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Prediction of the Secondary Structure of the *AgfA* Subunit of *Salmonella enteritidis* Overexpressed as an MBP-Fused Protein

WON, MISUN[1], SOYOUN KIM, SEUNGHWAN LEE, CHULJUNG KIM[2], HYUNSU KIM[2], MOOHYUNG JUN[2], AND KYUNG BIN SONG*

Department of Food Science and Technology, and [2]Department of Veterinary Medicine, Chungnam National University, Taejon 305-764, Korea
[1]Genome Research Center, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-600, Korea

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Abstract To examine the characteristics of the recombinant thin aggregative fimbriae of *Salmonella*, the *AgfA* subunit gene was amplified from *Salmonella enteritidis* using a PCR. The maltose binding protein (MBP)-*AgfA* fusion protein was overproduced in *E. coli* and purified. The secondary structure of *AgfA* was then elucidated from the difference CD spectra. An estimation of the secondary structure of *AgfA* using the self-consistent method revealed a mostly β-sheet structure.

Key words: Secondary structure, *Salmonella enteritidis*, *AgfA*

Salmonella enteritidis is a serious food-borne enteric pathogen in humans and poultry. It expresses three morphologically distinct fimbriae, which are filamentous surface structures composed of a repeated major subunit protein [9]. Three distinct fimbrial types have been reported in *Salmonella enteritidis*: SEF (*Salmonella enteritidis* fimbriae) 21, 17, and 14 [2]. SEF17 is tightly coiled and comprised primarily of *AgfA*, a 17,000-MW fimbrin protein, and a DNA sequence analysis has revealed three contiguous genes, *AgfBAC* [2-5]. All *Salmonella* spp. possess the *AgfA* fimbrin gene [6-7]. SEF17 fimbriae are responsible for the autoregulation and distinct colonial morphology of *Salmonella enteritidis*.

The excellent antigenic nature of these fimbriae indicates a potential for use in the development of vaccines. Accordingly, diagnostic tests using fimbriae or specific antibodies against them have been applied in the identification of *Salmonella enteritidis* infections [12]. However, due to their extremely aggregative nature and the existence of multiple isoforms, there is still little biophysical data available. Therefore, to understand the molecular basis for this aggregative nature and unusual stability, plus provide the molecular design for an *AgfA*-based heterologous *Salmonella* vaccine [13], the *AgfA* subunit protein was overproduced as an MBP-*AgfA* fusion protein and its secondary structure examined.

Due to their aggregative nature, the purification of thin fimbriae by conventional procedures is not easy. *AgfA* fimbrin exists in multiple isoforms and has an extremely aggregative nature [2]. As such, the only treatment that can effectively depolymerize it is exposure to 90% formic acid [2]. Therefore, an MBP-fusion system was introduced to solublize and overproduce the *AgfA* protein, since MBP can facilitate the folding of fimbrin, thereby resulting in a soluble form.

To overproduce the *AgfA* subunit protein of *S. enteritidis*, the DNA fragment of *AgfA* was cloned into an *E. coli* expression vector, pMAL-CR1, encoding IPTG-inducible MBP. The 393-base-pair of the DNA (Fig. 1) was amplified by a PCR using synthetic primers and chromosomal DNA

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1   ATGAAACTTTTAAAAGTGGCAGCATTCGCAGCAATCGTAGTTTCTGGCAGTGCTCTGGCT   60
      M  K  L  L  K  V  A  A  F  A  A  I  V  V  S  G  S  A  L  A
60 ↓GGCGTCGTTCCACAATGGGCGGCGGCGGTAATCATAACGGCGGCGGCAATAGTTCCGGC  120
      G  V  V  P  Q  W  G  G  G  G  N  H  N  G  G  G  N  S  S  G
121 CCGGACTCAACGTTGAGCATTTATCAGTACGGTTCCGCTAACGCTGGCGCTTGCTCTGCAA 180
      P  D  S  T  L  S  I  Y  Q  Y  G  S  A  N  A  A  L  A  L  Q
181 AGCGATGCCCGTAAATCTGAAACGACCATTACCCAGAGCGGTTATGGTAACGGCGCCGAT 240
      S  D  A  R  K  S  E  T  T  I  T  Q  S  G  Y  G  N  G  A  D
241 GTAGGCCAGGGTGCGGATAATAGTACTATTGAACTGACTCAGAATGGTTTCAGAAATAAT 300
      V  G  Q  G  A  D  N  S  T  I  E  L  T  Q  N  G  F  R  N  N
301 GCCACCATCGACCAGTGGAACGCTAAAAACTCCGATATTACTGTCGGCCAATACGGCGGT 360
      A  T  I  D  Q  W  N  A  K  N  S  D  I  T  V  G  Q  Y  G  G
361 AATAACGCCGCGCTGGTTAATCAGACCGCATCTGATTCCAGCGTAATGGTCGTCAGGTT  420
      N  N  A  A  L  V  N  Q  T  A  S  D  S  S  V  M  V  R  Q  V
421 GGTTTTGGCAACAACGCCACGGCTAACCAGTAT
      G  F  G  N  N  A  T  A  N  Q  Y
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Fig. 1. DNA sequence of *AgfA* of *Salmonella enteritidis*.
Arrow indicates the cleavage site of signal sequences.

*Corresponding author
Phone: 82-42-821-6723; Fax: 82-42-825-2664;
E-mail: kbsong@cnu.ac.kr

as the template. The sequence of the N-terminal or C-terminal primers were 5'-GCGGAATTCGGCGTCGTT-CCACAATGG-3', 5'-CGCGTCGACATACTGGTTAGC-CGTGGC-3', respectively. The PCR fragment was then cloned into a pGEM-T vector and the DNA sequence of *AgfA* confirmed by DNA sequencing. The DNA fragment of *AgfA* digested with *Eco*R1 and *Sal*1 was ligated into the plasmid pMalE-CR1, also digested with *Eco*R1 and *Sal*1. This ligation mixture was then used for the transformation of *E. coli DH5*. The subcloning of *AgfA* was confirmed by digesting the plasmid DNA with *Eco*R1 and *Sal*1.

The production of the MBP-*AgfA* fusion protein was induced by growing cells in the presence of 1 mM IPTG for 5 h. The presence of the MBP-*AgfA* fusion protein was detected in 12% polyacrylamide gels by SDS-PAGE (Fig. 2). To purifiy the MBP-*AgfA* fusion protein, a crude cell extract containing MBP-*AgfA* was prepared by sonicating cell suspensions in PBS followed by centrifugation. The MBP-*AgfA* in the *E. coli* crude cell extract was allowed to bind to amylose cross-linked to an agarose resin (NEB, Beverly, U.S.A.) for 2 h. The MBP-*AgfA* bound to the amylose resin was then released by the addition of a 20 mM maltose solution. In Fig. 2, a distinct band indicating an MBP-*AgfA* fusion protein of 60 kDa was detected (lane 5). The expression level of the MBP-*AgfA* fused protein was about 20%. Furthermore, since the MBP-*AgfA* fusion protein existed in a soluble form (lane 4), this indicated that the MBP affected the folding, thereby increasing the solubility of *AgfA*. The purity of the MBP-*AgfA* on the SDS-PAGE was more than 90%.

To elucidate the secondary structure of *AgfA*, CD spectroscopy was performed at 25°C using a JASCO J-720 spectropolarimeter according to the method reported

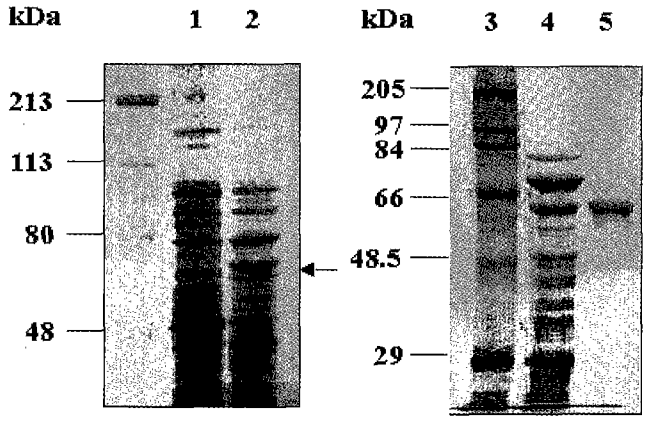

Fig. 2. Expression and purification of MBP-*AgfA* subunit protein. The production of the MBP-*AgfA* fusion protein was induced by growing cells in the presence of 1 mM IPTG for 5 h. A crude cell extract containing MBP-*AgfA* was prepared by sonicating cell suspensions. The MBP-*AgfA* was bound to an amylose resin, and then released by the addition of a 20 mM maltose solution. Lane 1, uninduced cell; 2, induced cell; 3, molecular weight marker; 4, supernatant after sonication of crude cell extract; 5, MBP-*AgfA*.

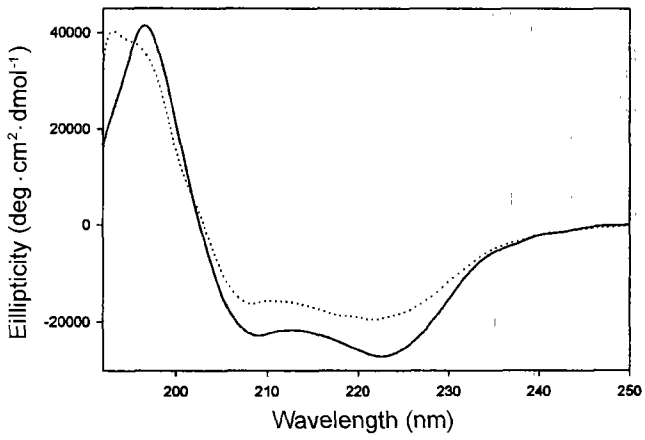

Fig. 3. CD spectra of MBP-AgfA and MBP.
A 1-mm-pathlength cell was used. The reported CD spectra were an average of five scans and were smoothed by a polynomial curve fitting program. The CD data were expressed as molar ellipticity in deg · cm^2 · $dmol^{-1}$. ___, MBP; -----, MBP-AgfA.

previously [1, 8]. Since *AgfA* is not soluble, the difference spectrum of the protein was obtained using the CD spectra of MBP-*AgfA* and MBP. The three-dimensional structure of MBP has been previously reported at 2.8 Å, which showed two globular domains by a deep groove [10]. Both domains consist of a central β-pleated sheet flanked on both sides with two or three parallel α-helices. The CD spectrum of MBP shows that it has a mostly α-helix structure with typical negative minimum ellipticity values at 207 and 221 nm (Fig. 3). Also, the CD spectrum of MBP-*AgfA* shows an α-helix structure.

The fimbrin subunit, *AgfA*, of *Salmonella entiritidis* consists of two domains: a protease-sensitive N-terminal region and a protease-resistant 109 residue C-terminal core [2]. Based on its sequence, the predicted secondary structure for *AgfA* is likely to be a mainly extended conformation with β strands linked by four to six residues. To explore the *AgfA* tertiary structure, Collinson *et al.* [2] applied three structural models. Among them, the parallel β-helix model, which is a compact coil of ten helically arranged β strands forming two parallel β-sheet faces, was favored. Figure 4 shows the difference spectrum of the MBP-*AgfA* fused protein and MBP, thereby indicating the CD spectrum of *AgfA*, assuming that MBP has no affect on the secondary structure of *AgfA*. The difference spectrum showed that *AgfA* had a mostly β-sheet structure. Its estimation using the self-consistent method [11] also indicated that it has a 46% β-sheet structure. This result agrees with the report that a cursory analysis of various bacterial fimbrins indicated a 31% to 69% predicted β-strand content with multiple β-strand motifs [2]. Also, this study clearly shows that the secondary structure of an insoluble protein can be predicted based on the difference CD spectra of the fusion protein using a spectropolarimeter.

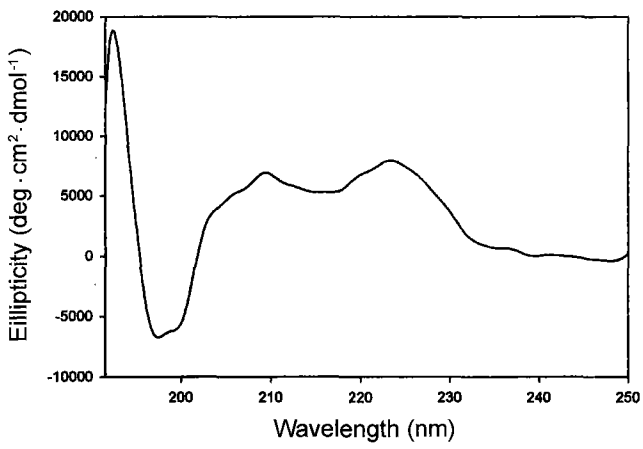

Fig. 4. CD spectra of *AgfA*.
The difference spectrum was obtained from Fig. 3.

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