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Identification of antigenic proteins of lymphocystis disease virus (LCDV) by MALDI-TOF mass spectrometry

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The antigenic proteins of Lymphocystis disease virus (LCDV) from tumors of olive flounder, *Paralichthys olivaceus,* are described following characterization by mass spectrometry. In SDS-PAGE, predominant protein bands were observed at 114, 88, 70, 54, 52, 47, 42 and 24 kDa. Western blot analysis showed that antisera reacted strongly at molecular weights of 114, 67 and 54 kDa, and reacted weakly at molecular weights of 74, 70, 36, 24 and 22 kDa. In the identification of LCDV antigenic proteins by matrix-assisted laser desorption ionization (MALDI) TOF mass spectrometry, 10 of 14 excised bands consisted mostly of proteins with amino acid sequences that matched LCDV-C (lymphocystis disease virus isolate China) ORFs. Strong antigens with molecular weights of 114, 67 and 54 kDa were identified as LDVICp236 (chromosome segregation ATPase), LDVICp033 (membrane bound metallopeptidase) and LDVICp157 (hypothetical protein), respectively. Minor antigens with molecular weights of 70, 36, 24 and 22 kDa proteins were identified as LDVICp160 (acetyl-coA hydrolase), LDVICp213 (hypothetical protein), LDVICp039 (hypothetical protein) and LDVICp213 (hypothetical protein). However, the major capsid protein (LDVICp043) did not react with the polyclonal antibody.

Key words: Lymphocystis disease virus (LCDV), Antigenic proteins, MALDI-TOF mass spectrometry, Olive flounder

Lymphocystis is a chronic, benign viral disease that affects approximately 100 species of marine and freshwater fish (Wolf, 1988). Fish affected with lymphocystis disease (LCD) exhibit characteristic external signs with clusters consisting of enormously hypertrophic dermal cells on their skin and fins. The hypertrophic cells, generally referred to as lymphocystis cells, posses thick hyaline capsules, enlarged nuclei, and prominent basophilic DNA cytoplamic inclusions (Peters and Schmidt, 1995).

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According to the International Committee on Taxonomy, *Iridoviridae* have been subdivided into five genera including *Chloriridovirus*, *Iridovirus*, *Megalocytivirus*, *Ranavirus* and *Lymphocystivirus*. Lymphocystis disease virus (LCDV), the causative agent of LCD, belongs to the genus *Lymphocystivirus* within the family *Iridoviridae*, and possesses an icosahedral capsid of approximately 200 ± 50 nm in diameter. The LCDV capsid is double-layered, with an outer envelope and a fringe consisting of fibril-like external protrusions (Walker, 1962; Zwillenberg and Wolf, 1968; Wolf, 1988). The viral genome is a single linear dsDNA molecule, the structure of which is cir-

cularly permuted and terminally redundant (Darai *et al*., 1983; Schnitzler *et al*., 1987; Schnitzler and Darai, 1993). The complete gene sequences of two LCDV strains, LCDV-1 isolated from flounder, *Platichtys flesus* in Europe and LCDV-C isolated from olive flounder, *Paralichtys olivaceus* in China, have been determined (Tidona and Darai, 1997; Zhang *et al*., 2004).

In characterizing any virus, its structural proteins and antigenic proteins are particularly important because they are the first molecules to interact with the host, and therefore play critical roles in cell targeting as well as triggering host defenses. However, it is not easy to exhaustively identify all the structural proteins and antigenic proteins with conventional tools alone. In the case of LCDV, the virions have a complicated sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profile, and so far, SDS-PAGE coupled with western blotting has determined only the molecular weights of structural proteins and antigenic proteins (Iwamoto *et al*., 2002; Cheng *et al*., 2006). A more comprehensive, global approach is provided by proteomics. In the field of functional genomics, proteomics is defined as the large-scale analysis of gene function (Pandey and Mann, 2000). For this kind of analysis, proteomics combines MALDI-TOF mass spectrometry with database searches of sequenced genomes. Viral proteins are amenable to this approach, since the complete genome sequences of many viruses are known, and viral particles have consistent and stable profiles. Using this approach, this study combined SDS-PAGE separation and western blotting with MALDI-TOF mass spectrometry, with focus on identifying antigenic proteins of LCDV.

Materials and Methods

Virus sample

Olive flounder exhibiting lymphocystis nodules on their body surfaces and fins, were collected from a culture farm in Wando, Korea in July 2007. The nodules were separated from the body surface using the back side of a knife and transported on ice to the laboratory. The samples were kept in a deep freezer at -80°C until use.

Purification

Viral suspensions were generated by homogenizing the lymphocystis cells adding 10 times volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The homogenate was passed through a 0.45 μm filter. The filtered viral suspensions were centrifuged for 1 h at 60000 *g* at 4°C using an ultracentrifuge (Hitachi himac CP 100α, P100AT Rotor) (Hitachi, Japan) (referred as 'concentrated'). Purification of the concentrated LCDV was performed under conditions similar to the protocol of Cheng *et al*. (2006). The concentrated LCDV were re-suspended in 500 μl of TE buffer and were then left overnight at 4°C. The virus (250 μl) was overlaid on a discontinuous gradient consisting of 20, 35 and 50% sucrose (in TE buffer) steps, and centrifuged for 1.5 h at 70000 *g*. Two viral bands (the 'upper band' between 20 and 35% sucrose; the 'lower band' between 35 and 50% sucrose) were harvested and diluted again in TE buffer and then centrifuged for 1 h at 115000 *g*. The pellets were re-suspended in TE buffer. The purified LCDV was stored at -80°C until use.

Immunization of rabbits

The protein concentrations of three types of LCDV antigens (concentrated virus and virus from the upper band and lower band) were adjusted to 50μg/500 μl in phosphate buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 0.88 mM KH_2PO_4, 7.6 mM $NaHPO_4$, pH 7.2). Each antigen was mixed with an equal volume (500 μl) of Freund's complete adjuvant (Sigma Chemical Co., USA) and injected subcutaneously into the backs of New Zealand white rabbits. Two weeks later, secondary immunizations were given to the rabbits in the same manner as the first immunization with

Freund's incomplete adjuvant (Sigma Chemical Co., USA). After 2 weeks, final immunizations were given into the ear vein with 500 μl of PBS (pH 7.2) containing 50μg of viral protein. Serum was collected from blood taken four days after the final injection in the marginal ear vein, and then stored at -80°C until use. Pre-immunized serum was used as a negative control.

Enzyme linked immunosorbent assay (ELISA)

ELISA was carried out according to the method described by Kim *et al*. (2007) with some modifications. Briefly, a 96-well immuno plate (Nunc, Denmark) was coated with filtered homogenate in TE buffer (1:10 dilution) over night at 4°C. Then, it was rinsed three times with PBS-Tween (PBS with 0.05% Tween 20), and blocked with 5% skim milk resolved in PBS (SM-PBS) at 25°C for 30 min. Each rabbit antiserum produced from the 3 different types of antigens (concentrated virus and virus from the upper band and lower band) were 2 fold diluted (1:40 to 1:20480) in 5% SM-PBS and incubated at 37°C for 1 h. Unimmunized rabbit antiserum was used as the negative control. The plate was rinsed and incubated with goat anti-rabbit immunoglobulin HRP conjugate (Dako) at 25°C for 30 min. After rinsing, peroxidase enzyme substrate was added. The color reaction was terminated after 30 min using 2N H_2SO_4. Absorbance was measured by a spectrophotometer (Bio-Rad, USA) at a wavelength of 492 nm.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) was performed under conditions similar to those of Laemmli, (1970). Filtered LCDV homogenate, concentrated LCDV, and the two purified products of LCDV (upper band and lower band) were mixed with 5X sample buffer (5% SDS, 5% mercaptethanol, 0.05% bromophenol blue) (Sigma, USA) and boiled in water for 5 min. The proteins mixed with SDS-PAGE sample buffer were separated by SDS-PAGE on 8 and 12% polyacrylamide gel at 50 mA, and then the gel was stained with 0.5% Coomassie Brilliant Blue R-250 (Acros, New Jersey, USA). The same set of SDS-PAGE gel was used for western blot analysis. Molecular weight markers (BenchMark^{TM} protein ladder, Invitrogen, USA) and pre-stained markers (SeeBlue® Pre-Stained Standard, Invitrogen, USA) were applied to compare the SDS-PAGE and western blot profiles.

Western blot analysis of viral proteins

The viral proteins of the filtered virus preparation were blotted onto 0.45 μm pore nitrocellulose membranes (Bio-Rad, USA) for 45 min at 75 V by a wet-type transblotter (Hoefer, USA). The membranes were blocked with blocking solution (Invitrogen, USA) for 30 min on a rotary shaker. After rinsing twice for 5 min in distilled water, the membranes were incubated for 1 h in the 3 types of antiserum diluted 1:640 in blocking solution. The membranes were washed for 5 min in an antibody wash solution (concentrated buffered saline solution containing detergent, Invitrogen, USA), which was repeated 3 times. After two additional rinsings for 2 min in distilled water, secondary antibody (alkaline phosphatase conjugated anti-rabbit, Invitrogen, USA) was applied for 30 min at room temperature and the membranes were washed 3 times for 5 min in the antibody wash solution. The membranes were then stained with NBT/BCIP solution (Invitrogen, USA) and staining was stopped by distilled water.

Identification of antigenic protein bands

The SDS-PAGE profile showed approximately 25 protein bands, and the bands between 40 and 100 kDa were very close to each other. To confirm the antigenic proteins, the blotted nitrocellulose membranes were stained with Ponceaus S staining solution [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid], and then the strong stained bands and were pointed out with a pencil and photos were taken. The Coomassie

Brilliant Blue stained bands on SDS-PAGE gel, Ponceaus S stained bands, and western blot positive bands were compared. Next, to confirm the antigenic proteins, candidate antigen bands on the SDS-PAGE gel were cut by a sharp knife and each band was inserted into a new comb in SDS-PAGE gel. SDS-PAGE and western blotting were conducted again, and then the antigenic proteins were confirmed by western blot reactivity.

Gel extraction

The bands of interest were excised manually and transferred into microcentrifuge tubes. For rehydration, the gel pieces were incubated in 100 mM ammonium bicarbonate and 10 mM DTT, and then alkylated in 100 mM ammonium bicarbonate and 55 mM iodoacetamide for 40 min at room temperature under dark conditions. After dehydration in acetonitrile, the gel pieces were dried under a vacuum, and the samples were digested with sequencing grade trypsin overnight at 37°C in 50 mM ammonium bicarbonate. The trypsinized gel pieces were extracted through a repeated process of hydration-dehydration and sonication. The supernatants were transferred into new tubes and then dried completely under a vacuum for 6 h. The resulting tryptic peptides were dissolved in 0.5% trifluoroacetic acid (TFA) solution, and then desalted using ZipTipC18 tips (Millipore, Bedford, MA). The peptides were eluted directly onto the MALDI target plate.

MLDI-TOF mass spectrometry

All mass spectra were acquired at a positive reflector mode by a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). External calibration was performed using a standard peptide mixture of des-Arg Bradykinin, Angiotensin I, Glu-Fibrino-peptid B, Adrenocortico-tropic hormone (ACTH) clip 1-17, ACTH clip 18-39, and ACTH clip 7-38. Internal calibration was also performed using two autolysis peaks of trypsin ([M+H]+ = 842.5099 and 2211.1046). When the protein spots were not identified by peptide mass fingerprinting (PMF), the fragmentation patterns of tryptic peptide molecular ions ([M+H]+) were analyzed by MS/MS methods to obtain their partial sequences using the MALDI-TOF/TOF technique. The protein bands identified by PMF were also confirmed by the MALDI TOF/TOF technique. All samples were irradiated with UV light (355 nm) from an Nd: YAG laser with a repetition rate of 200 Hz. One thousand and 3000 laser shots were averaged to normal mass spectra and MS/MS spectra, respectively. The samples were analyzed at 25 kV of source acceleration voltage with two-stage reflection in MS mode. In the MS/MS experiment, collision energy, which was defined by the potential difference between the source acceleration voltage (8 kV) and the floating collision cell (7 kV), was set to 1 kV.

Database search and protein identification

The proteins were identified by searching the NCBI nonredundant database using MASCOT Peptide Mass Fingerprint software (Matrixscience, London). All mass spectra were searched for within the rodent species database or in the all entry database. The search parameters were considered to allow modifications of N-terminal Gln to pyroGlu, oxidation of methionine, N-terminus protein acetylation, carbamidomethylation of cysteine and acrylamide modified cysteine. The criteria for positive identification of the protein were set as follows: (i) minimum for matching peptide masses, (ii) 50 ppm mass accuracy and (iii) molecular weight, and pI obtained from image analysis. For the MS/MS search, fragmentation of a selected peptide molecular ion peak was used to identify the protein by searching the NCBI nonredundant database using the MASCOT MS/MS ion search program.

Results

Virus purification

Two prominent bands were obtained in sucrose

gradients after ultracentrifugation. One band located between the 20 and 35%, and the other was in between the 35 and 50% sucrose concentrations (lower band).

Enzyme linked immunosorbent assay (ELISA)

The OD_{492} values for the antiserum against the 3 types of LCDV antigens ranged from 3.43 to 0.305 (Fig. 1). The highest OD value around 3.4 was sustained at up to 320 times serum dilution. The ELISA values against the non-immunized rabbit serum ranged from 1.182 to 0.032. A nonspecific reaction was shown at 40 to 320 times dilution for the non-immunized control serum. The 640 times dilution was chosen for the western blot analysis because it had high LCDV reactivity with less non-specific immune response.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

In SDS-PAGE, the 'concentrated' LCDV (by ultracentrifugation at 60000 *g*, 1 h) had more protein bands than the purified virus. The purified virus from the upper band and lower band exhibited similar patterns. The sizes of the predominant protein bands observed in all three samples were114 (band no. 1), 88 (band no. 4), 70 (band no. 6), 54 (band no. 8), 52 (band no.9), 47 (band no.11), 42 and 24 kDa (band no. 13) (Fig. 2).

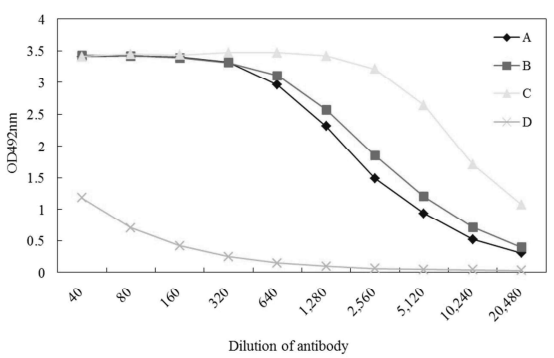

Fig. 1. ELISA values (optical density, OD) versus dilutions of different sera. Antiserum against lymphocystis disease virus collected from the virus band around 50% sucrose (▲), 30% sucrose (■) after ultracentrifugation and concentrated virus before loading on sucrose gradient (◆). Pre-immunized rabbit serum was used as negative control (X).

Western blot reactivity of LCDV-polyclonal rabbit antisera

In western blot analysis, the reaction activities of the LCDV protein filtrates with the 3 types of antiserum (antiserum against concentrated virus and virus from the upper and lower bands) showed similar polypeptide binding patterns with several additional bands in the antiserum against the concentrated virus. The antiserum against the upper and lower band virus ex-

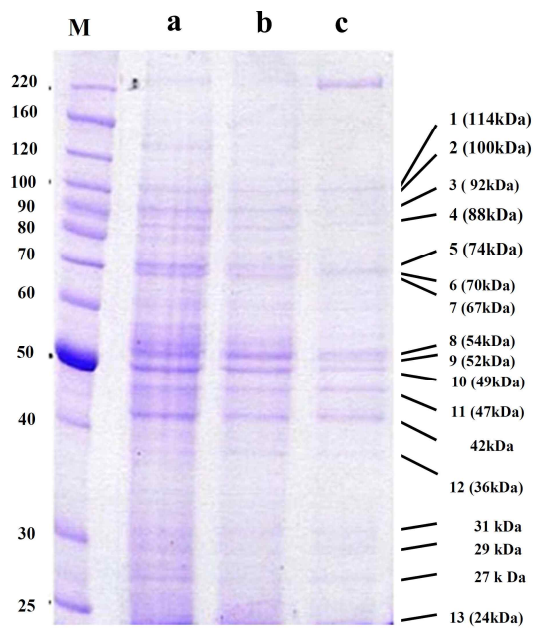

Fig. 2. SDS-PAGE of the proteins prepared from concentrated and purified LCDV in 10% gel. Proteins of the filtrate concentrate (LCDV) and purified virus bands between the 20 and 35% (a), and between the 35 and 50% sucrose concentrations (b). Protein band numbers and their molecular weights are shown on the right side of the SDS-PAGE profile. Numbered proteins were cut and sequenced using MALDI-TOF mass spectrometry. M: protein molecular mass marker (kilodaltons).

Table 1. Lymphocystis disease virus proteins identified in this study by MALDI-TOF mass spectrometry

Band No.[a]	Nucleotide position	Theoretical MW (kDa)[b]	Observed MW (kDa)[b]	Number of Amino acid	Queries matched	Mascot score	ID(NCBI)[c]	Protein Description[d]	Function	Antigenity
1	180717-180574	104.75	114	923	5	418	gil51870187	Chromosome segregation ATPase, LDVICp236	Cell division and chromosome partitioning	Strong
2	182066-182242	52.13	100	480	2	194	gil51870189	Hypothetical protein, LDVICp238		No
6	117323-118495	72.81	70	624	2	58	gil51870113	Acetyl-coA hydrolase, LDVICp160	Energy production and conversion	Weak
7	23615-24976	52.78	67	454	1	73	gil51869989	Membrane bound metallopeptidase, LDVICp033	Recombinant and DNA standard exchange inhibitor protein	Strong
8	114005-114463	50.83	54	457	2	141	gil51870110	Hypothetical protein, LDVICp157		Strong
9	32783-31407	51.17	52	459	5	492	gil48843722	Major capsid protein, LDVICp043	Structural protein	No
10	154959-155129	47.35	49	399	3	162	gil51870151	Hypothetical protein, LDVICp200	2-cystein adaptor Domain	No
12	166783-166446	21.83	36	185	2	104	gil51870164	Hypothetical protein, LDVICp213		Weak
13	28016-28684	26.02	24	223	3	218	gil51869994	Hypothetical protein, LDVICp039		Weak
14	166783-166446	21.83	22	185	3	325	gil51870164	Hypothetical protein, LDVICp213		Weak

[a]Band number from SDS-PAGE. Band numbers are identical in Fig. 1 and 2.
[b]Theoretical molecular mass of identified protein.
[C]Molecular mass observed by SDS-PAGE gel.
[d]Identification of protein first hit using non-redundant NCBI database.

hibited similar western blot patterns with very strong reactions at 114 (band no. 1), 67 (band no. 7) and 54 kDa (band no. 8) (Fig. 3). Other minor bands were shown at 74 (band no. 5), 70 (band no. 6), 36 (band no. 12), 24 (band no. 13) and 22 kDa (band no.14) (Fig. 3).

Identification of LCDV proteins by MALDI TOF mass spectrometry

After separation of the LCDV concentrated virus proteins by SDS-PAGE, all 14 bands exhibiting western blot positive reactions were excised from the gel. Following trypsin digestion of the reduced and alkylated LCDV proteins, the peptides of each band were sequenced using MALDI-TOF mass spectrometry. To identify the proteins, the peptide sequences obtained from the MS/MS data were searched for within the NCBI nonredundant database using the MASCOT MS/MS ion search program. Ten of the 14 excised bands consisted mostly of proteins with amino acid sequences that matched LCDV-C (Lymphocystis disease virus isolate China) ORFs (Table. 1). Four bands (bands 1, 6, 7 and 9) were identified by the NCBI nonredundant database as chromosome segregation ATPase, acetyl-coA hydrolase, membrane bound metallopeptidase and major capsid protein. The other six proteins were hypothetical proteins similar to LDVICp238, LDVICp157, LDVICp043, LDVICp200, LDVICp213 and LDVICp039. The hypothetical protein LDVICp213 was found at band 12 and band 14. However, four other bands (bands 3, 4, 5 and 11) could not be identified.

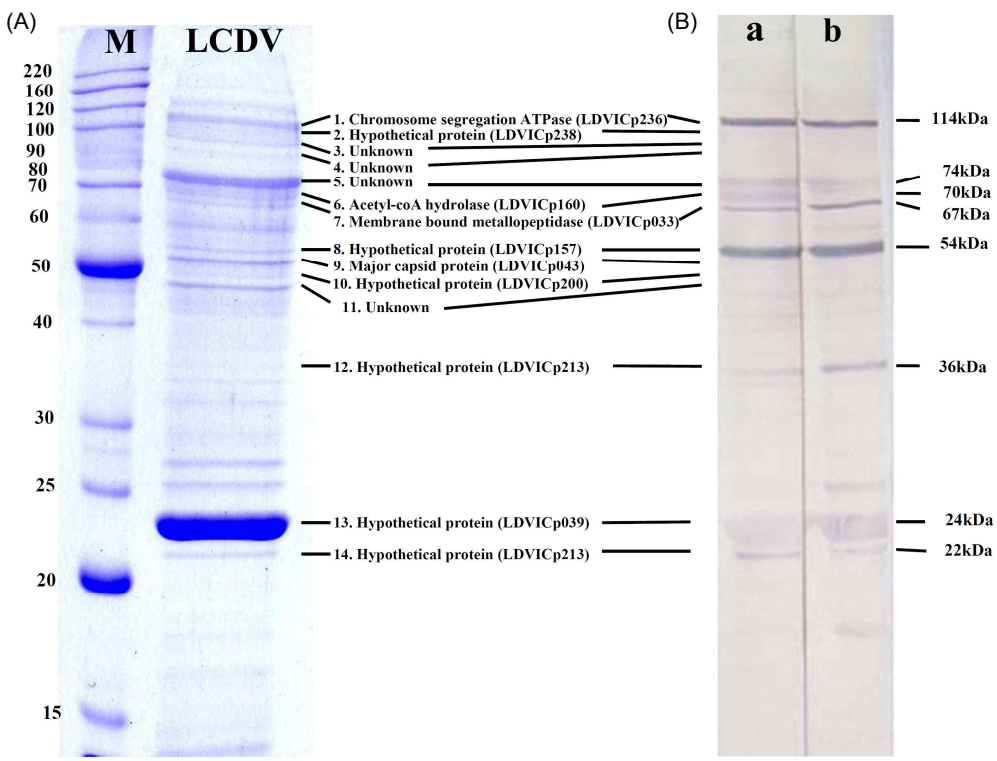

Fig. 3. SDS-PAGE of the structural proteins prepared from concentrated virus (A) and its western blotting (B) with antisera produced from concentrated virus antigen (a) and from the virus bands around 35% sucrose (b) and 50% sucrose (c) after ultracentrifugation. Numbers indicate the excised bands (no.1 ~ no.14) for MALDI-TOF analysis and molecular weights of the antigenic proteins were shown beside western blot positive bands.

Discussion

In this study, LCDV was separated from lymphocystis nodules by cell disruption, differential centrifugation, and density gradient centrifugation in sucrose. Generally, protocols for the purification of viruses are based on the size and density properties of the virus (Steiner *et al*., 1991). Purification protocols for LCDV have been described by several authors using 10 to 60% sucrose discontinuous or continuous gradients (Walker and Hill, 1980; Flugüel *et al*., 1982; Iwamoto *et al*., 2002; Cheng *et al*., 2006). However, the exact structural proteins of lymphocystis are still unclear because it has many polypeptides, and all reports show different SDS-PAGE profiles of the purified virus by using LCDV from different host species and virus strains. García-Rosado *et al*. (2004) reported 14 to 23 proteins from gilt-head seabrem, *Sparus aurata* (Linnaeus); blackspot seabream, *Pagellus bogaraveo* (Brünnich); and sole, *Solea senegalensis* (Kaup), with two major proteins (79.9 and 55.6 kDa). There were two studies on LCDV polypeptide and antigenic proteins isolated from Japanese flounder, which is the same host species used in our study. Iwamoto *et al*. (2002) reported 26 to 29 polypeptides by SDS-PAGE with different antigenic protein profiles in 3 strains [50 kDa (KU strain), 50 kDa and 90 kDa (YO strain), and 50 kDa and 73 kDa (KA strain)], and suggested antigenic differences among the different strains although the virus originated from the same fish species. Cheng *et al*. (2006) showed 22 LCDV structural proteins ranging from 26 to 124 kDa, and developed monoclonal antibody reactions with 116 and 90 kDa polypeptides. Using the monoclonal antibody, they showed that epitopes are located on the outer surface of the virions. Other antigenic proteins calculated from western blotting image of their study (Cheng *et al*., 2006) were around 97, 62, 54 and 25 kDa.

Polyclonal antibody is essential for the development of immunoassays that can analyze and detect viral antigens. In the case of LCDV, viral particles can exist at different density gradients of 37% sucrose and 50% sucrose (Cheng *et al*., 2006). To prevent the loss of antigenic proteins in purification steps, the present study used antisera produced from 3 different antigens of LCDV: those of concentrated virus (concentrated at 60000 *g*, for 1h) and of virus from purified bands (upper band around 35% sucrose and lower band around 50% sucrose). Then, the optimum antiserum dilution (640 times dilution) for western blotting analysis was pre-examined by ELISA. In this study, 8 virus proteins with molecular weights of 114 (band no. 1), 88 (band no. 4), 70 (band no. 6), 54 (band no. 8), 52 (band no.9), 47 (band no. 11), 42 (no band no.) and 24 kDa (band no. 13) were shown in all three of the concentrated and purified virus samples. Among them, the 88 (band no. 4) and 47 kDa (band no. 11) proteins were not matched in the MASCOT MS/MS ion search, supporting that they did not originate from LCDV. The antigenic reactive bands observed in this study were at 114, 74, 70, 67, 54, 36, 24 and 22 kDa, using rabbit antiserum. The strong antigenic 114 and 54 kDa bands would be the same as the 116 and 54 kDa proteins described by Cheng *et al*. (2006) and the 50 kDa protein described by Iwamoto *et al*. (2002). However, the 90 kDa antigenic protein reported in the YO strain by Iwamoto *et al*. (2006) and Cheng *et al*. (2006) was not found in this study. A possibility for this is strain differences for the 90 kDa protein as reported by Iwamoto *et al*. (2002). The 54 kDa antigen was found to be a strong common LCDV antigen in all three studies from Korea, Japan and China.

The comparison of amino acid sequences deduced from the LCDV-C proteins with entries in the protein databases led to the identification of several kinds of functionally characterized proteins in other species. Our target was antigenic proteins, and all 14 western blot positive proteins were identified by MALDI-TOF mass spectrometry. The western blot positive bands were cut out from the SDS-PAGE gel, and then each

band underwent SDS-PAGE and western blotting again to confirm its antigenicity and to make sure the correct target band was selected. Ten of the 14 proteins were identified as having similar amino acid sequences with lymphocystis disease virus-isolate China (LCDV-C). The other 4 un-identified proteins were thought to originate from host cell debris because these bands only existed in western blotting with antiserum produced from the concentrated virus. Among the 10 identified proteins, the observed molecular weights of 8 proteins were very similar with the theoretical molecular weights. However, band no.2 had a molecular weight of 100 kDa, which was twice that of the theoretical molecular weight possibly suggesting the protein existed as a dimer. In addition, LDVICp213 had two forms with apparent sizes of 36 (band no.12) and 22 kDa (band no.14) and a theoretical molecular weight of 22.83 kDa. This may be due to post-translational changes or the 36 kDa protein was a combined form with other protein(s). Strong antigens with molecular weights of 114 (band no.1), 67 (band no. 7) and 54 kDa (band no.8) were identified as LDVICp236 (chromosome segregation ATPase), LDVICp033 (membrane bound metallopeptidase) and LDVICp157 (hypothetical protein), respectively. Zhang *et al*. (2004) suggested that the majority of enzymes for LCDV-C represent homologues of cellular enzymes involved in virus replication and transcription and are shared by all iridoviruses. Chromosome segregation ATPase (LDVICp236) is concerned with cell division and chromosome partitioning, and membrane bound metallopeptidase (LDVICp033) is a DNA standard exchange inhibitor protein and concerned with recombination. However, the function of the 54 kDa protein, a common strong antigen of LCDV, is not clear.

The Iridoviridae family has been subdivided into five genera: *Chloriridovirus, Iridovirus, Megalocytivirus, Ranavirus* and *Lymphocystivirus.* One of the distinctive features of this family is the presence of major capsid protein (MCP), which is the main structural component of icosahedral virus particles. MCP comprises the largest part of the total viral polypeptides, and has a molecular weight of about 50 kDa (Flugüel *et al*., 1982). MCP is a structural protein and the MCP gene is one of the most important genes for analyzing genetic relationships among iridoviruses, because its nucleotide sequence is relatively conserved among viruses belonging to the *Iridoviridae* family (Tidona and Darai, 1997; Davison *et al*., 1992). The MCP of LCDV has been used for genotyping (Kitamura *et al*., 2006), phylogenetic analysis (Hossain *et al*., 2008), and virus detection (Kitamura *et al*., 2007a, b). In addition, the MCP gene was used for acquiring DNA vaccine against LCDV in japanese flounder (Zheng *et al*., 2006) and recombinant antigen of megalocytivirus in rock bream, *Oplegnathus fasciatus* (Kim *et al*., 2008). However, the rabbit antibodies in this study showed no immune reactive band at 52 kDa of the MCP (LDVICp043), similar to those found in other *iridoriridae* such as epizootic hematopoietic necrosis virus (Monini and Ruggeri, 2002), megalocytivirus (red sea bream iridovirus, RSIV) (Nakajima *et al*., 1998), insect iridovirues (Davison *et al*., 1992), and frog virus 3 (FV3) (Chinchar *et al*., 1984). These results suggest that major capsid protein is not an important antigenic protein in LCDV, and may not be a good candidate gene/protein for vaccine. In this study, strong antigens were shown at 114 (LDVICp236; chromosome segregation ATPase), 67 (LDVICp033; membrane bound metallopeptidase) and 54 kDa (LDVICp157), and their putative amino acid sequences were first identified. The data presented here will be useful to produce enhanced recombinant and DNA vaccine against LCDV, and will provide further understanding of the virus-host interaction and pathogenesis of LCDV.

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