Generation of antibodies against N-terminus fragment of AgI/II protein from *Streptococcus mutans* GS-5

Ji-Hye Han, Jae-Gon Kim, Byeong-Ju Baik, Yeon-Mi Yang, Jeong-Yeol Park

Department of Pediatric Dentistry and Institute of Oral Bioscience, School of Dentistry, Chonbuk National University

Abstract

Dental caries results from localized demineralization of tooth enamel by acids of bacterial origin produced from the fermentation of dietary sugars. A group of related oral bacteria, collectively known as mutans streptococci, are implicated as the primary etiological agents of human caries. Within this group, *Streptococcus mutans* has been known as a causative agent for dental caries. As well as acid production yielding the demineralization of tooth enamel, adherence and colonization of *S. mutans* to the teeth are also important for their virulence. Cell-surface fibrillar proteins, which mediate adherence to the salivary pellicle are virulence components of mutans streptococci, and primary candidates for a human caries vaccine. Here we report that the AgI/II gene from *S. mutans* GS-5 were cloned by PCR amplification of the bacterial chromosomal DNA and the integrity of cloned genes were confirmed by nucleotide sequencing. Sequence analyses showed the sequence alignment of 280 nucleotides between the cloned AgI/II and the reported sequence of *S. mutans* GS-5 showed the perfect match. The cloned genes which signal nucleotide was truncated, were transferred into bacterial expression vector and the recombinant proteins were purified as His-tag fusion proteins. In order to generate polyclonal antibodies against the recombinant proteins, AgI/II mr, some 100㎍ of the proteins was injected into mice three times. It can be used for an effective vaccine production to prevent dental caries caused by pathogenic *S. mutans*.

Key words : *Streptococcus mutans*, AgI/II, Vaccine

I. INTRODUCTION

Dental caries is considered to be one of the most prevalent and costly infectious diseases in the world[1], and despite the reports that this disease is on the decline in some developed countries[2,3], it continues to be a worldwide problem. A group of related oral bacteria known as *Streptococcus mutans* have been implicated as the primary etiological agents of caries in humans and experimental animals[4,5].

The first strain in mutans streptococci, *S. mutans* Clarke, was isolated in 1924 and recently the whole genomic sequence was revealed for *S. mutans* UA159, a Bratthall serotype c strain of *S. mutans*[6,7]. *S. mutans* is frequently isolated from human dental plaque, which can produce a cell surface protein antigen[4,8-11]. As well as acid production yielding the demineralization of tooth enamel, adherence and colonization of *S. mutans* to the teeth are also important for its virulence[4,12]. It has been reported that AgI/II[8], also known as protein B[13], P1[10], Pac[14], and SpaP[15], is

교신저자 : 김 재 곤

전북 전주시 덕진구 덕진동
전북대학교 치과대학 소아치과학교실
Tel: 063-250-2121 Fax: 063-250-2131
E-mail: pedodent@chonbuk.ac.kr

an important virulence factor that contributes to the pathogenesis of *S. mutans*-induced dental caries because of its involvement in the initial adherence of the organism to tooth surface and in bacterial aggregation[16]. Once *S. mutans* forms a bacterial biofilm, the bacteria can be better protected from the host defense system, sequestered into a nutrient-rich niche, and beneficial from the community mode of growth[17]. In addition, a well-established biofilm can be better resistant to antibiotic agents or phagocytosis. That is why the prevention of bacterial colonization is of very importance for the prevention of dental caries. In this respect, AgI/II will be good candidates for vaccine developments.

AgI/II adhesion protein weighs around 185 kDa and is expressed as a transmembrane protein with the help of signal peptide at its N-terminus and transmembrane segment at its C-terminus as shown in Fig. 1[11,18]. The gene encoding AgI/II had been sequenced and cloned successfully by Russell MW and Lehner T[8]. Recent studies suggested that the alanine-rich repeating region(A-region, amino acid residues 219-464) and the proline-rich repeating region(P-region, amino acid residues 867-967) of *agI/II* gene were important to the function and immunogenicity of AgI/II protein[19-21]. AgI/II is involved in many key steps of caries development and many previous reports suggest that induction of antibodies against mutans streptococci in oral cavity effectively prevent dental caries[22-26]. In oral cavity, secretory IgA(sIgA) antibodies act as safeguards against enormous challenges from oral bacteria and the principle role of AgI/II specific sIgA is to reduce the chance of colonization of pathogens at mucosal surfaces[27]. Therefore, studies aimed at the development of a caries vaccine have focused on the use of immunization regimens which stimulate the induction of IgA responses in saliva. Therefore, the determination of efficient epitopes in AgI/II will be helpful for the vaccine development.

Mucosal immunity can be induced at induction sites such as gut-associated lymphoid tissue (GALT) and nasal-associated lymphoid tissue (NALT) and then specific IgA can be produced at effector sites such as lamina propria of gastrointestinal and respiratory tracts and various exocrine glands including salivary gland. Owing to the common mucosal immune system, mucosal immunity can be induced at the remote area other than local pathogen-abundant region[28-33]. Because of homing of specific B cell and antigenic activation at the local area, mucosal immunity from the remote inductive sites can hold efficacy against the local infection. This property of mucosal immunity will be practical to develop vaccines against *S. mutans* by oral administration of bacterial antigens, such as AgI/II, to induce antigen-specific B cell population at GALT and to generate sIgA against bacterial pathogens in oral cavity.

The ultimate goal of this study is to develop an edible vaccine against *S. mutans* GS-5, a causative agent for dental caries. As an initial step, I cloned antigen genes of AgI/II protein from *S. mutans* GS-5 and generated polyclonal antibodies against recombinant amino-terminal half fragment of AgI/II protein.

Ⅱ. MATERIALS AND METHODS

1. Materials

All chemicals and plastic wares were purchased from Sigma (St. Louis, MO) and Falcon Labware (Frankline Lakes, NJ), respectively. Oligonucleotides were ordered from Genetech (Yusung, Korea) and T vector was supplied from Takara (Shiga, Japan).

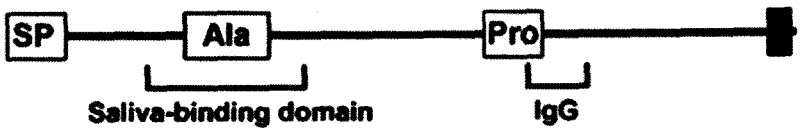

Fig. 1. Graphical presentation of a candidate for vaccine development against *S. mutans*. SP indicates signal peptides and the black box represents anchoring domain in bacterial membrane.

Restriction enzymes were obtained from KOSCHEM (Seongnam, Korea).

2. Strains and media

The genomic DNA of AgI/II was derived from *S. mutans* GS-5, *Escherichia* coli Top10 and M15 were used for transformation with the plasmids used in this study. Bacteria were grown in LB medium.

3. Preparation of *S. mutans* GS-5 genomic DNA

The genomic DNA from *S. mutans* GS-5 was prepared as described previously[34]. Briefly, 5㎖ of *S. mutans* GS-5 was collected and resuspended in 4㎖ of TE buffer containing 1㎖ of 25% glucose. After 30min incubation at 37℃, 1㎎ of lysozyme was added into the bacterial suspension and further incubated at 37 ℃ for 1h. RNAs in the lysates were removed by adding 0.5㎎ of RNase A and then proteins in the lysates were digested by 0.5㎎ of proteinase K. The cleared lysates was treated with SDS for 12h at 4℃ and then genomic DNA was precipitated by ethanol. Finally, the genomic DNA was resuspended in 1㎖ of TE buffer.

4. PCR amplification of *agI/II* gene

The genomic DNA was used as templates for amplifying *agI/II* genes in 50㎕ of PCR reaction containing Taq polymerase, dNTP, and oligonucleotides. The primer specific for *agI/II* gene was designed and synthesized as described in Table 1. The genomic DNA was denatured by 5 min incubation at 95℃ and then target genes were amplified for 30 cycles of PCR reaction (1 min 95℃, 1 min 55℃, 1 min 72℃). The PCR product was then treated for 5 min at 72℃.

5. Cloning of *agI/II* gene

About 2 ㎕ of PCR products were ligated into T vector and the ligation mixtures were transformed into competent Top 10 cells. The resulting antibiotics resistant bacteria were selected from agar plates and screened by checking the plasmid from each bacterial colony. The plasmids containing expected insert size were then sequenced to confirm the presence of target gene. The DNA sequencing was performed by BMRC (Daejeon, Korea).

6. Sequence analysis of *agI/II* gene from *S. mutans* GS-5

The DNA sequence of cloned genes was analyzed by comparing with the previously known sequences. For *agI/II* sequence analysis, the known sequence from *S. mutans* GS-5 (GenBank # D78181) was used as standard. The programs, Multalin and ESPript, were used to generate sequence alignment between two genes.

7. Expression of recombinant AgI/II protein in bacterial expression system

In order to express cloned genes in *E. coil* system, gene fragments of *agI/II* were subcloned to pQE expression vector. SphI and SalI were used to transfer N terminus of *agI/II* gene(*agI/II* mr), SalI and KpnI for C terminus of *agI/II* gene(*agI/II* mf). The ligation mixtures were transformed in Top10 and M15 bacte-

Table 1. List of oligonucleotides used to amplify *agI/II* gene. The upper case indicates the sequence from *agI/II*, while the small case shows the extra nucleotides to help gene cloning. The underline sequences are the location of restriction enzyme sites.

	sequence
agI/II f	acatGCATGCGGACAAAAGGTTTTGCCGAT
agI/II r	tggGGTACCTCAAGCATTGTTTGTTACTCCCG
agI/II mr	GTATACCAGAGCTAGCGAACCGGGAT
agI/II mf	GATCCCGGTTCGCTAGCTCTGGTATA

rial strains. Transformed M15 bacterial colonies were inoculated and grown overnight at 37℃ in Luria Broth containing 100㎍/㎖ of ampicillin and 25㎍/㎕ of kanamycin. The cells were further grown in the presence of IPTG to induce expression of the fusion protein. The cells were harvested by a centrifugation at 4,000g for 20-min and stored at -80℃, if necessary.

8. Purification procedures and SDS-PAGE analysis of AgI/II mr

The cell pellets were resuspended in lysis buffer. The suspension was sonicated with 30 second burst. The lysate was clarified by centrifugation at 15,000g for 30 min. The supernatant was loaded onto column preequilibrated with lysis buffer, which 50% Ni-NTA Superflow slurry(QIAexpressionist, Qiagen, Germany) was resuspended by. The column was washed successively with wash buffer, containing 20mM imidazole, and then was eluted with elution buffer, containing 250mM imidazole. The active fractions containing recombinant protein were dialyzed and identified by SDS-PAGE and pooled.

9. Production of anti-AgI/II mr antibodies in mouse

Female Balb/c mice about 6 weeks old were immunized with the recombinant *agI/II* mr protein. Four mice each were injected with the antigen. Mice were injected with a mixture of 100㎕ antigen solution(100 ㎍ antigen) and 100㎕ Freund's complete adjuvant. At 10-day intervals the mice were boosted twice with and without incomplete Freund's adjuvant. Three days after each boost, several eye bleeds were collected and tested for polyclonal antibodies against recombinant *agI/II* mr protein. To confirm the presence of the anti-AgI/II mr antibody, Western blot method was used to screen the reactivity of recombinant AgI/II mr. The serum was diluted 1:1000 as primary antibody, and goat-anti-mouse IgG, conjugated to HRP, was used as secondary antibodies. In these assays, the negative control was the purified recombinant protein, GST. By day 20, the mice were sacrificed with anesthetic and spleen cells were used for fusion with myeloma cells. Generation of monoclonal antibodies against to AgI/II mr antigen is in process.

Ⅲ. RESULTS

1. PCR amplification of agI/II gene

PCR was performed in three ways, yielding full-length, N-terminus, and C-terminus fragments of *agI/II* gene (Fig. 4). Smaller fragments were frequently amplified in a better quantity than the full-length counterpart. To my guess, this was caused by the PCR condition fitting better for about 2 Kbp DNA amplification.

2. Cloning of *agI/II* gene

PCR products were ligated into T vector, yielding T vector cloned with *agI/II* gene. The cloned T vectors were transformed into an *E. coli* strain, Top 10. Plasmids were prepared from antibiotics-resistant colonies and separated in agarose gel (Fig. 5). One or more colonies contained T vector with expected size of inserts from PCR products of partial fragments but none of colonies had full-length genes, meaning that antibiotic-resistant colonies from these

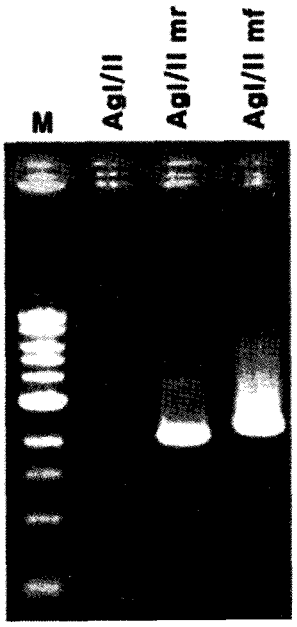

Fig. 2. PCR amplification of *S. mutans* GS-5 genes encoding AgI/II. The gene for AgI/II was amplified by PCR using the genomic DNA. In addition to full-length genes, truncated genes encoding *agI/II* mr or *agI/II* mf were also amplified.

samples resulted from self-ligated T vector without any insert. Finally, the sequences of cloned genes were confirmed by nucleotide sequencing.

3. Sequence analysis of cloned genes

To prove the integrity of the cloned genes, sequence analyses were accomplished using several programs such as Multalin[35] and ESPript[36]. For *agI/II* analysis, the corresponding sequence from *S. mutans* GS-5 was compared with that of the previously-cloned gene. The representative sequence alignment was shown in Fig. 4. In this 280 bp sequence, there was no miss match at all.

4. Expression and purification of *agI/II* in *E. coli* system

AgI/II gene cloned in pQE-30 vector was expressed and purified according to the procedures suggested by the manufacturer(Qiagen). M15 bacterial strains transformed with pQE-*agI/II* mr plasmid were isolated and purified for the induction of recombinant protein. Plasmids with expected *agI/II* mr gene were prepared from antibiotics-resistant colonies and separated in agarose gel (Fig. 5). Five colonies containing pQE-*agI/II* mr plasmid were obtained.

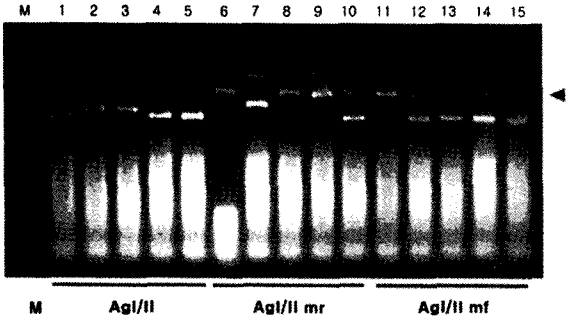

Fig. 3. Screening of plasmids cloned with PCR-amplified *agI/II* genes. The genes encoding full-length (*agI/II*) or partial segments (*agI/II* mr or *agI/II* mf) were cloned into T vector and bacterial transformants were screened for the presence of insert DNA. Arrow indicated the positions of plasmids with insert DNA.

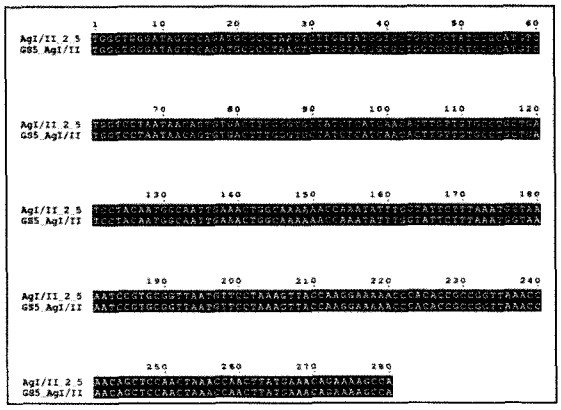

Fig. 4. Sequence alignment between cloned *agI/II* segment and the corresponding region in reported *agI/II* gene. The sequence of the cloned *agI/II* gene was compared to that of known *agI/II* gene (GenBank #D78181).

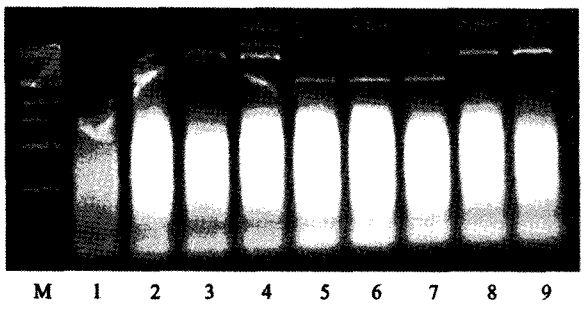

Fig. 5. Screening of plasmids of *agI/II* mr cloned in pQE-30 vector. The pQE-*agI/II* mr plasmid was transformed in M15 bacterial strains, and their transformants were screened for the presence of recombinant expression vector.

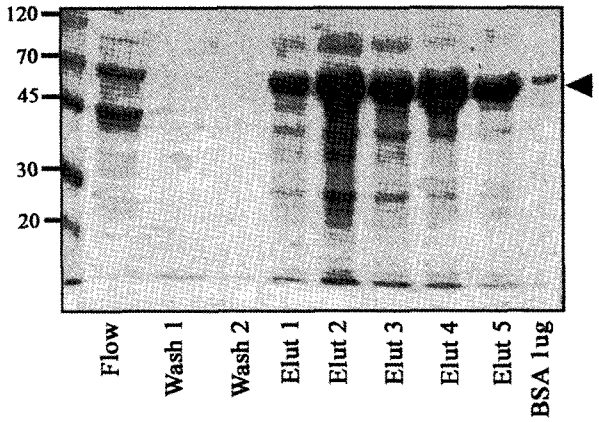

Fig. 6. Purification of recombinant AgI/II using Ni-NTA comlumn. Isolated AgI/II fragment was separated in SDS-PAGE and stained with Coomassie Brilliant Blue.

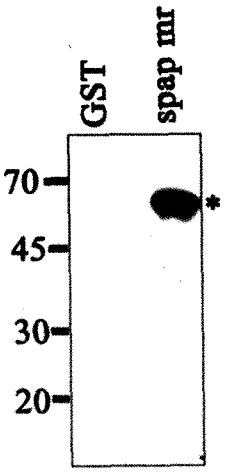

Fig. 7. Generation of polyclonal antibodies against AgI/II mr of *S. mutans* GS-5. Specific antibodies against amino-terminal AgI/II were produced using recombinant AgI/II fragment.

After purification using Ni-NTA resin, the purified protein was analysed by SDS-PAGE (Fig. 6).

5. Production of anti-AgI/II mr antibodies in mouse

In order to know if the polyclonal antibodies against recombinant protein AgI/II mr have the reactivity, the serum obtained from immunized mice were used as primary antibody in Western blot analysis. Figure 7 showed that the recombinant protein AgI/II mr was able to react with the specific antibody while no reactivity was seen in negative control of GST protein.

Ⅳ. DISCUSSION

As a candidate for vaccine against dental caries, AgI/II protein was always used in vivo study to prevent caries via mucosal or non mucosal routes in experimental animals such as rats, rabbits and monkeys with or without adjuvants, and the caries lesions were reduced[37]. S-IgA antibodies act as the first line of defense against dental caries by blocking of adhesin-receptor interaction[38]. So, in this study, we established the basis for a vaccination with defined epitope against AgI/II protein.

S. mutans AgI/II contains an alanine-rich tandem repeating region in the N-terminal third, and a proline-rich repeat region in the center of the molecule. These regions have been associated with the adhesin activity of AgI/II. Crowley and colleagues[39] and Nakai and co-workers[40] have each described a region within or near the alanine-rich region that can bind salivary components in experimental tooth pellicles. Lehner and co-workers[41] and Kelly and co-workers[42] suggested that the proline-rich central portion contains an adhesion epitope, basing their conclusions on adhesion inhibition assays involving recombinant fragments of AgI/II. Synthetic peptide approaches have also shown the alanine rich repeat region of AgI/II to be immunogenic and to induce protective immunity. For example, subcutaneous immunization with a synthetic peptide derived from the alanine-rich region of AgI/II from *S. mutans* (residues 301-319: PAcA) induced higher levels of serum IgG antibody reactive with recombinant AgI/II than a synthetic peptide derived from the proline-rich region (residues 601-629)[43]. Intranasal immunization with PAcA, coupled to cholera toxin B subunit, suppressed colonization of mouse teeth by *S. mutans*[43]. Fusion proteins containing PAcA also inhibited sucrose-independent adhesion of *S. mutans* to saliva-coated hydroxyapatite beads[44]. Thus, this *S. mutans* adhesin contains multiple functionally based epitopes that are sufficiently immunogenic to be considered for dental caries vaccines.

Designing vaccines permits one to eliminate regions which may induce unwanted antibody specificities. The AgI/II family of proteins shares extensive sequence homology with surface proteins of non-cariogenic *S. gordonii*[45], *S. intermedius*, and *S. oralis*[46]. These homologous sequences may induce cross-reactive responses that could influence colonization, attachment, or accumulation of commensal microbiota. Also, research showed that the C-terminal part of AgI/II contains an epitope which is cross-reactive with human IgG[47,48] as shown in Fig. 1. and, although the clinical significance of this observation is unknown it appears that this potentially harmful epitope should be excluded from a caries vaccine. Given this knowledge, vaccines can be designed to include the salivary-binding domain(s), but exclude sequence bearing the potential for induction of unwanted antibody responses. In the present study, the

genes for AgI/II mr were cloned, and expressed as fragmented form rather than full-length. In the previous mouse model, immunization with AgI/II mr conjugated to adjuvant generates polyclonal antibodies against *S. mutans* GS-5. By using smaller fragments, the specific polyclonal antibodies against target epitope could be better induced by avoiding unnecessary competition through epitope dominance. Another advantage of using small fragment of AgI/II is that the epitope adjacent to proline-rich region, mimicking human IgG can be discarded.

Currently, as well as generation of monoclonal antibodies against AgI/II mr, the expression of *agI/II* mf and gtfD genes are being tested.

In conclusion, *agI/II* genes from *S. mutans* GS-5 were cloned and sequenced. Sequence analyses of the cloned *agI/II* gene showed that the nucleotides sequence of cloned genes had perfect homology to the sequences previously reported from *S. mutans* GS-5. Recombinant protein of N-terminus of AgI/II protein was expressed and purified and polyclonal antibodies against protein was generated in mice. In consideration of the importance of these proteins in the pathogenesis of *S. mutans* GS-5, vaccine development against these proteins will be promising and also vaccination through oral administration will be reasonable approach to stimulate mucosal immunity of the host.

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국문초록

연쇄상구균(*Streptococcus mutans* GS-5)의 항원단백질 AgI/II의 N-terminus절편에 대한 항체형성

한지혜 · 김재곤 · 백병주 · 양연미 · 박정렬

전북대학교 치과대학 소아치과학교실 및 구강생체과학연구소

치아 우식은 구강 내 미생물에 의해 치아 석회 조직의 일부가 용해되고 파괴되는 감염성 질환이다. 치아 우식의 원인균은 *Streptococcus mutans*와 같은 Mutans streptococci로 알려져 있고, 이 미생물이 치면에 접착하여 군집을 형성하는 능력이 균의 독성에 중요한 역할을 한다. *S. mutans*가 치면의 타액성 피막에 부착하는 데에는 AgI/II와 같은 세포표면의 섬유성 단백질을 매개로 한다. 그러므로, AgI/II는 *S. mutans* GS-5에 대한 백신 개발에 적절한 목표가 된다. 본 실험은 *S. mutans* GS-5로부터 AgI/II 유전자를 복제하고 염기서열분석을 하였다. 복제된 AgI/II와 앞서 보고된 *S. mutans* GS-5의 해당 부위의 280개의 핵산은 완벽하게 일치하였다. 복제된 유전자를 두 부위로 절단하여 형질전환을 통해 재조합 단백질인 AgI/IImr을 얻었고, 정제된 재조합 단백질을 쥐에게 주입하여 다클론 항체를 얻었다. 추출된 다클론 항체는 AgI/IImr항원단백질에 반응하였고, 대조군으로 쓰인 단백질에는 반응하지 않았다.

주요어 : 연쇄상구균, AgI/II, 백신