by a Combination of ELISA Screening and Sephadex Column

A combination of ELISA screening and Sephadex column selection was used to further enrich M1114-89 for dextran binding. Those phages selected by ELISA screening were passed through sephadex columns. After propagation, dextran binding was determined by indirect sandwich ELISA on culture supernatants. A second set of ninety four phages, selected after a combination of ELISA screening and sephadex column,
Figure 16. The effect of dextran size on detection using a paper-dip assay. Phage (SD-3, 4.4 x 10^7 t.u./ml) was tested to determine the effects of dextran size. Horse radish peroxidase anti-M13 antibody conjugated and TM Blue™ substrate produced blue color, which was scanned by NucleoTech's (San Mateo, CA) NucleoVision scanning densitometry system with GelExpert software. The error bars represent standard deviation of triplicate experiments (n = 6). R². The coefficient of determination represents of how well the regression model predict the dependent variables from the independent variables.
Table 7. Determination of dextran binding affinity of phages obtained after ELISA screening

<table>
<thead>
<tr>
<th>Phage (M1114-#)</th>
<th>Absorbance</th>
<th>Phage (M1114-#)</th>
<th>Absorbance</th>
<th>Phage (M1114-#)</th>
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<td>94</td>
<td>0.17</td>
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</tbody>
</table>

Bold numbers indicate phages showing 2.5 fold the absorbance ($A_{430} > 0.27$) of the control and number *89 indicates phage (M1114-89) which produced highest absorbance (0.36) at $A_{430}$. The phages were propagated individually from ninety four E.coli TG1Tr colonies. The control which does not contain dextran showed less than 0.11 at $A_{430}$. Phages with equal values to or below control are not listed.
were tested for dextran binding. These 53% were 2 fold over control ($A_{430} 0.11$) and 29% were more than 2.5 fold control (Table 8). A seven fold increase was seen in the percentage of phages that produced an absorbance greater than 0.27 at 430nm (Table 9). This indicates that those phages propagated from single colonies of *E.coli* TG1Tr were mixtures of phages with different dextran binding affinities and that combination screening improves the chances of selecting for the desired property. After combination screening, phages (M1114-m74) screened with Sephadex bead-agarose electrophoresis (SBAE).

3.6. Selection by Sephadex Bead-Agarose Electrophoresis (SBAE)

Selection of dextran binding phages by TLC, ELISA screening or Sephadex columns is both time consuming, requiring repeated procedures and the selected phages generally produce low color intensity on indirect sandwich ELISA. Screening using Sephadex bead-agarose electrophoresis (SBAE) produced highly enriched phage preparations, AE-M1114-m74-1R and AE-M1114-m74-2R after only two rounds. The color intensity produced by AE-M1114-m74-2R, obtained after the 2nd round selection was 3.5 times higher than that of phages AE-M1114-m74-1R obtained after the 1st round selection and approximately 30 times higher than the starting phage (M1114-m74). AE-M1114-m74-2R was used in the paper dip assay (Fig. 17) and all further work.

3.7. Indirect Sandwich ELISA for Dextran Analysis Using Phages

Detection of various concentration of dextran using indirect sandwich ELISA with phages, M1114-m74 and AE-M1114-m74-2R, were tested. ELISA plate wells were each coated with 200 µl of an anti-dextran polyclonal antibody (goat origin) and then 200 µl of various concentration of dextran solutions (T2000, 1.4, 6.3, 25, 100, and 200 µg)
Table 8. Determination of dextran binding affinity of phages obtained after a combination screening by ELISA and Sephadex column.

<table>
<thead>
<tr>
<th>Phage (M1114-m#)</th>
<th>Absorbance</th>
<th>Phage (M1114-m#)</th>
<th>Absorbance</th>
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<td>0.25</td>
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Bold numbers indicate phages showing 2.5 fold the absorbance (A$_{430}$ > 0.27) of the control and number *74 indicates phages (M1114-m74) which produced the highest absorbance (0.40) at 430 nm. The phages were propagated individually from the ninety four E.coli TG1Tr colonies tested. The control, which does not contain dextran, showed less than 0.11 at A$_{430}$. Phages with equal values to or below control are not listed.
Table 9. Comparison of a combination of ELISA screening and sephadex column with ELISA screening for dextran binding phages.

<table>
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<tr>
<th>Screening method</th>
<th>% Phages(^1) ((A_{430} &gt; 0.22))</th>
<th>% Phages(^2) ((A_{430} &gt; 0.27))</th>
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<td>A single ELISA screening</td>
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<td>4%</td>
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<tr>
<td>A combination of ELISA screening and Sephadex column</td>
<td>54%</td>
<td>29%</td>
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</table>

Percentage of phages showing 2.0\(^1\) or 2.5\(^2\) fold higher absorbance increase than control out of ninety four phages.
Figure 17. Determination of dextran binding affinity using a paper dip assay of phages, AE-M1114-m74-1R and 2R. Phage (AE-M1114-m74-1R and 2R, $10^5$ t.u./ml) were tested to detect dextran (T2000, 1000ppm). Horse radish peroxidase anti-M13 antibody conjugated and TM Blue™ substrate produced blue color, which was scanned by NucleoTech's (San Mateo, CA) NucleoVision scanning densitometry system with GelExpert software. The error bars represent standard deviation of triplicate experiments. (A) A paper-dip assay using phages (AE-M1114-m74-1R and 2R, $10^5$ t.u./ml) obtained after first and second round of Sephadex bead agarose electrophoresis (SBAE) (B) Comparison of color densities of first and second round of phages (AE-M1114-m74-1R and 2R) by a paper-dip assay. 1R-test (or control), 1st round SBAE test (or control); 2R-test (or control), 2nd round SBAE test (or control). The error bars represent standard deviation of triplicate experiments.
were added and then phages were added to each well. Phages (10^5 t.u./ml) showed saturation at 25 µg of dextran. The smallest amount of dextran detected was 6.3 µg. Phage (AE-M1114-m74-2R) produced the highest absorbance (A_{430} 0.83) against 100 µg of dextran (T2000) (Fig. 18).

3.8. Transmission Electron Microscopy for Dextran Analysis

Dextran binding by the phage was confirmed by enumeration of phage on TEM grids, which had been coated with anti-dextran monoclonal antibody and dextran (T10). The grids were dipped in solution of AE-M1114-m74-2R or of the Fab 2lox library as a control. Phages were quantitated by counting phages bound on 48 fields for each of 8 grids. Phages (AE-M1114-m74-2R) were distributed over the dextran coated grids with 39 ± 25 phages/µm² on the grids (Fig. 19). Phages were not seen on dextran coated grids exposed to phage library (Fig. 20). The small marks on the TEM photographs are uranyl stain artifacts. They are also seen in the negative control grids.

3.9. Characterization of Phage AE-M1114-m74-2R

3.9.1. Effects of Dextran Size and Dextran Concentration. The effect of dextran molecular size on binding of dextran by AE-M1114-m74 was determined for the paper-dip format. High molecular size dextran (T10000, 10^7) produced the highest color intensity (59 ±5), T2000 (2x10^6), 40 ±8 and T40 (4x10^4) showed the lowest intensity, 15 ±1 (Fig. 21). As with phage SD3, there was a linear relationship (R²=0.9712) between dextran size and color intensity.

Serial dilutions of dextran (T2000) solution were used to determine the detection limits of phage (AE-M1114-m74-2R, 2.5 x10^5 t.u./ml). The phage produced 54±3 color normalized intensity (N.I.) from 125 ppm to 1000 ppm of dextran and 5 ±1 (N.I.) for 63
Figure 18. Detection of dextran concentration with an indirect sandwich ELISA. Dextran (1.4, 6.3, 25, 100, and 200 µg) was bound to the ELISA plates coated with 200 µl of an anti-dextran polyclonal antibody and then the presence of phages that bound to these dextran was determined using horseradish peroxidase anti-M13 antibody conjugated and a substrate solution (500 µg/ml 2,2’-azino-bis 3-ethylthiazoline-6-sulfonic acid, 4 µl of 30% hydrogen peroxide in 12 ml of 0.1 M citrate buffer, pH 3.8) producing a blue color and then plates were read using a SPECTRAmax Plus microtiter plate reader (Molecular Devices Corporation, Sunnyvale, CA) at A$^\text{430}$. The error bars represent standard deviation of duplicate experiments (n = 4).
Figure 19. Transmission electron microscopy photograph of the phages (AE-M1114-m74-2R) bound to dextran (T2000, 0.13 µg) coated grids. Grids were placed on the drops (10 µl) of anti-dextran antibody diluted 1:500 with sterile distilled water for 5 min. After washing twice with distilled water, they were placed on a drop (10 µl) of dextran (T10, 500 ppm) dissolved in filtered water, air dried for 5 minutes, blotted with filter paper, then touched to a 20 µl drop of phage solution and rinsed once in filtered water. The grids were exposed to 50 µl of 2% uranyl acetate stain. Arrows indicate phages bound to dextran on grids. The small dots are uranyl staining marks. The marker bar is 1 µm.
Figure 20. Transmission electron microscopy photograph of Fab 2lox library. Grids were placed on the drops (10 µl) of anti-dextran antibody diluted 1:500 with sterile distilled water for 5 min. After washing twice with distilled water, grids were placed on the drop (10 µl) of dextran (T10, 500 ppm) dissolved in filtered water, air dried for 5 minutes, blotted with filter paper to drain excess solution, then touched to a 20 µl drop containing control Fab 2lox library and rinsed once in filtered water. The grids were exposed to 50 µl of 2% uranyl acetate stain. Small dots are uranyl staining marks. The marker bar is 1 µm.
Figure 21. Effects of dextran molecular size on dextran binding.
The effect of dextran molecular size on dextran binding was determined for a paper-dip assay with phage (AE-M1114-m74-2R, 10^5 t.u./ml). Horse radish peroxidase conjugated anti-M13 antibody and TM Blue™ substrate were used to detect the presence of phages bound to dextran adhere to the paper and the blue color was scanned by NucleoVision scanning densitometry system. The error bars represent standard deviations of triplicate experiments (n = 6). R^2. The coefficient of determination represents of how well the regression model predict the dependent variables.
ppm of dextran. The phage showed saturation below 125 ppm of dextran and 63 ppm of test dextran solution produced the lowest dextran detection in this paper-dip assay (Fig. 22). This corresponded approximately to the visual detection limit.

3.9.2. Phage Antibody Concentration and Specificity Using Dip Stick Assay

Phage (AE-M1114-m74-2R) was serially diluted with PBS and the effects of phage concentration on detection of dextran (T2000, 1000 ppm) on the paper-dip phage-enzyme-linked assay were determined. Peak interesting was found at a phage concentration of $6.5 \times 10^4$. Above this level there was decreased N.I., possibly due to blinding (Fig. 23). Any commercial application will have to take this into account.

Phage was tested for specificity against dextran, corn starch (Sigma, St. Louis, MO), sucrose (Fisher Scientific, Fair Lawn, NJ), glucose (Fisher Scientific, Fair Lawn, NJ), and chitin (Fisher Scientific, Fair Lawn, NJ) (Table 10). The N.I. for dextran was at least 18 fold higher for the other carbohydrates. The negative control, PBS, produced an N.I. of 3.5 ±5.

3.10. Application of the Paper-Dip Assay to Sugar Mills Samples

The paper-dip assay was used to detect dextran in mixed juices from four Louisiana sugar mills. The results were compared to results obtained with the Midland SucroTest™ (Fig. 24). Both assay methods were rapid and showed linear relationships between dextran concentrations. Dextran concentrations above 50 ppm on sugar juices were visually identified in the paper-dip assay, but only 10 ppm of dextran in solution was required for detection with the Midland SucroTest™. The Midland SucroTest™ was approximately 5 fold more sensitive than the paper-dip assay. However, the paper dip assay shows promise as a semi-quantitative rapid dextran method.
Figure 22. Detection of dextran. Serial dilutions of dextran (T2000) solution were prepared and the effects of dextran concentration on dextran binding were determined by a paper-dip assay using phage (AE-M1114-m74-2R, $10^5$ t.u./ml). Horseradish peroxidase conjugated anti-M13 antibody and TM Blue™ substrate were used to detect the presence of phages bound to dextran on the paper and a developed blue color was scanned by NucleoVision scanning densitometry system. The error bars represent standard deviations of triplicate experiments ($n = 6$).
Figure 23. Effect of phage concentration on dextran bound in a paper dip assay. Phage (AE-M1114-m74-2R, $5 \times 10^5$ t.u./ml) diluted with PBS was used for detection of dextran (T2000, 1000 ppm). Horse radish peroxidase conjugated anti-M13 antibody and TM Blue$^{TM}$ substrate were used to detect the presence of phages bound to dextran on the paper and a developed blue color was scanned by NucleoVision scanning densitometry system. The error bars represent standard deviations of triplicate experiments ($n = 6$).
Table 10. Specificity of phage (AE-M1114-m74-2R) for polysaccharide

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<th>Normalized intensity (N.I.)</th>
<th>Standard deviation</th>
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<td>Glucose</td>
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<td>8.5</td>
</tr>
<tr>
<td>PBS (Control)</td>
<td>3.5</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Phage (AE-M1114-m74-2R, $10^5$ t.u/ml) was tested for specificity against dextran (T2000, 1000 ppm), which was compared with 1000 ppm of other carbohydrates (corn starch, sucrose, glucose, chitin). Phosphate buffered saline (PBS) was used as a control. Horse radish peroxidase conjugated anti-M13 antibody and TM Blue™ substrate were used to detect the presence of phages on the paper and a developed blue color was scanned by NucleoVision scanning densitometry system. Standard deviations were obtained from triplicate experiments ($n = 6$).
Figure 24. Comparative detection of dextran in sugar juice between the paper-dip assay and MCA-SucroTest™. 1, 2, 3, and 4 represent samples obtained at different sugar mills. Normalized intensity is the units from the NucleoVision scanning densitometer. ΔTurbidity (NTU) is the unit of immunonephelometric assay using anti-dextran polyclonal antibody. The error bars represent standard deviations of triplicate experiments (n = 6).
3.11. PCR Analysis of an Fab Insert in Phage Vector

Phage DNA expressing Fab fragments were amplified by PCR. Phage (AE-M1114-m74-2R) DNA produced two bands, (2.1kb and 1.6kb) in 1% agarose gel (Fig. 25-A). The 1.6 kb bands are considered to be Fab DNA (Medical Research Council, 1995) and were cut out for purification. The purified DNA was separated on 1% agarose gels by electrophoresis for confirmation of the presence of single bands and for heavy chain DNA sequencing (Fig. 25-B).

3.12. Fab DNA Sequencing

The PCR products of both light chains (λ, κ) and the heavy chain from phage (AE-M1114-m74-2R) were approximately 420 bp, 550 bp and 600 bp (Fig. 26-A and B). The phage was found to display mixed antibodies with \( V_H-V_\kappa \) and \( V_H-V_\lambda \) as Fab inserts. Cloning vectors (pCR® 2.1 TOPO®, Invitrogen, Carlsbad, CA) for the light chain inserts (4 kappa chains and 3 lambda chains) were run on 1% agarose gel (Fig. 27-A) and topoisomers were detected in the gel (Fig. 27-A). The presence of light chain inserts in the cloning vector was confirmed by EcoRI enzyme digestion of the TOPO® cloning vector with inserts. The lambda and kappa light chain DNA were 500 bp and 550 bp, respectively (Fig. 27-B). 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; Lambda chain with Homo sapiens anti-rabies SO57 immunoglobulin lambda light chain mRNA (E= 2e-3); and the heavy chain with Homo sapiens cDNA FLJ40046 fis, clone SYNOV2001300, immunoglobulin heavy chain (E= 8e-08). The lambda chain homology was poor.
Figure 25. PCR Analysis of Fab inserts in phage (AE-M1114-m74-2R).

(A) PCR confirmation of a Fab insert in phage (AE-M1114-m74-2R): Template DNA was obtained from phage infected bacterial stocks using a Wizard Plus Mini-preps DNA purification system (Promega). Fab DNA amplification was carried out at 94°C, with 5 min for pre-soaking, followed by 30 cycles of 94°C, 1 min for denaturation, 60°C, 1 min for annealing, 72°C, 1.5 min for extension and finished by incubation at 72°C for 5 min in 50 μl PCR reaction mixture using primer, Fdperback-1 (GCGATGGTTGTTGTCATT) and primer, Fdseq1 (CCTCATACAGAAAATTC), 5 mM of dNTP, 25 mM of MgCl₂, Taq polymerase and Taq buffer. The PCR products were separated by electrophoresis at 70 volts for 60 min on 0.8 % agarose gel using TAE buffer. (B) 1.6 kb DNA obtained after agarose gel purification. M, lambda Hind III size marker.
Figure 26. PCR products of light (κ and λ) and heavy chains in phage (AE-M1114-m74-2R) (A) Kappa (lane 1, 2 and 3) and lambda light chain (lane 4, 5, and 6) PCR products (B) Heavy chain PCR products (lane 1 and 2). M, 100 bp DNA ladder size marker. DNA amplification was carried out at 94°C, with 5 min for pre-soaking, followed by 30 cycles of 94°C, 1 min for denaturation, 60°C, 1 min for annealing, 72°C, 1.5 min for extension and finished by incubation at 72°C for 5 min in 50 µl PCR reaction mixture using primers, 5mM of dNTP, 25mM of MgCl₂ (0 to 2 µl), Taq polymerase and Taq buffer.
Figure 27. (A) Agarose gel electrophoresis of plasmid containing kappa and lambda light chain DNA of phage, AE-M1114-m74-2R after gel purification DNA: Kappa light chain (K1, K3, K4, and K7) and lambda light chain (L1, L2, and L3) and M, lambda Hind III size marker. (B) Restriction enzyme analysis of the plasmid containing kappa and lambda light chain DNA of phage, AE-M1114-m74-2R using EcoRI: K1, Kappa light chain #1; L1, lambda light chain #1; M1, lambda Hind III size marker; M2, 100 bp DNA ladder size marker.
4. DISCUSSION

Bacterial action on sucrose can produce dextran. Dextran produces a number of significant problems during sugar production. Dextran can be found in sugar mill process streams at greater than 5000 ppm on volume in juice and at higher than 750 ppm in raw sugar. The presence of dextran in sugar process juices is normally indicative of stale cane, as much of the dextran is generated in damaged plant (sugarcane) material. Stale juice is immediately obvious as it requires more lime to raise the pH and longer heating times for adequate clarification (Chung, 2000). If the dextran levels are high it may not clarify. Dextran in syrups cause production losses due 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 (Clarke, et al., 1988). The proper management of a sugar cane processing facility would benefit from targeted exclusion of loads of stale cane from the process.

Current methods for determining dextran are either slow, generally affected by the presence of other polysaccharides, require special instrumentation or have high per analysis cost. This research was conducted to further the development of a simple screening method suitable for determining dextran in each load of sugar cane delivered to sugar factory. The factories currently sample each load of cane for sugar content and a number of other parameters. A rapid, low cost method for dextran can be applied at this point to screen loads of sugar cane prior to delivery to the mill yard. The use of an anti-dextran phage suitable for use in a simple assay format, such as a paper-dip stick was targeted as an appropriate reagent for dextran analysis in this application.
Phage display antibody is an efficient production system that provides an alternative to standard antibody methodology. Phage display systems allow the omission of animal immunization and hybridoma production in acquiring specific haptens. Classical antibody production methods require specialized equipment and procedures and normally use high-cost serum-containing media, all of which is unnecessary for phage production using bacteria (Clackson, et al., 1991, Emanuel, et al., 2000 and Harrison, et al., 1996). Phages displaying fusion proteins, or antibody fragments (Fab), have the ability to bind epitopes, antigenic determinants (Harrison, et al., 1996). These phages can be maintained in and produced by *E.coli*, simplifying production to fermentation. The selection of specific antigens, as against tumor cells, virus, toxins and self antigens, with this technology is also simpler than classical antibody technology (Emanuel, et al., 2000 and Itoh, et al., 2001). Phage display library sizes can be expanded to larger repertoires by combinatorial infection, chain shuffling and mutations. Increased library size provides increased opportunity for selection of suitable haptens for non-immunogenic compounds (Winter, et al., 1994). There are a few reported instances on the use of phage display antibodies for carbohydrates detection. Phage displaying Fab or scFV, fused with capsid protein, were used for detection of a rhamnogalacturonan, *Samonella* serotype B O-polysaccharide and of cancer cell related-carbohydrate antigens, including sialyl Lewis^x^ and Lewis^x^ (Deng, et al., 1994, Yamamoto, et al., 1999, and Williams, et al., 1996).

A phage library (Fab 2lox) was screened for dextran binding phages using five different methods: thin layer chromatography (TLC), Sephadex column, enzyme-linked immunosorbent assay (ELISA) screening, a combination of ELISA and Sephadex column screening and a technique developed in the course of this research, Sephadex bead
agarose electrophoresis (SBAE). The basis for TLC screening is that dextran binding phages bind to dextran (T2000) adsorbed, as bands, on silica plates, preventing these phages from moving beyond the dextran region. Only those phages (Silica 426) collected from the dextran band closest to the origin, showed dextran binding activity in immunonephelometric assays. A Sephadex column is an alternative to biopanning with immunotubes, and was preferred because unmodified dextran is water soluble and will not bind to immunotubes. Sephadex consists of beads of cross-linked dextran, which both provide sites for anti-dextran phage binding and concurrently separate salts and small impurities from the preparations. Immunonephelometric assays were used to determine the dextran binding affinity of all new phage preparations (Silica 426, M710-1R and M710-3R). The immunonephelometric assay measures turbidity by light scattering caused by lattice formation between antigens and antibodies (Whicher, et al., 1983). Phages, M710-3R, and Silica 416 showed increases greater than 21 fold over control values after 60 min-reaction times. Day et al. (2002) reported that turbidity formation by a mouse anti-dextran monoclonal antibody was concentration dependent, with the reaction going to completion in 3 min at high antibody concentrations. The reaction rate of phage antibody presumably was slow in comparison, because lower concentrations of phage were used.

Six phage collections (SD-1, SD-2, SD-3, SD-4, SD-5, and SD-6) were enriched by Sephadex column. They were from six different E.coli TG1Tr colonies that had been infected with phage collection (M710-3R). They each produced different color densities on paper chromatography, indicating that each one was a mixture with different dextran binding capabilities. One, (SD-3), was selected for use in development of a paper-dip
assay. Protein coated polvyinylidene difluoride membranes (PVDF) were dipped into
dextran solution (T2000) and dried at room temperature to use as the matrix.  Polyvinylidene difluoride membranes (PVDF) are recommended over cellulose acetate membranes because PVDF is more efficient in blocking non-specific binding of antibody on membranes (Dewey, et al., 1989). Test solution was applied to the membrane and dried at room temperature. Then 5 µl of phage SD-3 was applied to the paper, incubated for 1 min, washed once by dipping in 1% PBST (PBS + 1% Tween 20) and dried at room temperature. One µl of a dilution (1: 10000) of HRP-anti-M13 in 5% MPBST (PBS + 1% Tween 20 + 5% skim milk) was applied for 5 sec and then washed three times by dipping into 1% PBST. TM Blue™ substrate (0.5 ml) was applied to develop color. The intensity of the spots that was formed was determined using scanning densitometry. The normalized intensity was calculated as scanned intensity divided by area of the blot. The effects of the phage concentration, dextran concentration, and dextran size were determined using this assay. This assay was independent of phage concentration between $4.4 \times 10^5$ t.u./ml and $4.4 \times 10^7$ t.u./ml. Saturation with dextran was achieved below 500 ppm of T2000, the normal intensities did not increase with concentration above 500 ppm of dextran. Saturation of antigen on the polyvinylidene difluoride membranes (PVDF) limits the upper range of the assay. The normalized intensity increased as the molecular size of dextran increased because high molecular size dextran has more epitope sites that can react with a paratope, antigen binding sites on antibodies, than low molecular size dextran. The SD-3 phage was not stable on propagation, it required further selection. Phages can lose their insert (Fab) during propagation and phages without insert propagate rapidly, which enriches the population for those phages.
Phage (M1114), a mixture of phages (Silica 426 and M710-3R) were screened for dextran binding by ELISA and then further screened by sephadex column. ELISA screening provides for selection by using dextran bound to a polyclonal antibody coated on the plate wells in a Sandwich ELISA format. After ELISA screening, ninety four *E.coli* TG1Tr colonies carrying phages were chosen and these phages propagated. The dextran binding of each phage was determined by indirect sandwich ELISA. Four % of the 94 phages produced greater than 2.5 fold more color than the control ($A_{430}$ 0.11). Sephadex column screening of M1114-89 produced phages where 29% were more than 2.5 fold control ($A_{430}$ 0.11). This indicates that phages propagated from single colonies of *E.coli* TG1Tr are mixtures of phages, each with different dextran binding affinities and that combination screening improves the chances of selecting for the desired property.

The enriched population (M1114-m74) was then screened by sephadex bead-agarose electrophoresis.

Sephadex bead-agarose electrophoresis (SBAE) provided a novel and simple screening method to select and enrich for a high dextran binding phages. Steptovidin attached beads or magnetic beads are commercially available to select specific protein bound to a specific ligand such that this procedure can potentially be used for any antigen. In SBAE, after incubation of antigen attached to beads (sephadex beads) with phage, agarose gel electrophoresis is used to separate specific phages. Phages (AE-M1114-m74-2R) selected by SBAE produced about 30 fold higher color intensities than phages M1114-m74. A paper dip assay was performed using phage (AE-M1114-m74-2R). The intensity produced in paper-dip assay was again proportional to dextran concentration as was seen with SD-3. Phages, AE-M1114-m74-2R showed high
specificity against dextran but not corn starch, sucrose, glucose or chitin. Dextran concentrations greater than 50 ppm in sugar juices could be visually detected using a paper-dip assay.

Serologically specific electron microscopy was used for confirmation of phage binding to dextran. Derrick et al. (1975) pioneered this technique for diagnosis of plant viruses in corn. Maize mosaic virus, in extracts from corn, was quantitated on serologically specific electron microscopy grids, with potato virus Y antiserum diluted from 1:100 to 1:3,200,000. The highest number of the virus was found on grids with an antiserum diluted between 1:100 and 1:1000. Phage (39±25 phages/µm²) bound to dextran was quantitated on TEM grids coated with a goat anti-dextran antibody diluted 1:500. This dilution was found to produce a low background. Serologically specific TEM was also found to be a useful diagnostic assay for the quantifying dextran binding phage.

Because the phages can easily lose the inserts after each round of selection, they were checked for the presence of an Fab insert in the vector using a β-galactosidase assay and polymerase chain reaction. If the lac DNA of a phage is interrupted by Fab DNA, the defective β-galactosidase of the host E. coli, induced by IPTG, will not complement the β-galactosidase fragment of the phage. The resulting cells infected with phage containing Fab DNA will produce no color in a β-galactosidase assay (Messing, 1983). Antibody inserts in the phage vector were confirmed by PCR using DNA templates from single colony infected cells. The single colonies were plated on tetracycline containing media and checked for deletion of the Fab DNA insert (Harrison, et al., 1996). The insert was approximately 1.6 kb (Medical Research Council, 1995) and a 2.1 kb band was present which probably contains phage cloning vector sequence.
B-cells produce 100 millions of different antibodies by a combination of antibody DNA and somatic hypermutation, which is introduced into rearranged immunoglobin genes by point mutations during activation and proliferation. It provides diversity of antibody genes. Antibody engineering used to construct gene libraries uses this somatic hypermutation to produce a large number of antibodies. The human synthetic phage antibody library (Fab 2loox) was constructed from gene repertoires of variable domains of the heavy and light chains produced by PCR, which causes a high synthesis error rate in all the CDRs through mutation, synthesis errors and PCR artifacts. This synthetic library replaces CDRs with random sequences from synthetic oligonucleotides to express all antigen binding fragments (Fab) (Breitling and Dubel, 1999). Therefore, DNA sequences of phage inserts are matched partially with DNA of human origin antibodies. The selected phages (AE-M1114-m74-2R) showed two formats of Fab (VH-Vκ and VH-Vλ) and nucleotide sequence alignment analysis by BLAST search showed that the phages (AE-M1114-m74-2R) contain human origin antibody DNA with two light chains (κ and λ) and a heavy chain DNA.

For use of a paper-dip assay in sugar mills, mixed juice solutions needed to be diluted with distilled water prior to assay because maximum detection limit of this assay was 125 ppm of dextran (T2000). After dipping each test paper into a serially diluted mixed juice samples and drying at room temperature, then 5 µl of phage solution containing 6.5x10⁴ to 5x10⁵ t.u./ml was applied to the paper, incubated for 1 min, washed once by dipping in 1% PBST (PBS + 1% Tween 20) and dried at room temperature. One µl of a dilution (1: 10000) of HRP-anti-M13 in 5% MPBST (PBS + 1% Tween 20 + 5% skim milk) was applied for 5 sec and then washed three times by dipping into 1% PBST.
TM Blue™ substrate (0.5 ml) was applied to develop color. The test strips were compared to standard papers, which had previously been dipped into known dextran solutions ranging from 0 to 125 ppm. Dextran concentration in mixed sugar juices was estimated by multiplying the dilution factor of the sample paper by the concentration of the standard paper that showed similar color intensity. The paper dip assay was used to detect dextran in mixed juices from sugar mills. The results were compared to those obtained with the Midland SucroTest™. This test was used to analyze the dextran contents in mixed juices from four different sugar mills of Louisiana, detecting greater than 50 ppm of dextran on solids in juice and showed high correlation with Midland SucroTest™, which is a rapid test using an anti-dextran monoclonal antibody. The paper-dip stick assay was specific to dextran. This system has the potential of becoming a method that can be used for routine screening of incoming loads of sugar cane, as well as this being a case study for the value of phage display technology in analytical method development.
REFERENCES


APPENDIX A

NUCLEIC ACID SEQUENCES OF ANTI-DEXTRAN PHAGE ANTIBODY
(AE-M1114-m74-2R)

(A) Kappa light chain DNA sequences
5’GCACCTTGACATCCAGACCGATGCTCCATCCATCCCTCCTCCCCAAGTGCTCTG
GTATCTGCAAATCTCCCCCCTTAACACTCAGATGGCCTATTTACTCTGCTAA
AGTATAACAGTGCAAGTCTTCGCTCTGCTCTGCTGAAATTCCTGGCCTGCC
CAATATCTGGGTAACGCGTACGCCTCGAGCGAAATATCAGGCAGGCGATTTCTCTC
CGCTGTTCCGCTATAACGTGCGATATTCTATTGGCGATAGCGCTTACAA
AGTATAACAGTGC
C
AAGTTCGCCTGGTCGTCGGCTGAAAACCTGGCTGGCC
CAATATCTGGGTACGCGTGCCGCTGCAGCAAATTCATCAGGGCGATTTCTCTC
CGCTGTTCCGCTATACGCTGGCGATTATGCTATTGGCGATAGGCGGGGCTGG
CTGTTTATTCGTAACGACAACCGACCACCCGTTGCTGATCGAACACACAGCCTT
GCAAGGTGGTTAAGGGGATATTTCCGCTATCGAAGGCGGGCTTCGATCTCTC
CACGGCTGACGCCTTGCTGCTAGGGCGACTAGATGGGAACACGCCTCTGAGAG
CAAT3’

(B) Lambda light chain DNA sequences
5’GTGGCCTTGTGGGCAAGGCTTGAGCTCTCTCCTCTCCTGTCCGCAGTACATGCTCTG
TGCGTGACTGCGCGATGTTAGGTGGCTGCTCGGCTGCTGACTGCGTTGATCTT
CTCTGTGGGTGCTTGTCTCATACCCGCGATTTCCGCTATTCTAATTATTATG
CGAATACCCCGAGAGGCAACATCACGCAATTGTAGTTGAGTCTGTCAGG
AGTACCCGCGCCGGCTTCCGCTAATAATGGAATACCATAGCCGATAGCGTGCCTG
TG
GATAGCAAGCGACGTCAATTCGGAAAGTTTGAATTTGAGATCCCTGAGACACCCCGA
TATTGGTCTGATGACTTCTGGGTCTGAGTCTCAGTACACACAGAGTCTTTGGTCTGAGGA
AAGAGATAAGAGTCAGACCTCGGCGGACAAAGTTTCAAGCAGCCACAGAAGCGCCAC3’

(C) Heavy chain DNA sequences
5’GGGTGCTGGTGGCCATGGAATCTTACGACTGGTTCCGAGCCTTGTGGCAAGACGCGC
GGGCGTGGCTGACGACCTTCTGCAAATTTGAGCATGTTTCTGACTAGACACAGT
GATATTACTGGGCAAGAGTGAAGACTGCGCCAGAAGGTGGCCCATCGAAGGTGGACAAAGATGCTTGTAATACGAGTTCATCGAGCTGCTGGAGTATGCTCCTAGC
TCGGCATGCTGGCTGACATCCTCTGCTTCTGAGTTAAAGTGATCATCCTGATCTCAGTACCCGCA
TCACCAGCAGTGATACACACAAATGGCATGTAGTTTAGTATGCTGATACATAGAG
ATAGTACGACTGGAATGTTGATGGTTTCTGGACAGCATAGTTGTAACACTGATGCTGCTGACGAGTACTATCAACGAGTTTGATGTTTCTGAGCTGACAGCTTAA
ACACTGATGCTGCTGCAACACTACGAGCAGCATACATGAGCCATGACGTGTG
GCGTGACAGGCGACGCAACAGACGACTGTGATGTTGCTACAGGACTGATATTG
ATTGTGTTCTGACTCGAAGTCTGAAACAGTAGACGTGCTGCGACAGCG
ACGACGAATCGAAGCTCGG3’
APPENDIX B

DNA SEQUENCE ALIGNMENTS OF ANTI-DEXTRAN PHAGE ANTIBODY
(AE-M1114-m74-2R) BY BLAST SEARCH

(A) Kappa light chain DNA sequence alignments

Homo sapiens partial mRNA for immunoglobulin light chain variable, E= 2e-08

Query: 1 gcacttgacatccagatgacccagtctccatcctccct 38
Sbjct: 4 gcactcagctccagatgacccagtctccatcctccct 41

Homo sapiens immunoglobulin kappa locus, proximal V-cluster and J-C cluster (IGK-proximal) on chromosome 2, E= 7e-08

Query: 6 tgacatccagatgacccagtctccatcctccct 38
Sbjct: 128022 tgacatccagatgacccagtctccatcctccct 128054

Query: 93 tattactgtcaaaagtataacagtgcc 119
Sbjct: 128278 tattactgtcaaaagtataacagtgcc 128304

Homo sapiens BAC clone RP11-316G9 from 2, complete sequence, E= 7e-08

Query: 6 tgacatccagatgacccagtctccatcctccct 38
Sbjct: 182643 tgacatccagatgacccagtctccatcctccct 182611

(B) Lambda light chain DNA sequence alignments

Homo sapiens anti-rabies SO57 immunoglobulin lambda light chain mRNA, E = 2e-3

Query: 1 gttgcctttgtgctggctgaagc 21
Sbjct: 482 gttgcctttgtgctggctgaagc 462

Homo sapiens immunoglobulin lambda light chain C2 region (IGLC2) gene, E= 0.22

Query: 1 gttgcctttgtgctggctgaagct 22
Sbjct: 102 gttgcctttgtgctggctgaagct 81

Query: 427 gcttcaagccaacaaggccac 447
Sbjct: 82 gcttcaagccaacaaggccac 102
(C) Heavy chain DNA sequence alignments

Homo sapiens cDNA clone MGC:39273 IMAGE:5440834, Expect = 8e-08

Query: 147 gcctccaccaagggcccatcg 167
   |||||||||                  |
Sbjct: 487 gcctccaccaagggcccatcg 507

Query: 168 aaggtggacaagaaagtgagcccaaatcttgt 200
   |||                  ||||                          |
Sbjct: 763 aaggtggacaagaaagtgagcccaaatcttgt 795

Homo sapiens cDNA FLJ40046 fis, clone SYNOV2001300, immunoglobulin heavy chain, Expect = 8e-08

Query: 147 gcctccaccaagggcccatcg 167
   |||||||||                  |
Sbjct: 441 gcctccaccaagggcccatcg 461

Query: 168 aaggtggacaagaaagtgagcccaaatcttgt 200
   |||                  ||||                          |
Sbjct: 717 aaggtggacaagaaagtgagcccaaatcttgt 749

Homo sapiens IgH mRNA for anti-HBs antibody heavy chain, Expect = 8e-08

Query: 147 gcctccaccaagggcccatcg 167
   |||||||||                  |
Sbjct: 412 gcctccaccaagggcccatcg 432

Query: 168 aaggtggacaagaaagtgagcccaaatcttgt 200
   |||                  ||||                          |
Sbjct: 688 aaggtggacaagaaagtgagcccaaatcttgt 720
APPENDIX C

QUANTIFICATION OF PHAGES, AE-M1114-m74 OR FAB 2LOX LIBRARY ON TRANSMISSION ELECTRON MICROSCOPY GRIDS COATED WITH DEXTRAN (T10)

<table>
<thead>
<tr>
<th>No. of Grid</th>
<th>Phages/ No. of µm² Field</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
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<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

Phages were counted on 28 fields of 4 grids (#1 – 4) or for Fab 2lox library, from 20 fields of 4 grids (#5 – 8).
APPENDIX D

LETTER OF PERMISSION

Reply From: Duwoon Kim on 03/04/2004 01:58 PM

To: "KoSFoST" <kosfost2@kosfost.or.kr>
cc: 
Subject: Urgent permission letter

Dear Dr. Hae Koung Byon,

I want permission to use portion of my paper, "Determination of Dextran in Raw Sugar Process Streams" pressed On Food Science & Biotechnology Vol. 13 No. 1 on February 29 in my dissertation for Louisiana State University. I plan to graduate May 2004. This permission letter is required to finish my dissertation work.

Could you send me a letter stating permission to use portion of my paper in Food Science & Biotechnology for my dissertation?

Sincerely,

Duwoon Kim

---

DR. Duwoon Kim

Your paper will be pressed On Food Science & Biotechnology Vol. 13 No. 2. (Will be pressed April 30, 2004)

stating: Food Science & Biotechnology has given Duwoon Kim permission to use portions of article, "Determination of Dextran in Raw Sugar Process Streams" for his dissertation in Louisiana State University.

---

Korean Society of Food Science and Technology

The President Lee, Young-Chun
VITA

Duwoon Kim was born in Seoul, Republic of Korea, on May 5, 1971. After graduating from Kwang-Ju Seo-Seok High School, he entered Chonnam National University in 1990. He obtained a Bachelor of Science degree in food science and technology in 1997. During the school he served the army for 32 months and attended the English language program of Mississippi State University in 1994. He worked as a graduate research assistant in Chonnam National University in 1997. He attended Louisiana State University in Baton Rouge, Louisiana, in January 1998. In December of 1999, he received the degree of Master of Science in food science. In May of 2004, he will receive the degree of Doctor of Philosophy in biological sciences.