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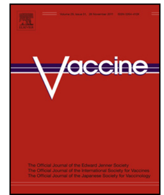

Oral immunization of fish against iridovirus infection using recombinant antigen produced from rice callus

Y.J. Shin [a], T.H. Kwon [a,1], J.Y. Seo [b], T.J. Kim [b,*]

[a] *Jeonju Biomaterials Institute, Jeonju 561-360, Republic of Korea*
[b] *College of Veterinary Medicine, Chonnam National University, 300 Yongbong-dong, Buk-gu, Gwangju 500-757, Republic of Korea*

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ABSTRACT

Fish iridoviruses cause systemic diseases with high morbidity and mortality in various species of wild and farm-raised fish, resulting in severe economic losses, and no large-scale protective vaccine program or therapy is currently available. In this study, we expressed a recombinant major capsid protein (rMCP) of rock bream iridovirus in transgenic rice callus. The rMCP in lyophilized rice callus powder was added to feed to induce intestinal mucosal immunity for protection against and/or to reduce the severity of the iridovirus infection. We found that fish (Rock bream) immunized orally with rMCP underwent successful induction of antibodies ($P < 0.05$) and were protected ($P < 0.001$) against viral challenge. These results suggest that oral administration of rMCP as an antigen is a useful method to implement a vaccine program against iridovirus and other marine viral diseases.

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1. Introduction

Iridoviruses in the genus *Megalocytivirus* (Iridoviridae) cause systemic disease in infected animals and are associated with high morbidity and mortality [1]. Since 1990, outbreaks of rock bream iridovirus have resulted in high mortality in cultured bream [2]. Unfortunately, no large-scale protective vaccine program or therapy is currently available, although some injectable methods are applicable. Furthermore, parenteral immunization methods are very stressful, and individual immunization of thousands of fish is very labor, time, and cost intensive.

The concept of using plants to produce high-value pharmaceuticals such as vaccines is 20 years old and is only now on the brink of realization as an established technology. Routine production of inexpensive vaccines in plants has been an idealistic and largely unrealized dream [3]. Plant-based expression systems have received much attention as an alternative platform for producing recombinant proteins because of their relatively low capital cost, easy scale-up, efficient storage, and they are risk-free for animal pathogen contamination [4]. Moreover, the potential of plant-based expression systems to be used as edible vaccines is one of their greatest advantages [3]. The major capsid protein (MCP) of fish iridovirus, which is well-conserved [5], has been used as an antigen in previous studies [6,7]. Our aim was to produce a codon-optimized recombinant MCP (rMCP) in rice callus, which could be provided as a feed additive to immunize fish. We evaluated its availability as an oral vaccine candidate against viral infection.

2. Materials and methods

2.1. Viral DNA preparation and MCP cloning

Standard DNA, RNA, and protein manipulations were performed as described previously [8,9]. All enzymes and the pGEM®-T Easy cloning vector were purchased from Promega (Madison, WI, USA). Viral DNA was prepared as described previously [7], following MCP gene amplification and cloning. The MCP gene (GenBank #AY849393) was codon-optimized according to rice codon usage data from the Kazusa DNA Research Institute. A series of overlapping primers encoding MCP with 80 mer with 20 mer overlaps as described in Table 1. F1, F2, and F3 oligonucleotides were combined by overlapping with each reverse primer pair. A polymerase chain reaction (PCR) amplification was performed for each pair, and the resulting fragment was combined with the neighboring overlapping PCR fragment, and the PCR reaction was performed again. Then, three PCR fragments were combined by overlapping PCR to yield the 1362 bp synthetic MCP (sMCP) gene. To fuse the signal peptide, the RAmy3D signal peptide (RAmy3Dsp) was amplified from PMYN44 [10] and combined with sMCP by overlapping PCR to yield 1527 bp RAmy3Dsp/sMCP. The resulting synthetic fragment was ligated into pGEM®-T Easy vector to generate pJKB12. pJKB12

* Corresponding author. Tel.: +82 62 530 2859; fax: +82 62 530 2857.
E-mail address: tjkim@chonnam.ac.kr (T.J. Kim).
1 Current address: Natural Bio-Materials, Wanju 565-904, Republic of Korea.

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Table 1
Primers used to optimize the major capsid protein (MCP) codon.

Primer name	Sequence (5′-3′)
IV(opt)-F1	ATGTCTGCcATCTCAGGTGCcAACGTgACCtcaGGcTTtATtGACATCTCtGCaTTcGATGCGATGGAGACtCACTTGTA
IV(opt)-R1	CTGTACCAtGAgCTcCtCACGGTCTCgCGaGCgAAGTAGGTCACGGCgTTaTCGCCtCCgTACAAGTGaGTCTCCATCGC
IV(opt)-R2	CTgAACTCCTGGCCGAAgTTAGCATGGCCAGTCTGTTTgGAgAGtGTcACtGGaGGCTTGCTGTACCAtGAgCTcCtCAC
IV(opt)-R3	GGGATCTTcACgCGgAGCCACACgTTgATGAGGTAaTCGCCtCCtCTaGCCACtGTCACgCTgAACTCCTGGCCGAAgTT
IV(opt)-R4	TTGTGCATCAAgTTgTCgCACCAGCGAATGTAGCTGTTCTCCTTGCTGGACGTGATcGAcGGGATCTTcACgCGgAGCCA
IV(opt)-R5	TCGCTGGTgAGtGTCTGaGCgACgAGGTCGTTgAAgctCACtGAgACtTCCTCcACgAGATTGTGCATCAAgTTgTCgCA
IV(opt)-R6	TTGTTGTAGCCAGAcTGTTTGCTtCCAGGCATCATGCAcGCaTTCCAgAAGTCgAGaAACTCGCTGGTgAGtGTCTGaGC
IV(opt)-R7	GCaGGCATgGTtTGACCaTTGGTGATtCCGCCCACCAGaTCagaGCGCATGCCAATCATCTTGTTGTAGCCAGAcTGTTT
IV(opt)-F2	AAtGGTCAaACcATGCCtGCCGCtTACCTcAAccTGCCgATTCCgCTGTTCTTcACaCGcGACACAGGCCTcGCcTTGCC
IV(opt)-R8	TCCTCCCAGCGCCtCAaCTTGAAGTGGATtCtCACCTCgTTGTAtGGCAGgctCACtGTcGGCAAgGCgAGGCCTGTGTC
IV(opt)-R9	TTgGCgAGGGTGACgGTgGAgATGGCCATGTCGGCCTGaGTagaCTGGCTGATcAagAGGTCCTCCCAGCGCCtCAaCTT
IV(opt)-R10	AacACgGCGTAtGTGCCCATCACGGACACGTTGGTCAacGCtGGcGCcACATTGCCgATGTTgGCgAGGGTGACgGTgGA
IV(opt)-R11	CACTGtTCgATCAGCATGgacCtGgaAGACTGaGCaACgACCTCgCGtTCCTCgCTcGTCAacACgGCGTAtGTGCCCAT
IV(opt)-R12	AaGTCaAGATGgACCAAGGAgTTGTCaACtGGaGTcACcGGCACtCttGGaGCaACCTGgCACTGtTCgATCAGCATGga
IV(opt)-R13	TTGCGaTGcGTcACgTTCTTcACgGCgAAGAACAAcGCCTTCACAGGaTGgaGAACCTCAaGTCaAGATGgACCAAGGA
IV(opt)-R14	GGgAGgTTCACCTTGTTGTTGACATACACcGGtgaGGCaGCGGTGTAgTTGgaTTGgACGTTGCGaTGcGTcACgTTCTT
IV(opt)-F3	AACAACAAGGTGAAcCTcCCgTTGCTcGCCACCAATCCgCTGTCtGAaGTcTCaCTCATcTACGAGAACACaCCTCGcCT
IV(opt)-R15	ATGCTcGGaGCgAAGTAGTAcGGaTCGACgGAgGTGAAGCAGTCcACgCCCATCTGGTGGAGgCGAGGtGTGTTCTCGTA
IV(opt)-R16	GGaTTGATgTTGCCCATGTCgAGCGTgTAgCAGTAGGTCATAACACCATCCATCTCAGGCATGCTcGGaGCgAAGTAGTA
IV(opt)-R17	TCgGACACCTTACAgGAgAGGGTcACGTTGGAgAGcCGGCCGTAGTTGGTCGAGCCCATcGGaTTGATgTTGCCCATGTC
IV(opt)-R18	TGGGCGACCGTGTAGCCtGTGCCGTTGCCTCCtCCtCCgGCaGCtGTcGTCTTGGCgTTGTCgGACACCTTACAgGAgAG
IV(opt)-R19	GCtCCGTCgGCgATCTTCATGATGTTGTGGTTcACgGCgATcACcACCAGcTCgAACTTcTGGGCGACCGTGTAGCCtGT
IV(opt)-R20	GGTACCTCATCACAACTCGTCTTTgAGGATcGGGAAGCCaGCAGCtCCGTCgGCgATCTTCAT
3DspF1	TTGGATCCATCAGTAGTGGTTAGCAGCAAC
3D-IV-F	CAAGCCCAGGTCCTCGAGACTCACTTGTAC
3D-IV-R	GTACAAGTGAGTCTCGAGGACCTGGGCTTG

was digested with *Bam*HI and *Sac*I, then sub-cloned into the same sites of the pMYN75 plant expression vector, which contained the sugar starvation inducible rice α-amylase 3D promoter (pRAmy3D) and a 3'-untranslated region (UTR) terminator [10]. The resulting recombinant vector, pJKB13 (Fig. 2B), was utilized to transform the rice calli via microprojectile bombardment.

2.2. Plant transformation and screening of transgenic rice calli

Rice calli (*Oriza sativa* L. cv. Dongjin) were prepared and transformed by particle bombardment-mediated transformation [11]. Control plants were obtained by transformation with the pMYN75 empty binary vector. Transgenic calli were selected on 50 mg/l hygromycin B. Transgenic calli harboring the sMCP gene were used to establish cell suspension cultures. These cultures were grown at 25 °C in the dark in N6 + S media containing 2,4-D (2 mg/l), kinetin (0.02 mg/l) and 3% sucrose and on a shaking incubator with a rotation speed of 110 rpm [12]. The medium was changed every 7 days for sub-culturing. The N6 + S medium was removed from the cell suspension by aspiration, and the cells were transferred to fresh N6-S medium (sucrose-free medium) at 10% (weight of wet cells/volume of N6-S medium) density to induce MCP expression.

2.3. Northern and Western blot analyses

Total RNA from each cell line was extracted from the rice callus with TRI Reagent® (MRC, Cincinnati, OH, USA), according to the manufacturer's instructions. The RNA pellet was dissolved in 100 μl DEPC-treated water. Total RNA (30 μg) was run on a 1.2% formaldehyde agarose gel and transferred to a Hybond N+ membrane (GE Healthcare, Piscataway, NJ, USA). sMCP fragments were labeled with [α-^{32}P]-dCTP using Prime-a-gene labeling system (Promega) and then hybridized for 16 h at 65 °C. The membranes were washed twice in 2× SSC containing 0.2% SDS for 20 min and subsequently washed twice in 0.1× SSC containing 0.2% SDS for 20 min. Autoradiography was then performed using X-ray film. Spot intensity corresponded to the amount of the transcript. After a 7-day incubation of the transgenic rice cells in N6-S liquid medium, the cells were harvested, ground into a fine powder with mortar and pestle in liquid nitrogen, and total protein was extracted in protein extraction buffer [13]. After centrifugation, the resulting supernatant was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, stained with Coomassie Brilliant Blue, and transferred to a PVDF membrane (GE Healthcare) using a semi-dry transfer unit (BioRad, Hercules, CA, USA) at 5 V for 60 min. The membrane was blocked overnight with 5% skimmed milk in PBS (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, and 135 mM NaCl, pH 7.4) at 4 °C and was then probed with anti-MCP monoclonal antibody (11A40 hybridoma supernatant, which was prepared separately) diluted 1:10 in PBS. Subsequently, the blot were incubated with goat anti-mouse IgG-AP conjugated (Sigma, St. Louis, MO, USA) for 60 min at room temperature. The NBT/BCIP substrate (Sigma) was used for color development.

Table 2
Immunization design and protection study results following viral challenge.

Groups	Feed details (oral immunization every Monday at one week intervals)	Trial number (fish)	No. of survivors (%)
#1	Expended pellet + Callus (W/T) 10% in 2.5 g of feed/fish, 3 treatments at 1-week intervals	10	0 (0%)
#2	Expended pellet + Callus (transformant) 10%* in 2.5 g of feed/fish, 3 booster treatments at 1-week intervals	10	8 (80%)**
#3	Expended pellet + Callus (transformant) 30%* in 2.5 g of feed/fish, 3 booster treatments at 1-week intervals	10	9 (90%)***

*10% contains "10 μg" of rMCP/10 g fish body weight, while 30% contains 30 μg. Two-sided *P*-value is 0.0007** or 0.0001***, which is extremely significant based on Fisher's exact test.

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