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Immune Characterization of C-Type Lectin-Like Protein in Rainbow Trout

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qRT-PCR;
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Abstract

C-type lectins play crucial roles in innate immunity and pathogen recognition. In this study, a C-type lectin-like protein (*Om-CTLTP*) gene was cloned from rainbow trout, *Oncorhynchus mykiss* by rapid amplification of cDNA ends (RACE) PCR. The full-length cDNA of *Om-CTLTP* was composed of 1,153 bp with a 723 bp open reading frame (ORF) that encodes a 240-residue protein with an estimated molecular mass of 27 kD and a predicted isoelectric point of 5.48. The deduced amino acid sequence of *Om-CTLTP* possessed all conserved features crucial for the fundamental structure, such as the four cysteine residues (Cys¹²⁸-Cys²³⁰, Cys²⁰⁷-Cys²²²) formed the carbohydrate recognition domain (CRD) internal disulfide bridges and the potential Ca²⁺/carbohydrate-binding sites. *Om-CTLTP* contains a transmembrane domain and a single CRD. *Om-CTLTP* showed high similarity of 44%-84% with C-type lectins from other fish species. Quantitative RT-PCR (qRT-PCR) analysis showed *Om-CTLTP* mRNA was constitutive expression in gill, heart, liver, kidney, intestine, spleen and muscle tissues in healthy fish and up-regulated under infection by bacteria or viruses. After being infected by *Aeromonas hydrophila* or viral haemorrhagic septicemia virus (VHSV), the expression of *Om-CTLTP* were up-regulated from 12 h post-injection and lasted until the fish became moribund ($P<0.05$). The recombinant *Om-CTLTP* CRD can agglutinate both Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*A. hydrophila*) in a Ca²⁺-dependent manner. Further analysis showed that the recombinant *Om-CTLTP* can directly bind to all tested microorganisms (*S. aureus*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Escherichia coli*, *Vibrio natrie-gens*, *Vibrio parahaemolyticus*, and *A. hydrophila*). These results suggest that *Om-CTLTP* may have important roles in anti-bacterial and anti-viral immunity of rainbow trout.

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Иммунная характеристика лецитиноподобного белка типа С у радужной форели

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Ключевые слова:

лектин типа С;
лектин-подобный белок типа С радужной форели;
ОТ-ПЦР;

Реферат

Лектины типа С играют определяющую роль в формировании врожденного иммунитета и распознавании патогенов радужной форели. В ходе данного исследования, из радужной форели (*Oncorhynchus mykiss*) был выделен лектин-подобный белок типа С (аббр. CTLTP) методом ПЦР с быстрой амплификацией комплементарной ДНК (кДНК). Идентифицированная кДНК лектин-подобного белка типа С радужной форели состояла из 1153 пар нуклеотидных оснований, из которых 723 пары с от-

экспрессия иРНК;
врожденный
иммунитет

крытой рамкой считывания (аббр. ORF) кодировали остаток 240 белка с расчетной молекулярной массой 27 кД и изоэлектрической точкой 5,48. Установленная аминокислотная последовательность лектин-подобного белка типа С радужной форели обладала свойствами, необходимыми для базовой структуры, такими как четыре цистеиновых остатка (Cys¹²⁸–Cys²³⁰, Cys²⁰⁷–Cys²²²), образующих доменоносители углеводов (CRD); внутренние дисульфидные связи и участки потенциального связывания Ca²⁺ и углеводов. Лектин-подобный белок типа С радужной форели содержит трансмембранный домен и одиночный доменоноситель углеводов и продемонстрировал высокое (44–84 %) сходство с лектинами С-типа других видов рыб. Количественный анализ ОТ-ПЦР (qRT-PCR) показал, что лектин-подобный белок типа С радужной форели является результатом конститутивной экспрессии в жаберных, сердечных, печеночных, почечных, кишечных, селезеночных и мышечных тканях здоровых рыб и активируется при инфицировании бактериями или вирусами (патогенами). После заражения гидрофильной аэромонадой (*Aeromonas hydrophila*) или вирусной геморрагической септициемией (VHSV) количество лектин-подобного белка типа С радужной форели активировалось в течение 12 ч после дополнительного впрыска и продолжалось до момента гибели рыбы ($P < 0,05$). Рекombинатные доменоносители углеводов лектин-подобного белка типа С радужной форели могут агглютинировать как грамположительные (золотистый стафилококк), так и грамотрицательные бактерии (*A. hydrophila*) путем связывания Ca²⁺. Дальнейший анализ показал, что рекомбинантный лектин-подобный белок типа С радужной форели может напрямую связываться со всеми тестируемыми микроорганизмами (*S. aureus*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Escherichia coli*, *Vibrio natriegens*, *Vibrio parahaemolyticus*, *A. hydrophila*). Эти результаты свидетельствуют о том, что лектин-подобный белок типа С радужной форели может играть значительную роль в антибактериальном и противовирусном иммунитете радужной форели.

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Introduction

Lectins are a superfamily of carbohydrate-binding proteins which are widely distributed throughout the animal kingdom (Holmskov et al., 2003). Based on the structure of the carbohydrate-recognition domains (CRDs), structural fold, distinct sugar specificities, functions and requirement of divalent cations, lectins are classified as C-type, F-type, I-type, L-type, P-type, R-type and S-type lectins and others (Bianchet et al., 2010; Lai et al., 2013; Mukherjee et al., 2014). Numerous lectins involved in defense against various infectious pathogens and reproduction have been identified and characterized within tissues and eggs of several species of fish, including Atlantic salmon (*Salmo salar*) (Zhang et al., 2001), rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*) (Bergsson et al., 2005), common carp (*Cyprinus carpio*) (Gonzalez et al., 2007), Japanese flounder (*Paralichthys olivaceus*) (Kondo et al., 2007), ayu (*Plecoglossus altivelis*) (Watanabe et al., 2008), Nile tilapia (*Oreochromis niloticus*) (Argayosa and Lee, 2009), sea bass (*Dicentrarchus labrax*) (Salerno et al., 2009), turbot (*Scophthalmus maximus*) (Zhang et al., 2010), bighead carp (*Aristichthys nobilis*) (Pan et al., 2010), grass carp (*Ctenopharyngodon idellus*) (Liu et al., 2011), channel catfish

(Zhang et al., 2012), rock bream (*Oplegnathus fasciatus*) (Park et al., 2012), tongue sole (*Cynoglossus semilaevis*) (Zhou and Sun, 2015), and so on.

C-type lectins were originally defined as a group of Ca²⁺-dependent (C-type) carbohydrate-binding proteins (Drickamer, 1988). Most C-type lectins consist of one CRD, whereas some have several or more CRDs. Each CRD usually possesses a characteristic double-loop stabilized by two conserved disulfide bridges (Zelensky and Gready, 2005). As C-type lectins can recognize and bind the specific carbohydrate patterns present on the surface of invading microorganisms, they are considered as pattern recognition receptors (PRR) that play important roles in non-self-recognition and clearance of invading pathogens (Poisa-Beiro et al., 2009). Besides functioning as pattern recognition receptors, C-type lectins can take part in various kinds of immune responses, including cytokine release (Tsutsui et al., 2007; Wei et al., 2010), phagocytosis (Luo et al., 2006), encapsulation and melanization (Ling and Yu, 2006), cell adhesion (Odintsova et al., 2001), aggregation (Zhang et al., 2009), antibacteria, fungi or virus activity (Willment and Brown, 2008; Liu et al., 2011; Huang et al., 2013), activating the comple-

ment pathway, activating apoptosis by a complement-independent style (Zhang et al., 2012), and many other biological responses.

Rainbow trout are one of the most intensively studied fish species because of their importance as a food and sport fish (Young et al., 1998). To efficiently manage disease problems and provide knowledge for long-term enhancement in rainbow trout aquaculture production, the rainbow trout immune response to pathogen exposure should be studied thoroughly. The C-type lectin of teleost fish are important as tools for research and as targets for novel therapeutants. However, at present our understanding on the immunity of rainbow trout is poor. Considering that a number of various C-type lectin have been isolated and characterized in other fishes, C-type lectins may be primarily involved in rainbow trout immunity against a great variety of pathogens. In this study, we reported the cloning and characterization of a full-length cDNA of a C-type lectin-like protein gene from *O. mykiss* (designated as *Om-CTLP*). Furthermore, *Om-CTLP* mRNA expression profiles in different tissues and following both viral haemorrhagic septicemia virus (VHSV) and *Aeromonas hydrophila* (*A. hydrophila*) infection, and characteristics of recombinant *Om-CTLP* protein following immunity response were also investigated.

Materials and Methods

O. mykiss with body weight of 150–200g were obtained from an aquatic product farms and acclimatized to laboratory pathogen-free conditions for two weeks. Before experiments, the pathogen-free status of the fish was confirmed by analysis for bacterial, parasitic and viral pathogens. For challenge experiments with VHSV or *A. hydrophila*, fish were injected with 10 µl PBS per gram body weight, 10 µl VHSV suspended in PBS (2×10^8 PFU ml⁻¹) or 10 µl *A. hydrophila* resuspended in PBS ($OD_{600}=0.2$) intraperitoneally. The control group received individually an injection of 10 µl PBS solution per gram body weight. The non-injected fish were used as blank group. Five individuals were killed and tissues including gill, heart, liver, kidney, intestine, spleen, and muscle were collected at 0, 12, 24, 36, 48, 72, 96, 144 and 192 h after injection. Tissue samples were collected and frozen at –70 °C until analysis.

All fresh tissues (50–100 mg) were homogenized in the Trizol Reagent (Invitrogen, Carlsbad, USA), and total RNA was prepared according to the manufacturer's instruction, and then was digested with RNase-free DNase I (Promega, Madison, USA) to remove genomic DNA contamination. The cDNA was synthesized from 5 µl of total RNA by ExScript RT reagent kit (TaKaRa, Dalian, China) with oligo d (T) 18 as primer according to the manufacturer's instruction.

Using the cDNA prepared above as a template, highly conserved region of *Om-CTLP* was amplified with a pair of degenerate primers (*Om-CTLP-F* and *Om-CTLP-R*, Table 1) based on known C-type lectin gene sequence alignment from other species. The PCR amplification conditions of temperature profile was 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 50 s, and the final extension step at 72 °C for 10 min. The PCR products were gel-purified and cloned into PMD18-T Vector (TaKaRa, Dalian, China) and sequenced.

The 3'-RACE and 5'-RACE were performed using the TaKaRa 3'-Full RACE Kit and the 5'-Full RACE Core Set Kit according to the kit manufacturer's manual, respectively. A total of 5 µg RNA was reversely transcribed using the MMLV reverse transcriptase (TaKaRa) to obtain the first-strand cDNA. The 3' and 5' region sequences of *Om-CTLP* were both amplified by nested PCR using 3'-RACE primers (3' *Om-CTLP-1* and 3' *Om-CTLP-2*, Table) and 5'-RACE primers (5' *Om-CTLP-1* and 5' *Om-CTLP-2*, Table) which were based on the above amplified highly conserved region of the *Om-CTLP* cDNA fragment, respectively. The full-length cDNA sequences were confirmed by sequencing the PCR product amplified by primers *FL-Om-CTLP-F* and *FL-Om-CTLP-R* (Table) within the predicted 3' and 5' untranslated regions, respectively.

Results

A highly conserved core region sequence of 328 bp was amplified with primers mentioned above, and using the 3'-RACE and 5'-RACE method, two cDNA 3' ends of 461 and 227 bp, two cDNA 5' ends of 484 and 267 bp were amplified by 3'- and 5'-RACE as well, respectively. The five nucleotide sequences above were assembled with DNAMAN software, and a 1,153 bp of single contig was acquired. To confirm that the resulting 1,153 bp consensus sequence represented a single mRNA of rainbow trout, primers *FL-Om-CTLP-F* and *FL-Om-CTLP-R* were designed spanning the coding region. Using these primers, a single 853 bp product was amplified. The full-length cDNA of *Om-CTLP* was 1,153 bp, containing a 5' untranslated region (UTR) of 62 nucleotides, a 723 bp open reading frame (ORF) which encoded 240 amino acids, and a 3' UTR of 368 nucleotides containing a canonical polyadenylation signal site (AATAAA) 18 bp upstream of the polyadenylation tail. The full-length cDNA sequence of *Om-CTLP* has been deposited in the GenBank database under accession number FJ607865. Blastp analysis indicated that the deduced protein sequence has significant homology to previously characterized C-type lectin proteins.

The expression of *Om-CTLP* mRNA in various tissues at 36 h post-injection was analyzed using real-time RT-PCR to assess the levels of *Om-CTLP* tran-

Oligonucleotide primers
Олигонуклеотидные праймеры

Name	Sequence (5'–3')	Application
<i>Om-CTLP-F</i>	GGGTGGYTTTCNTGTACTRGT	Gene cloning
<i>Om-CTLP-R</i>	GGACYACACVGNCTCRCT	
5' <i>Om-CTLP-1</i>	TTGCTGTGCAGGATGCTAAG	5'RACE
5' <i>Om-CTLP-2</i>	TACTCAGATCCAACTTCAGG	
3' <i>Om-CTLP-1</i>	CAGAGTAACACTCATGCCTG	3'RACE
3' <i>Om-CTLP-2</i>	TGTAAACGCGGTGGTAATCA	
<i>FL-Om-CTLP-F</i>	TGATTGGCCTCTTGGTATCC	Confirming sequence
<i>FL-Om-CTLP-R</i>	AATGCACGTTAGGGAATGC	
<i>Q-Om-CTLP-F</i>	ACTGGAGATGGGTGGACAAC	qPCR
<i>Q-Om-CTLP-R</i>	ATGCGGTGGTAGAGGAATTG	
<i>Q-β-actin-F</i>	TATGTGCAAAGCCGGATTCCG	qPCR
<i>Q-β-actin-R</i>	GTGACGATGCCATGCTCAAT	
<i>RE-Om-CTLP-F</i>	TACTCAGAATTCTGTCTGAGGGGTGGCTTCAT	Recombination
<i>RE-Om-CTLP-R</i>	TACTCACTCGAGTCAAGGAGAAGTAGTGGGGAA	

Note: Y=C/T; R=A/G; V=A/G/C; and N=A/G/C/T; restriction enzymes of EcoRI and XhoI sites were underlined.

scripts. *Om-CTLP* mRNA was detected in all tissues tested at low expression levels, and was dramatically up-regulated after bacterial or viral injection ($P<0.05$) (Fig. 4). In two infected groups, the strongest expression were found all in the spleen, liver and kidney (Fig. 1).

Each bar represented the mRNA expression level of target gene relative to those in control group, *Om-CTLP* mRNA expression was measured in the gill, heart, liver, kidney, intestine, spleen, and muscle of rainbow trout 36 h after infection with VHSV or bacteria. The control groups were injected with PBS. Data are the means $\pm$ SE of five rainbow trouts. Asterisks represent significant difference between treated group and control ($*P<0.05$). Error bars showed the standard error.

The *Om-CTLP* expression in spleen was most up-regulated 8.5-fold by bacterial stimulation or 6.9-fold by viral. For fish VHSV disease, liver is one of the main affected organs (Liu et al. 2011), and *Om-CTLP* in liver was the second most up-regulated 8.4-fold by bacterial stimulation or 6.6-fold by viral. Therefore, liver was selected to investigate the temporary expression profile of *Om-CTLP* mRNA after microorganisms challenge.

To analyze the effects of bacterial or viral stimulation on *Om-CTLP* gene expression in liver tissue, five fish from each group were dissected at each time point (0, 4, 12, 24, 36, 48, 72, 96 and 144 h post-injection) and qRT-PCR assays were employed. No significant differences were observed between control and blank groups at any of the time points ($P>0.05$) (data not shown). In treated groups, the *Om-CTLP* mRNA expressions levels were significantly in-

creased at 12 h post-infection, reached highest level at 36 h post-infection ($P<0.05$), and then gradually decreased, but they still kept relatively high levels until the fish became moribund ($P<0.05$) (Fig. 2).

The control groups were injected with PBS, while the treated groups were infected with VHSV or *A. hydrophila*. Asterisks represent significant difference between treated group and control ($*P<0.05$). Error bars showed the standard error.

The data obviously showed that *Om-CTLP* expression levels were stimulated in infected animals, for both bacterial and viral stimulation ($P<0.05$). The temporary expression profile of *Om-CTLP* between bacterial and viral stimulation was similar, just up-regulation extent was relatively less in viral stimulation.

The agglutination activity against *S. aureus* and *A. hydrophila* of *Om-CTLP* was tested. In the presence of Ca^{2+} , *Om-CTLP* can agglutinate Gram-positive and Gram-negative bacteria tested (Fig. 7). No agglutination activity was observed in the BSA plus Ca^{2+} control (Fig. 3).

A Gram-positive (*S. aureus*) and a Gram-negative bacteria (*A. hydrophila*) were shown.

Discussion

C-type lectins play crucial roles in innate immunity and pathogen recognition. In this study, a C-type lectin-like protein (*Om-CTLP*) gene was cloned from rainbow trout, *Oncorhynchus mykiss* by rapid amplification of cDNA ends (RACE) PCR. The full-length cDNA of *Om-CTLP* was composed of 1,153 bp with a 723 bp open reading frame (ORF) that encodes a 240-residue protein with an estimated molecular mass of 27 kD and a predicted isoelectric point of

Fig. 1. Tissue distribution of Om-CTLp transcripts in rainbow trout

Рис. 1. Распределение лектин-подобного белка типа С в соединительной ткани радужной форели

Fig. 2. The temporary expression profile of Om-CTLp mRNA after microorganisms challenge in liver using qRT-PCR

Рис. 2. Временный профиль экспрессии иРНК лектин-подобного белка типа С радужной форели после введения микроорганизмов в печень с помощью ОТ-ПЦР

Fig. 3. Agglutination of bacteria by Om-CTLp

Рис. 3. Агглютинация бактерий лектин-подобным белком типа С у радужной форели

5.48. The deduced amino acid sequence of *Om-CTLP* possessed all conserved features crucial for the fundamental structure, such as the four cysteine residues (Cys¹²⁸-Cys²³⁰, Cys²⁰⁷-Cys²²²) formed the carbohydrate recognition domain (CRD) internal disulfide bridges and the potential Ca²⁺/carbohydrate-binding sites. *Om-CTLP* contains a transmembrane domain and a single CRD. *Om-CTLP* showed high similarity of 44–84 % with C-type lectins from other fish species. Quantitative RT-PCR (qRT-PCR) analysis showed *Om-CTLP* mRNA was constitutive expression in gill, heart, liver, kidney, intestine, spleen and muscle tissues in healthy fish and up-regulated under infection by bacteria or viruses. After being infected by *Aeromonas hydrophila*

or viral haemorrhagic septicemia virus (VHSV), the expression of *Om-CTLP* were up-regulated from 12 h post-injection and lasted until the fish became moribund ($P < 0.05$). The recombinant *Om-CTLP* CRD can agglutinate both Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*A. hydrophila*) in a Ca²⁺-dependent manner. Further analysis showed that the recombinant *Om-CTLP* can directly bind to all tested microorganisms (*S. aureus*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Escherichia coli*, *Vibrio natriegens*, *Vibrio parahaemolyticus*, and *A. hydrophila*). These results suggest that *Om-CTLP* may have important roles in anti-bacterial and anti-viral immunity of rainbow trout.

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