Development of oral vaccination for preventing streptococcosis disease in nile tilapia by using algae as a delivery system

Sugunya Maneenin[1], Atittaya Hoihuan[1], Ansaya Pumchan[1], Thararat Phurahong[1], Nontawith Areechon[2], Thanyanan W. Brocklehurst[3],Sasimanas Unajak[1,*]

[1] Department of Biochemistry, Faculty of Science, Kasetsart University, 50 Ngam Wong Wan Road, Chatuchak, Bangkok 10900, Thailand
[2] Department of Aquaculture, Faculty of Fisheries, Kasetsart University, 50 Ngam Wong Wan Road, Chatuchak, Bangkok 10900, Thailand
[3] Department of Biology, Faculty of Science, Silpakorn University, 6, Rajamankha Nai Rd., Amphoe Muang, Nakhon Pathom 73000, Thailand.
*E-mail: Sasimanas.u@ku.th

Abstract

Streptococcus agalactiae is the major infectious bacterial of streptococcosis disease in many fish species including Nile tilapia (*Oreochromis niloticus*). Although multiple types of vaccines were developed and used to control Streptococcosis disease, the principal method for vaccine application in fish is based on injection method. However, stress introduction to injected animals and skill labor requirement are the major drawback of this method. Hence, in this study, we interest in the development of orally vaccination method for fish by using subunit vaccines specific to streptococcosis disease as a model. Therefore, surface immunogenic protein (Sip protein), potential universal vaccine for streptococcosis disease, was used. Characteristic of Sip protein was analyzed by bioinformatics analysis indicated total 19 epitopes that have antigenic properties with antigenic value of 0.6910 from 0.4. Moreover, cellular localization analysis of Sip protein demonstrated that the protein was located as extracellular protein which also related to the cell-surface association mechanism.

Chlamydomonas reinhardtii is widely used for express many complex recombinant proteins. This system is potentially used as a vaccine delivery system for orally administration. For expression of protein in microalgae's chloroplast, codon usage of gene should be optimized. The sequence of *Sip* gene was optimized to be compatible with the *C. reinhardtii* codon usage table from Kasuza database by using bioinformatics. The sequence of *Sip* gene after optimized contains 1,042 bp of nucleotides sequence and 343 of amino acid residues. After that, the optimized *Sip* gene was inserted into pASapI vector, specific vector of *C. reinhardtii,* and introduced to algae cell by glass bead transformation. The result of the PCR product that harboring optimized *Sip* gene showed approximately ~1,200 bp by using specific primer. This result indicated that the optimized *Sip* gene was successfully constructed. Then, the SDS-PAGE and western blot was used to analyze the protein expression in Chloroplast of *C. reinhardtii*. From this study, It is expected that the novel vaccine delivery system will be useful to the tilapia aquaculture industry for reduce a loss from Streptococcosis disease.

Introduction

Nile Tilapia (*Oreochromis niloticus*) is a world important economic farmed fish. In Thailand, Nile Tilapia aquaculture showed significant increasing resembled to the requirement for local consumption and for exportation. This fish species is recognized as an important source of nutritious protein. However, during cultivation, tilapia aquaculture always faces with disease that caused devastation especially bacterial disease such as streptococcosis disease. Thus, this disease introduces seriously invasive infection and causes of mass mortality throughout the world.

Streptococcosis is majority disease in fish aquaculture, not only in fresh water fish but also marine fish. This disease causes by Gram-positive bacteria *Streptococcus* spp. especially *Streptococcus agalactiae* in tilapia. In Thailand, it was reported three *S. agalactiae* serotypes that cause disease in tilapia which are serotypes Ia, Ib and III.[1] However, it is important to control the infectious disease in aquaculture to increase production yield and sustain tilapia aquaculture. Currently, multiple types of vaccine were developed and used to the control and treatment of Streptococcosis disease including inactivated vaccines, activated vaccines, and DNA vaccine, for example. In this study we interested in protein-based subunit vaccines produced from antigenic protein of particular pathogen that are necessary to elicit a protective immune response.

Surface immunogenic protein (Sip) has recorded as a potential universal vaccine for streptococcosis. Sip is highly conserved protein among Group B streptococcus (GBS) isolates and shows the efficacy to develop a cross-reactive immune response.[2] Previous results shown that the immunized of mice with purified recombinant Sip protein elicited protective immunity against lethal infection with *S. agalactiae* strains of several serotypes.[2] However, vaccine application method is currently relied on the injection. It is showed some of disadvantage as it may cause stress, injury and inflammatory reactions to fish.[3] Oral vaccination is alternative method to transfer vaccine to living organism. This method has proven for many advantageous such as convenient administration, lower number of laborious, effectively stimulate immune response, and increasing in frequently of application – continuously stimulate immune response.[4] However, plant-produced vaccines are interested because easily in construction, more attractive for recombinant protein production and cannot cause disease.[5,6]

Green microalgae are widely used for express many complex recombinant proteins such as protein vaccines, antibodies, and enzymes.[7-11] This system is potentially used as a vaccine delivery system for orally administration. A significant immune response was detected in surface protein E2 from classical swine fever virus (CSFV) expressed in *Chlamydomonas reinhardtii*, algal-produced vaccine antigen. This system have been tested and demonstrated that it can prevent classical swine fever virus (CSFV) in mice by oral administration.[12]

Therefore, in this research aimed to develop an oral vaccination method for prevent Streptococcosis disease in Nile tilapia. The candidate antigen for subunit vaccine will apply for oral vaccination system by using *Chlamydomonas reinhardtii* to mediate transportation of subunit vaccine to the fish. It is expected that the novel vaccine delivery system will be useful to the tilapia aquaculture industry for reduce a loss from Streptococcosis disease.

Methodology

The experiment will be divided into two parts. First part, aim to identify an antigenic proteins of *S. agalactiae* and the second part is to produce of recombinant algae for using as an oral delivery system for study of the immune response in the fish.

Identification of antigenic regions from immunoreactive protein of S. agalactiae

The programs for analyzed antigenic protein that used in the study are the following:

- Antigenic determinant software (http://imed.med.ucm.es/Tools/antigenic.pl) for determined location on the protein sequence that has ability of the antigenic protein.

- VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) for prediction of protective antigens.

- CELLO v.2.5: subcellular localization predictor (http://cello.life.nctu.edu.tw) for predicted localization of genes in cell.

Production of recombinant algae for using as an oral delivery system for control Streptococcosis disease in Tilapia

Codon optimization of Sip gene to protein expression in chloroplast of C. reinhardtii by using bioinformatics analysis

For Sip gene codon optimization, the COU Beta 0.92: Codon Usage Optimizer program (http://codonusageoptimizer.org/download/) was used to calculate and optimize codon of target protein to be expressed in algal chloroplast. This analysis used codon usage database (http://www.kazusa.or.jp/codon/) and Codon Adaptation Index (CAI) (http://genomes.urv.es/OPTIMIZER) for predict level of gene expression in algal chloroplast. Then, the secondary structure and Folding Free Energy of mRNA of *Sip* gene was determined using RNAfold Web Server program (http://rna.tbi.univie.ac.at/cgi-bin/ RNAWebSuite/ RNAfold.cgi).

In this study, N-terminal signal peptide and LysM domain were removed from full length of *Sip* gen.[12] This gene was synthesized by GeneArt Gene Synthesis and Subcloning Service at Gibthai Co., Ltd. However, *Sap*I and *Sph*I recognition sequence were added to the 5' end and the 3' end of *Sip* gene for cloning. In addition, Histidine-tag and Flag-tag were added for detection and purification.

Construction and production of the plasmid for protein expression in Chloroplast of C. reinhardii in the bacterial system

To produce the recombinant algae expression vector carrying *Sip* gene, *Sip* gene was excised from parented vector and transferred to pASapI vector, shuffling vector of *C. reinhardtii* (Saul Purton, University College London, UK). The pASapI vector was cut with corresponding restriction sites (section 2.1) and purified by HiYield™ Gel/PCR Fragments Extraction Kit (RBC Bioscience, Israel). Quality and quantity of products were determined by using Nano Drop spectrophotometer (Thermo scientific, USA) at $A_{260/280}$ or analyzed on agarose gel electrophoresis. The purified products were then stored at -20 °C until used.

To construct of plasmid, the ligation reaction including *Sip* gene and vector was ligated with T4 DNA ligase (Promega Corporation, USA). Then, the ligation products were transformed into *E. coli* (DH5α) competent cells by heat shock method. For screening positive plasmid, colony PCR was used. The specific primer vector; pASapI-FW and pASapI-RV (Table 1) were used in PCR reaction. The PCR products was analyzed on agarose gel electrophoresis and detected by ImageQuant™ (LAS500 Gel documentation). The validity of the nucleotide was determined by DNA sequencing (Macrogen, Korea).

Introduction of plasmid to chloroplast vectors of Chlamydomonas by glass bead transformation

According to the Kindle (1990), the algal cells (*C. reinhardtii* strain TN72 mutant) were cultured in 400 ml of TAP medium (2.42 g Tris base, 25 ml TAP-salts, 1 ml Phosphate solution, 1 ml Hutner trace elements solution and adjust pH with acetic acid)[13] to obtain final concentration of 2×10^8 cells/ml. Then, cell suspension was centrifuged at 3,500 rpm for 10 min. After that, 300 µl of the algal cells was transferred to 5 ml tube contain with 0.3 g of glass beads (Sigma-Aldrich, USA). 5 µg of recombinant plasmid was added to cell suspension and vortexed at maximum speed for 15 second. 3 ml of 0.5% washed agar was added to tube and culture on Sueoka's high salt medium (HSM) agar (5 ml Beijerinck's solution,

5 ml phosphate, 1 ml hunter trace elements solution and 1% Agar) at 23°C for overnight in dark condition and light condition for 14-30 days. Single colonies from the transformed plate were taken to culture and extracted DNA to verify the recombinant clone in algae.

Screening the recombinant algae harboring antigenic protein

Single colony of recombinant algae was streaked on HSM agar which incubated at 23 ° C for 7 days at light. Then, single colony of algae that grown on HSM agar was re-streak for 3-5 passage. Therefore, the algae cell was cultured in 50 ml of volume of TAP medium to get the cell density of 2×10^8 cells/ml. The DNA from algae was extracted by CTAB method[14].

For screening of inserted gene in recombinant vector, 3 primers: trnE2-F, psbH-R and atpa-R were used in PCR analysis (Table 1). The condition for PCR were performed as followed: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 second, annealing at 56.1°C for 30 second, extension at 72°C for 30 second and final extension at 72°C for 10 min. The cycle for PCR was proceeded for 29 cycles. PCR products was analyzed on 1% agarose gel electrophoresis and detected by ImageQuant™ (LAS 500 Gel documentation).

TABLE 1. Sequence of primers used in this study

Primer	Sequence (5' → 3')
pASapI-FW	CAAGTGATCTTACCACTCAC
pASapI-RV	CAAACTTCACATGCAGCAGC
trnE2-F	GTCATTGCGAAAATACTGGTGC
psbH-R	ACGTCCACAGGCGTCGTAAGC
atpa-R	GATGACGTTTCTATGAGTTGGG

Results and Discussion

Identification of antigenic regions from immunoreactive protein of S. agalactiae

From previous reports, surface immunogenic protein or Sip was shown highly conserved among GBS isolates that has ability to induce the cross-reactive immune response.[2] From this reason, *Sip* gene was suggested to use as a subunit vaccine for control streptococcosis disease in fish. In this research, amino acid sequence of *Sip* gene was taken from genomic DNA of *S. agalactiae* (Accession number AEK06226.1). Then, the bioinformatics analysis was used to characterize the properties of protein. The program was used in the study as described in methodology section.

CELLO RESULTS

SeqID: gi|339779223|gb|AEK06226.1| Sip [Streptococcus agalactiae]

Analysis Report:

SVM	LOCALIZATION	RELIABILITY
Amino Acid Comp.	Extracellular	0.449
N-peptide Comp.	Extracellular	0.662
Partitioned seq. Comp.	Extracellular	0.726
Physico-chemical Comp.	OuterMembrane	0.683
Neighboring seq. Comp.	Periplasmic	0.559

CELLO Prediction:

Extracellular	2.278 *
Periplasmic	1.168
OuterMembrane	1.136
Cytoplasmic	0.246
InnerMembrane	0.172

Figure 1. The localization of Sip gene in cell by using CELLO v.2.5 program. (http://cello.life.nctu.edu.tw)

For determining the localization of epitope on particular protein, antigenic determinant software was used to analyze. The results showed 19 epitopes in Sip protein that has potentially function in activate host immune response (data not shown). The VaxiJen was used to predict of protective antigens ability of protein. The results showed that Sip protein has antigens value about 0.6910 from threshold 0.4 (70% accuracy) (data not shown). Localization of protein in the cell was identified by CELLO v.2.5 program. Result showed that Sip protein locates on extracellular part of bacteria (Figure 1) which also related to the cell-surface association mechanism.[15] All of the results indicated that Sip was an interested candidate for the development of a subunit vaccine.

Optimization of antigenic protein for expression of recombinant protein in microalgae.

Codon usage of gene should be optimized to improve the ectopic expression of protein in microalgae's chloroplast.[16] Nucleotide sequence of *Sip* gene was optimized to be compatible with the *C. reinhardtii* codon usage table by using COU beta 0.92 programs. However, previous research showed that Sip could not be produced in *E. coli* if N-terminal signal peptide and LysM are remain which both regions may contribute in Sip toxicity.[12] Therefore, in this research, both domains were removed from Sip gene. Nucleotide sequence of optimized *Sip* gene was 1,042 bp with could translate to 343 of amino acid residues.

In addition, the Codon Adaptation Index (CAI) or expected codon adaptation index was verified to measure the synonymous codon usage bias of optimized gene. The score of CAI would be ranging from 0 to 1 if score reaches one, it will indicate that every amino acid is encoded by the most common codon in the reference codon table.[17] These results indicated that the optimized *Sip* gene shown the high score of CAI approximated 0.982 while non-optimized *Sip* gene shown the CAI value approximately 0.638 (Figure 2). These results represented that every amino acid were encoded by the most common codon in the reference codon table and it would be concluded that the amino acid sequence of optimized *Sip* gene were produced the protein.

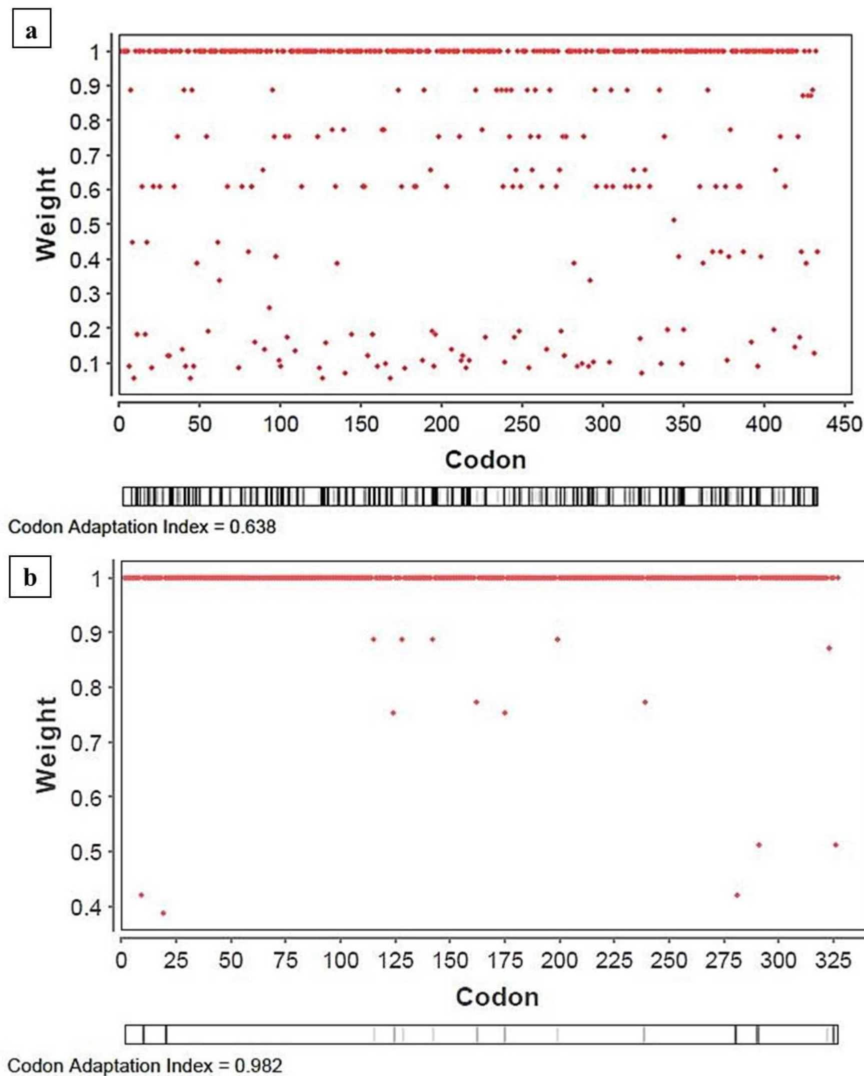

Figure 2. The Codon Adaptation Index (CAI) of gene, red spot represented each codon, horizontal axis represented number of codon and vertical axis represented CAI value Non-optimized *Sip* gene (a) and optimized *Sip* gene (b)

The mRNA secondary structures also involves in genes expression. It can impair the expression by interfering with the binding of ribosome during translational initiation.[18] In this research, the mRNA secondary structure and free energy for folding of optimized *Sip* gene were analyzed by RNAfold Web Server program. The folding free energy show - 241.08 kcal.mol^{-1} that can acceptable free energy for admit translation (Figure 3). Therefore, this structure was suitable for translation and express to the recombinant Sip. Then the optimized *Sip* gene was synthesized.

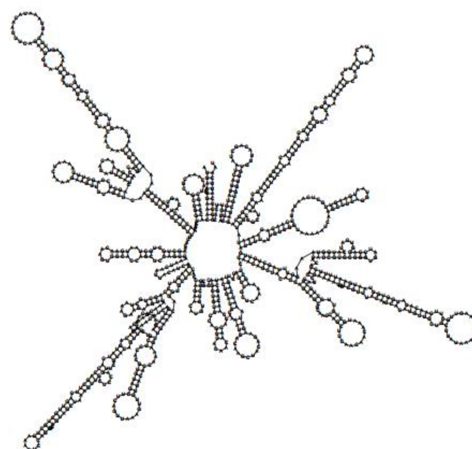

Figure 3. The mRNA secondary structure of optimized Sip gene by RNAfold Web Server program (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

Production and construction of recombinant algae for using as an oral delivery system

To construct the specific vector for recombinant Sip production in *C. reinhardtii*, optimized *Sip* gene were digested from parental vector with appropriated restriction enzymes as *Sap*I and *Sph*I, respectively. Then, the gene was ligated into the corresponding site of pASapI vector and transformed into *E. coli* DH5α competent cell by heat shock transformation. The colony were extracted and verified of the nucleotide sequence by DNA sequencing (Macrogen, Korea) (data not shown). Then, the plasmid was transferred to chloroplast vectors of *Chlamydomonas* by glass bead transformation.

The recombinant clones were confirmed by PCR screening by pASapI-FW and pASapI-RV primer according to Table1. The result revealed that positive clone shown band approximately 1.2 kb regard to the size of empty vector 237 bp and optimized *Sip* gene 1,042 bp (Figure 4). These result indicated that optimized *Sip* gene were successfully integrated to *C. reinhardii* chloroplast genome.

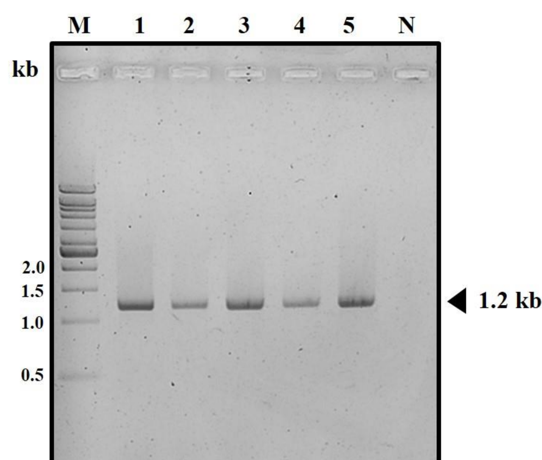

Figure 4. Analysis of production of the amplified homologous recombination clone. 1% agarose gel electrophoresis was performed. The expected size about 1.2 kb in lane 1-5 and lane N is negative control. Lane M is VC 1kb DNA Ladder marker (Vivantis, USA).

The SDS-PAGE and western blot analysis was used to determine of recombinant protein in Chloroplast of *C. reinhardtii*. Further work is to produce of large-scale algae cell culture and mixed with commercial feed for used in fish feed. The efficiency to prevent streptococcus disease will examine in fish and challenge with *S. agalactiae*.

Conclusion

This research aims to develop an oral vaccination method by using *C. reinhardtii* as a system to mediate subunit vaccine to Nile tilapia. The antigenic protein, Sip from *S. agalactiae*, was interested to be used as subunit vaccine model in this study. Results from bioinformatics analysis indicated that Sip protein has various characteristic of antigenic protein which could be used as a subunit vaccine candidate. Codon of Sip protein was optimized to be suited with chloroplast of *C. reinhardtii*. The optimized Sip was successfully constructed in pASapI vectors, which generated the recombinant protein. The plasmid was transferred to chloroplast of *C. reinhardtii* by glass bead transformation and result indicated that the plasmid ere successfully integrated to *C. reinhardii* chloroplast genome. Taken together, transgenic *C. reinhardtii* harbouring Sip proteins were successfully produced and will further mixed with commercial feed for used in fish feed to prevent streptococcus disease. The novel vaccine delivery system will be solved for many disadvantage of route vaccination. It will be useful in the application to prevent the other disease in aquatic animals.

References

1. Suanyuk N, Kong FR, Ko D, Gilbert GL and Supamattaya K. Aquaculture. 2008;284: 35-40.
2. Brodeur BR, Boyer M, Charlebois I, Hamel J, Couture F, Rioux CR, Martin D. Infect Immun. 2000;68(10):5610-5618
3. Horne MT, Roberts RJ, Tatner M, Ward P. J fish Dis. 1984;7:91-99
4. Zhu Q, Berzofsky JA. **Gut Microbes**.2013;4(3):246–252.
5. Harris EH, Burkhart BD, Gillham NW, Boynton JE. Genetics. 1989;123: 281-292
6. Ruf S, Karcher D, Bock R. Proc Natl Acad Sci USA. 2007;104:6998-7002
7. Ishikura, K., Takaoka, Y., Kato, K., Sekine, M., Yoshida, K., and Shinmyo, A.J. Biosci. Bioeng. 1991;87(3):307-314
8. Mayfield, S. P., Franklin, S. E., and Lerner, R. A. Proc. Natl. Acad. Sci. U.S.A. 2003; 100:438-442
9. Tran, M., Zhou, B., Pettersson, P. L., Gonzalez, M. J., and Mayfield, S. P. Biotechnol. Bioeng. 2009;104, 663-673
10. Rasala, B. A., Muto, M., Lee, P. A., Jager, M., Cardoso, R. M. F., Behnke, C. A. Plant Biotechnol. J. 2010;8:719-733
11. Georgianna, D. R., Hannon, M. J., Marcuschi, M., Wu, S., Botsch, K., Lewis, A. J. Algal Res. 2013;2: 2–9.
12. He Y., Wang K.-Y., Xiao D., Chen D.-F., Huang L., Liu T., Wang J., Geng Y., Wang E.-L., Yang Q. Vaccine. 2014;32:7025-7032.
13. Harris, E Academic Press, 1989:780
14. Worden, A. Z., Lee, J. H., Mock, T.,Rouze, P., Simmons, M. P., Aerts, A. L. Science. 2009;324:268-272
15. Rioux S, Martin D, Ackermann H-W, Dumont J, Hamel J, Brodeur BR. Infect Immun. 2001;69(8):5162-5165
16. Campbell WH, Gowri G. Plant Physiology. 1990 Jan;92(1):1-11
17. Sharp PM, Li WH. Nucleic Acids Res. 1987;15:1281-1295.
18. Meyer, M. M. WIREs RNA.2017;8:e1370

Acknowledgements

This research is supported by Kasetsart University Research and Development Institute (KURDI) and the Thailand Research Fund (TRF) for M.S student scholarship.

I gratefully thank Dr. Thanyanan W. Brocklehurst, Department of Biology, Silpakorn University for generously expression vector of algae and *Chlamydomonas* cell.