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Expression of New Recombinant IgG-Binding Polypeptides and Analysis of Their Capacity to Bind Human IgG

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Protein G is present in group G streptococcus strain (G4223); the IgG-binding part of this protein contains three IgG-binding domains and binds human IgG with very high activity. We obtained two recombinant polypeptides G4223 and $G_1$4223 with high IgG-binding activity. Polypeptide $G_1$4223 consisting of three IgG-binding domains and W region has higher molecular weight and is characterized by higher affinity for IgG than polypeptide G4223 consisting of only three IgG-binding domains. It was shown that polypeptide affinity depends on its structure and size.

Key Words: *group G streptococcus; IgG-binding polypeptide; IgG-binding domain*

Many streptococcal strains bind two main human plasma proteins, IgG and albumin. Protein G isolated from group C and G streptococci has special binding sites for these proteins. Streptococcal protein G interacts with IgG due to capacity of IgG-binding part of protein G to bind the IgG Fc part at the site of contact between CH2 and CH3 domains, mainly in the loop regions [5]. Protein G isolated from group G streptococcus has two or three IgG-binding domains [4]. The protein G with three IgG-binding domains demonstrates one order of magnitude higher affinity for IgG than in protein G variants with two IgG-binding domains [4]. PCR allows cloning of only the target fragment of the gene (in our case, the fragment of protein G gene sequence encoding the region with three IgG-binding domains). Recombinant protein G was produced by cloning protein G gene from group G streptococcus strain G148 isolated from the upper airways mucosa of a healthy individual [3].

Protein G binds IgG Fc fragment and can be used in clinical diagnosis, biotechnology, and proteomics. These diverse practical applications necessitate creation of a recombinant producer strain for the production of high amounts of the protein capable of binding human IgG with high activity.

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Group G streptococcus strain G4223 has been isolated from human upper airways mucosa in 1999 [4]. Protein G expressed by this strain has three IgG-binding domains and activity of its IgG binding is higher.

Here we obtained new recombinant IgG-binding polypeptides from group G streptococcus strain G4223 and analyzed their capacity to bind human IgG.

MATERIALS AND METHODS

S. gordoni strain G4223 was cloned. Chromosomal DNA of strain 4223 served as a matrix for PCR. In order to isolate the chromosomal DNA, bacterial cells were lyzed with 50 mM EDTA (Serva) and lysozyme (Amresco) in a concentration of 1 mg/ml, DNA was deproteinated with phenols and chloroform and then extracted with ethanol.

IgG is the main class of serum immunoglobulins (80% of all antibodies). It can be isolated and purified using a recombinant polypeptide with high IgG-binding capacity. Amino acid sequence of streptococcal strain G148 protein G consists of a signal sequence, region E, three albumin-binding domains, three IgG-binding domains, region W (cell wall anchor), and region M (plasmalemma anchor) [3].

Fragments of DNA corresponding to different regions of IgG-binding protein gene were amplified in PCR with the use of Dream Tag Green PCR Master

TABLE 1. Oligonucleotide Primers Used in the Study

Primer	Orientation	Nucleotide sequence 5'→3'
PG1	Forward	CG**GGATCC**CGAGCTGTGCATTACCTAAGACTG
PG2	Reverse	GG**GGTACC**CCTAACCATTTCAGTTACCGT
PG3	Forward	GG**GGTACC**CCACCAGCCATTACTGCAAGC
pQE1	Reverse	GCGGATAACAATTTCACACAGAA
pQE2	Forward	CCGTAATATCCAGCTGAACG

Note. Restriction sites for endonucleases used in creation of the construct are shown with bold letters.

Mix (Thermo Fisher Scientific) and an amplifier (Bio-Rad). Amplified DNA site was isolated with the use of QIAquick Gel Extraction Kit (Qiagen). Oligonucleotide primers were presented in Table 1.

Construction of recombinant plasmids. Amplified DNA fragments were cloned using a system of expression vectors pQE30/31/32 (The QIAexpress System; Qiagen) by restriction sites incorporated in the primers during their construction. The DNA hydrolysis products were separated by electrophoresis in 1% agarose gel, isolated using QIAquick Gel Extraction kit (Qiagen), ligated, and transformed into a heterologous *E. coli* M15 system. Ligation and transformation were carried out as described previously [1].

The media for selection of transformants contained 100 μg/ml ampicillin and 25 μg/ml kanamycin. Antibiotic-resistant transformants were separated and inoculated into two parallel Petri dishes with antibiotics. Colonies from one dish were transferred onto a nitrocellulose membrane and plunged in a lyzing solution containing 0.2N NaOH, 0.1% SDS, and 0.5% β-mercaptoethanol. The membrane was washed in PBS and incubated for 1 h at ambient temperature in blocking solution (3% milk and PBS, 2:1) to block nonspecific binding and then in blocking solution, containing IgG labeled with HRP (Sigma). The membrane was then washed in blocking solution, then in PBS. Peroxidase activity was visualized by tetramethylbenzidene (TMB) solution for membranes (Sigma). Recombinant plasmids were isolated with the use of Mini-prep plasmid DNA purification kit (Qiagen) from colonies with the highest IgG binding activity.

Production and analysis of recombinant proteins. *E. coli* M15 clones, containing recombinant plasmids, were cultured in liquid LB medium with antibiotics (100 μg/ml ampicillin and 25 μg/ml kanamycin) till attaining the late logarithmic growth phase (OD_{600}=0.7-0.9). Expression of recombinant protein was induced by isopropyl-β-D-thiogalactopyranoside (Helicon), and the cells were cultured for 4 h more. The bacteria were precipitated by centrifugation, washed in buffer A (in mM: 20 Na_2HPO_4, 20 NaH_2PO_4, 500 NaCl, 20 imidazole; pH 8.0) and suspended in the same buffer with protease inhibitor phenylmethylsulfonyl fluoride added to the concentration of 1 mM. Cell suspension was sonicated and centrifuged. Supernatants were applied onto columns packed with Ni-sepharose (Qiagen). After polypeptide binding to Ni-Sepharose, the columns were washed with buffer A in order to remove free proteins. Recombinant polypeptides were eluted with buffer B (in mM: 20 Na_2HPO_4, 20 NaH_2PO_4, 500 NaCl, 250 imidazole; pH 8.0). After affinity chromatography, the polypeptides were dialyzed for 18 h in distilled water. Protein concentration was measured by the method of Lowry. The molecular weights of the recombinant polypeptides were determined after electrophoresis in 12% PAAG with SDS.

Analysis of nucleotide and amino acid sequences. Nucleotide sequences of the resultant fragments of IgG-binding protein gene were determined at the Syntol Company. Amino acid sequences of polypeptides were determined based on nucleotide sequences encoding these polypeptides using ExPASy translate tool.

Study of recombinant polypeptide interactions with IgG. Binding of recombinant polypeptides with IgG was studied by direct receptor enzyme analysis (REA) [2]. Analysis was carried out in NUNC Max Sorb plates. Each polypeptide in a concentration of 1 μg/ml dissolved in PBS was sensitized for 18 h at 4°C. Control wells contained PBS instead of polypeptide solution. Free polypeptide was removed by triple washing with 150 ml PBS with 0.05% Tween-20 (PBST). Serial 2-fold dilutions of HRP-labeled IgG were added into all wells and the plates were incubated for 1 h. Conjugation of HRP with IgG was carried out by the periodate method. Excess of IgG was removed by 3-fold washing with PBST. HRP activity was measured using a substrate mixture consisting of TMB, 0.1 M phosphate-citrate buffer (pH 5.0), and 0.0065% H_2O_2. The reaction was stopped by adding 2N H_2SO_4. Optical density was evaluated on a SmartSpecPlus spectrophotometer (Bio-Rad) at λ=450 nm.

Strain G148 recombinant IgG-binding polypeptide served as the reference sample for comparison of IgG binding activities.

RESULTS

Fragments of G4223 strain IgG-binding protein gene encoding the sequences of only three IgG-binding domains and IgG-binding domains with W region were cloned.

Obtaining IgG-binding protein gene fragments from group G streptococcus strain G4223 and construction of recombinant plasmids. Oligonucleotides PG1, PG2, and PG3 were constructed for cloning gene fragments. They served as primers in PCR, while strain G4223 genomic DNA served as the matrix.

Both amplified DNA fragments and pQE30 plasmid were restricted by BamHI and Kpnl endonucleases. The resultant fragments were ligated and used for transformation of *E. coli* M15. Plasmids were extracted from the clones exhibiting pronounced IgG-binding capacity. The presence of recombinant DNA was verified by PCR with the initial primers. Recombinant plasmid denoted as pQE30-*pG4223* was restricted by Kpnl and BamHI, and a 696 bp DNA fragment of was produced. Plasmid DNA pQE30-*pG4223*, pQE30-*pG$_1$4223* served as matrices for PCR with pQE1 and pQE2. Amplifiers were isolated after electrophoresis in 1% agarose gel and sequenced.

The nucleotide sequence of plasmid DNA pQE30-*pG4223* (696 bp) that encodes polypeptide G4223 amino acid sequence consisting of 232 amino acid residues and corresponding to the region of three IgG-binding domains covalently bound with a fragment of 22 amino acid residues encoded by pQE30 is presented in Figure 1, *a*. The nucleotide sequence of plasmid DNA pQE30-*pG$_1$4223* (939 bp) encoding polypeptide G$_1$4223 consisting of 313 amino acid residues and corresponding to the region with three IgG-binding domains and region W covalently bound to a fragment of 22 amino acid residues encoded by pQE30 is presented in Figure 1, *b*.

Isolation and purification of recombinant polypeptides. Polypeptides G4223 and G$_1$4223 were iso-

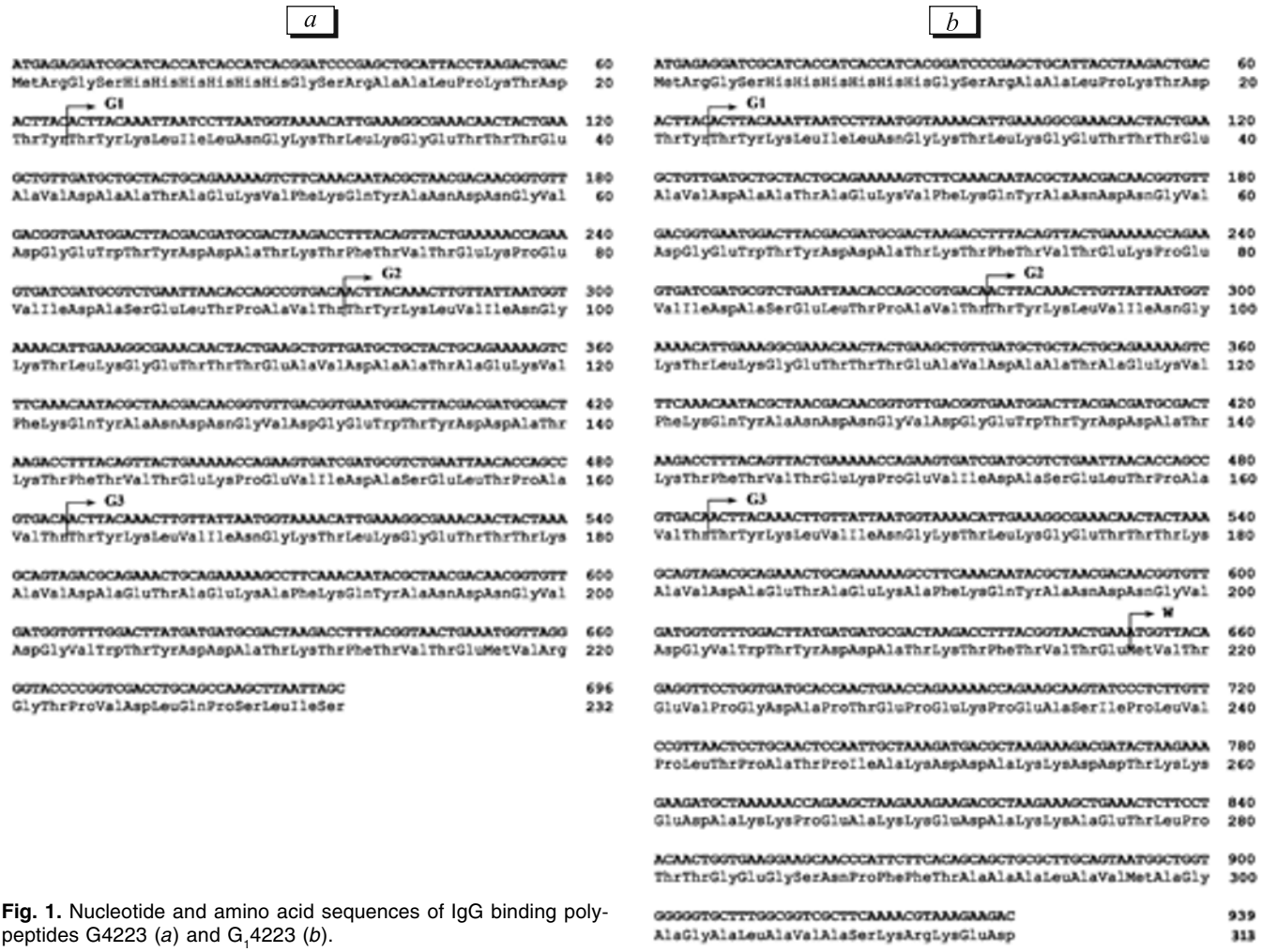

Fig. 1. Nucleotide and amino acid sequences of IgG binding polypeptides G4223 (*a*) and G$_1$4223 (*b*).

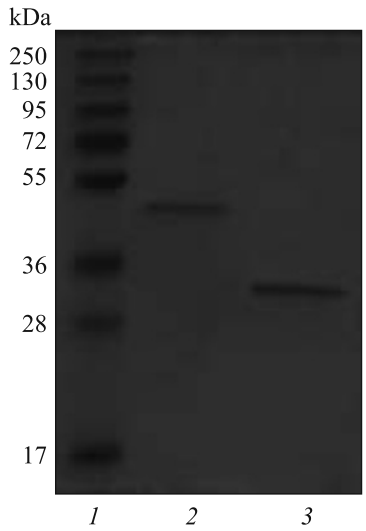

Fig. 2. SDS-PAAG electrophoresis of recombinant polypeptides. *1*) Molecular weight marker; *2*) polypeptide $G_1$4223; *3*) polypeptide G4223.

lated from the strains carrying recombinant plasmids pQE30-*pG4223* and pQE30-*pG$_1$4223* and purified. For the resultant producer strains, the yield of both polypeptides was 60-70 mg purified protein per 1 liter culture. The molecular weights of the polypeptides assessed by electrophoresis in 12% PAAG were 30 and 50 kDa for G4223 and $G_1$4223, respectively (Fig. 2), which coincided with the data of computer analysis.

Comparison of selectivity of recombinant polypeptide interactions with IgG. Selective interactions of polypeptides with IgG were evaluated by direct REA. Equal quantities of polypeptides G4223, $G_1$4223, and strain G148 IgG-binding polypeptide in a concentration of 0.4 μg/ml were adsorbed on the plate. It was shown in a preliminary experiment that no more than 0.3 μg/ml polypeptide could be adsorbed on the plate. The studied polypeptides exhibited IgG-binding activity towards labeled IgG, the quantity of IgG bound by polypeptides G4223 and $G_1$4223 was virtually the same (Fig. 3, *a*). The IgG-binding polypeptide isolated previously from strain G148 exhibited lower IgG-binding activity.

Hence, recombinant polypeptides G4223 and $G_1$4223 were characterized by high IgG-binding activity and included a region with three IgG-binding domains, but differ by size and structure. Therefore,

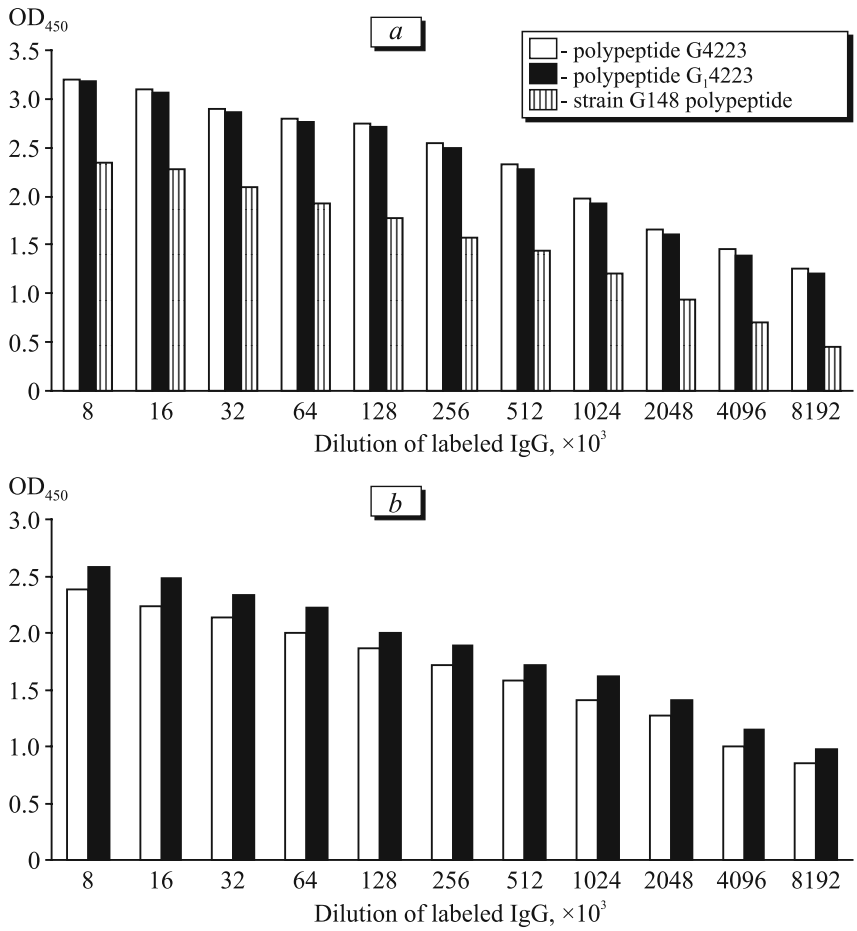

Fig. 3. Comparison of the capacity of polypeptides G4223 and $G_1$4223 and strain G148 polypeptide to interact with IgG (*a*) and capacity of polypeptides G4223 and $G_1$4223 to bind IgG adsorbed on the plate (*b*). Mean optical density for several experiments are presented.

it was hypothesized that affinity of these polypeptides for IgG depended on their structure and size.

In order to verify this hypothesis, equal quantities of molecules of each polypeptide were adsorbed on a plate. This quantity was calculated with consideration for the molecular weight: 0.18 μg/ml for polypeptide G4223 and 0.3 μg/ml for polypeptide $G_1$4223. Further analysis (carried out similarly as in the direct REA) showed that polypeptide molecule with region W and a region with three IgG-binding domains and hence, a higher molecular weight than polypeptide G4223 molecule was characterized by higher affinity for IgG (Fig. 3, *b*). Affinity of the polypeptide for IgG depended on the structure and size of the entire polypeptide.

Each of the polypeptides can be used with various purposes in biotechnology, clinical diagnosis, and proteomics. Both polypeptides can be labeled by fluorescent, radioactive, or enzymatic probes and used in the direct and indirect ELISA for identification of infectious diseases. Polypeptides G4223 and $G_1$4223 can be used for creation of adsorbents in affinity chromatography for IgG removal from the serum and for the production of highly purified IgG preparations.

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