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Enzymic Degradation of Fucoidan by Fucoidanase from the Hepatopancreas of *Patinopecten yessoensis*

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A fucoidanase from the hepatopancreas of *Patinopecten yessoensis* was purified by ammonium sulfate precipitation, anion exchange chromatography, isoelectric focusing, and gel chromatography. The purified enzyme gave a single band on polyacrylamide gel electrophoresis. The fucoidanase was practically free from α-L-fucosidase and arylsulfatase activities. The molecular weight of the enzyme was estimated to be 85,000 by gel filtration on TSKgel G3000SW and 84,000 by SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis. The enzyme hydrolyzed fucoidan to produce sulfated oligosaccharides as the reaction products.

Fucoidan, a common sulfated polysaccharide in brown seaweeds, has been investigated to clarify the biological activities of its moieties. Anticoagulant activities of fucoidans from *Eisenia bicyclis*,[1] *Ecklonia kurome*,[2-4] *Hizikia fusiforme*,[5] and *Laminaria angustata* var. *longissima*[6] have been reported.

Enzymes capable of degrading fucoidan have been purified and used in investigating the structure of fucoidan.[7-13] In the study of the degradation of fucoidan from *Nemacystus decipieus* through enzymic hydrolysis using a homogenate of hepatopancreas of *Patinopecten yessoensis*, several sulfated oligosaccharides were detected in the reaction mixture. It seemed quite probable, therefore, that some kind of enzyme (sulfated-oligosaccharide-forming enzyme) was present in the homogenate. To explain the characteristics of the enzyme, we attempted the purification of the enzyme from a homogenate that cleaved fucoidan to produce the sulfated oligosaccharides. This paper describes the purification of the sulfated-oligosaccharide-forming enzyme and its use in investigating the structure of fucoidan.

Materials and Methods

Fucoidan. Fucoidan from the brown alga *Nemacystus decipieus* was a generous gift from Towa Chemical Industry Co.

Hepatopancreas of Patinopecten yessoensis. Hepatopancreas of *Patinopecten yessoensis* was a generous gift from Shoji Store Co. (Hokkaido).

Enzyme assay.

Fucoidanase assay. Fucoidanase activity was estimated by measuring the viscosity reduction of fucoidan. The reaction mixture consisted of 2 ml of 0.5% fucoidan solution, 2.95 ml of 50 mM acetate buffer (pH 5.5), and 0.05 ml of the enzyme solution. After incubation at 30°C in an Ostwald-type viscometer, the flow time was measured. The value of viscosity reduction, α_{obs} was expressed as $\alpha_{obs}=(t_r-t_w)/(t_0-t_w)$, where t_0 was the flow time of the reaction mixture at 0 time, t_r was the flow time of the reaction mixture after incubation, and t_w was the flow time of water. The activity was measured graphically, by plotting the reciprocal values of the viscosity reduction against the amount of enzyme applied. The resulting slopes of the straight lines provide the values of the activity. A unit of enzyme activity was defined as follows: $1-\alpha_{obs}$.[14]

α-L-Fucosidase and arylsulfatase assay. α-L-Fucosidase and arylsulfatase activities were measured under the following conditions. *p*-Nitrophenyl α-L-fucoside was used as a substrate for α-L-fucosidase, and *p*-nitrophenyl sulfate was used as a substrate for arylsulfatase. The reaction mixture contained 0.25 ml of 2 mM substrate in 50 mM acetate buffer (pH 5.5) and 0.05 ml of the enzyme solution. After incubation at 30°C, 2.2 ml of 0.55 M sodium carbonate was added to stop the reaction. The *p*-nitrophenol released was measured by its absorbance at 400 nm. One unit of the enzyme was defined as the amount which liberated 1 μmol of nitrophenol per min under these assay conditions.

Protein assay. Protein was measured by the method of Lowry *et al.*[15] or by absorbance at 280 nm.

Preparation of enzymes. All operations were done at 4°C unless otherwise stated. Enzyme and protein assays were done at every step in the purification procedure.

Step 1. Crude enzyme extraction. The hepatopancreas from *Patinopecten yessoensis* (360 g) were homogenized using a Waring blender for 5 min in 360 ml of a mixture containing 0.1 M phosphate buffer (pH 7.0), 3% KCl, and 0.45 M $MgSO_4$ (10:85:5, by volume).[8] The homogenate was centrifuged at 10,000 × *g* for 20 min.

Step 2. Ammonium sulfate precipitation. The supernatant solution was brought to 30% saturation with ammonium sulfate. The solution was centrifuged at 8,000 × *g* for 30 min to remove the precipitates. The supernatant solution was brought to 70% saturation with ammonium sulfate. The precipitate was collected by centrifugation at 8,000 × *g* for 30 min, dissolved in 0.01 M phosphate buffer (pH 7.0), and dialyzed against 0.01 M phosphate buffer (pH 7.0).

Step 3. Anion exchange chromatography. The dialyzed solution (25.2 ml) was put on a column (3.2 × 45 cm) of DEAE Toyopearl 650M equilibrated with 0.01 M phosphate buffer (pH 7.0). The column was washed with the same buffer, and then eluted with a linear gradient of 0—0.2 M sodium chloride. The unadsorbed fractions on DEAE Toyopearl 650M were pooled and concentrated by ultrafiltration with an Ultra Filter 10,000 membrane (Advantec).

Step 4. Isoelectric focusing. The concentrated solution (19.7 ml) was used for isoelectric focusing in a 110-ml column (LKB). The pH gradient was formed by mixing glycerol (0—50%) and Bio-Lyte 3/10 solution (pH 3—10). A constant power of 2 W was applied for 48 hr. The column was drained with a 10-ml fraction per tube. The fractions were pooled according to their enzyme activities and designated as Fraction I (No. 19—20) and Fraction II (No. 26—36). Fractions I and II were dialyzed against 0.1 M phosphate buffer and concentrated by ultrafiltration with an Ultra Filter 10,000 membrane.

Step 5. Gel filtration column chromatography. Fraction II (3.0 ml) was put on a column (2.2 × 90 cm) of Sephacryl S-300HR equilibrated with 50 mM phosphate buffer containing 0.1 M sodium chloride (pH 7.0) and eluted with the same buffer. The volume of each fraction was 3 ml. The fractions were pooled according to the enzyme activities and designated as Fraction II-1 (No. 68—71) and Fraction II-2 (No. 83—87), respectively.

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Electrophoresis.

Cellulose acetate membrane electrophoresis. Cellulose acetate membrane electrophoresis was done under the following conditions: (a) 1.0 M acetic acid–pyridine (pH 3.5) and (b) 0.1 N hydrochloric acid.[16]

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was done in 10% polyacrylamide gel with Tris–glycine buffer (pH 9.5).[17]

SDS (Sodium dodecyl sulfate) polyacrylamide gel electrophoresis. SDS polyacrylamide gel electrophoresis was done in 10% polyacrylamide and 0.1% SDS. Marker kit proteins used were α_2-macroglobulin, reduced (MW 170,000), phosphorylase *b* (97,400), and glutamate dehydrogenase (55,400).

Isoelectric focusing. Isoelectric focusing was done with Ampholine 8101 (LKB). The pH and density gradient were formed by mixing glycerol (0—50%) and Bio-Lyte 3/10 solution (pH 3—11).

Gel filtration chromatography. The molecular weight measurement of the enzyme was done with TSKgel G3000SW column. Phosphate buffer (100 mM, pH 6.8) containing 0.3 M NaCl was used as the elution buffer. Marker kit proteins used were aldolase (MW 158,000), hexokinase (100,000), and bovine serum albumin (68,000).

Sugar content. Total sugar content was measured by the phenol–sulfuric acid method[18] using fucose as the standard.

Gas liquid chromatography (GLC). Gas liquid chromatography was done on a Shimadzu GC-4B fitted with FID. A glass column packed with 3% ECNSS-M on Uniport HP was used. Neutral sugars composed of fucoidan and oligosaccharides were measured as alditol acetate derivatives after acid hydrolysis.[19]

High performance liquid chromatography (HPLC). HPLC was conducted using a Refractive Index Detector 504 (GL Sciences). Fucoidan and oligosaccharides were analyzed by TSKgel G4000SW using molecular weight marker dextrans (MW 480,000, 66,300, 39,500, and 10,400). Neutral sugars were analyzed by TSKgel Amido-80.

Ion chromatography. Ion chromatography was done using Tosoh CCP & 8010 series apparatus. The sulfate contents of fucoidan and sulfated oligosaccharides, after acid hydrolysis, were analyzed by Shim-pack IC-A1.

Infrared (IR) spectrum. IR spectrum was done with JASCO IRA-1, using sodium chloride as a tableting agent.

Blood anticoagulant activity. Blood anticoagulant activity was measured by anti-thrombin activity with bovine thrombin according to the method of Shimada *et al.*[20] The anti-thrombin activities of fucoidan and oligosaccharides were compared with a commercial heparin (140 units/mg) as a standard.

Materials. All of the materials were purchased from commercial sources: *p*-nitrophenyl α-L-fucoside, *p*-nitrophenyl sulfate, and bovine thrombin were from Sigma Co. DEAE-Toyopearl 650 M, TSKgel G3000PW, TSKgel G4000SW, and TSKgel Amido-80 were from Tosoh Co. Bio-Lyte 3/10 was from Bio-Rad Co. Sephacryl S-300HR was from Pharmacia Fine Chemicals Co. Shim-pack IC-A1 was from Shimadzu Co. The α-L-fucosidase from *Fusarium oxysporum* was from Seikagaku Co. Heparin was from Eastman Kodak Co.

Results

Enzyme purification

A summary of the purification procedure is shown in Table I. Fucoidanase, α-L-fucosidase, and arylsulfatase activities were detected in the precipitate of 30—70% saturated ammonium sulfate. In the DEAE Toyopearl 650M column chromatogram, the peak of arylsulfatase activity was observed in the eluent at 0.11 M NaCl. Both the fucoidanase and α-L-fucosidase were not adsorbed on the DEAE Toyopearl 650M column at pH 8 (0.01 M phosphate buffer). The pass-through solution of this column was used for isoelectric focusing. The elution profile of isoelectric focusing is shown in Fig. 1. Fucoidanase and α-L-fucosidase activities were detected at Fraction II (No. 26—36). In the pass-through solution of the DEAE Toyopearl 650M column, two kinds of α-L-fucosidase were observed. On the isoelectric focusing, one of the α-L-fucosidase activities was detected at Fraction I

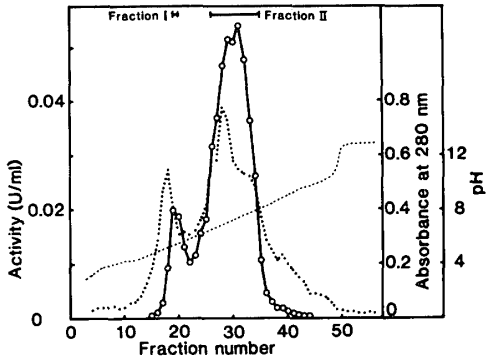

Fig. 1. Isoelectric Focusing Column Chromatography.

Isoelectric focusing was done in a 110-ml column. The pH gradient was formed by mixing glycerol (0—50%) and Bio-Lyte 3/10 solution (pH 3—10). A constant power of 2 W was applied for 48 hr. The volume of each fraction was 10 ml. Fraction numbers 19—20 (Fraction I) and 26—36 (Fraction II) were pooled. Symbols: —○—, α-L-fucosidase activity; ·····, absorbance at 280 nm; -----, pH.

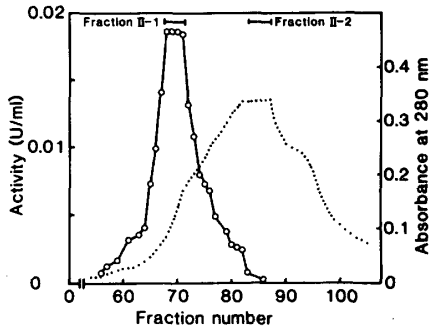

Fig. 2. Gel Filtration Column Chromatography.

A Sephacryl S-300HR column (2.2 × 90 cm) was equilibrated with 50 mM phosphate buffer containing 0.1 M sodium chloride (pH 7.0). The volume of each fraction was 3 ml. Fraction numbers 68—71 (Fraction II-1) and 83—87 (Fraction II-2) were pooled. Symbols: —○—, α-L-fucosidase activity; ·····, absorbance at 280 nm.

Table I. Summary of Purification of Fucoidanase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	α-L-Fucosidase activity	Arylsulfatase activity	Yield (%)
$(NH_4)_2SO_4$ precipitation	320	90.7	0.28	+	+	100
DEAE-Toyopearl 650 M	80.8	50.7	0.63	+	−	56
Isoelectric focusing	27.3	33.7	1.2	+	−	37
Sephacryl S-300	6.2	24.2	3.9	−	−	27

(No. 19—20), and the other was detected at Fraction II. Fraction I was designated as α-L-fucosidase I. The concentrated Fraction II was put on a gel filtration column of Sephacryl S300HR. Fucosidanase and α-L-fucosidase were separated on this column. The elution profile of gel filtration is shown in Fig. 2. Fucoidanase activity was detected at Fraction II-2 (No. 83—87), and α-L-fucosidase activity was detected at Fraction II-1 (No. 68—71). Fraction II-1 was designated as α-L-fucosidase II. The purified fucoidanase was free from α-L-fucosidase activity. The fucoidanase was purified about 14-fold from the 30—70% saturated ammonium sulfate precipitation with a recovery of 27%.

The purified fucoidanase gave a single band on polyacrylamide gel electrophoresis and on SDS polyacrylamide gel electrophoresis (Fig. 3). The molecular weight of the fucoidanase was estimated to be about 85,000 by TSKgel G3000SW gel filtration and 84,000 by SDS polyacrylamide gel electrophoresis (Fig. 4). It was supposed that the enzyme was of a monomeric structure. The isoelectric point of the enzyme was pH 7.4.

Fig. 3. Polyacrylamide Gel Electrophoresis of Fucoidanase.

Arrows show the position of the purified enzyme. (A) electrophoresis of the native enzyme; (B) electrophoresis of the enzyme treated with SDS.

Enzymic hydrolysis of fucoidan

On the viscosity reduction of fucoidan, such as the fucoidanase, α-L-fucosidase I, and α-L-fucosidase II, were monitored for 24 hr (Fig. 5). α-L-Fucosidase II could hydrolyze only *p*-nitrophenyl α-L-fucoside but not fucoidan. α-L-Fucosidase I slowly hydrolyzed fucoidan, like the α-L-fucosidase from *Fusarium oxysporum*[13] (data not shown). During the hydrolysis of fucoidan by the fucoidanase, rapid viscosity reduction was observed. The final value of viscosity reduction α_{obs}, was 0.2. These results indicated that the enzyme seemed to lack the ability to completely degrade fucoidan.

Products of enzymic hydrolysis of fucoidan

The HPLC pattern of unhydrolyzed fucoidan is shown in Fig. 6-A. The unhydrolyzed fucoidan was eluted at the void volume and the approximate molecular weight was estimated to be over 480,000 from the elution profile of the marker dextrans (MW 480,000, 66,300, 39,500, and 10,400). The fucoidanase, α-L-fucosidase I, and α-L-fucosidase II were reacted with fucoidan for 24 hr. In the

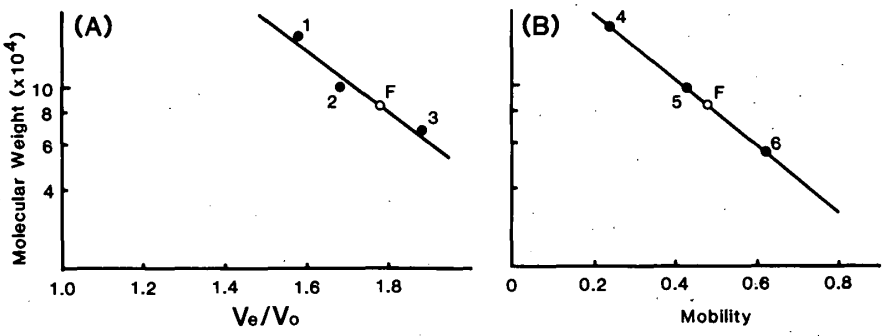

Fig. 4. Estimation of the Molecular Weight of Fucoidanase.

(A) TSKgel G3000SW gel filtration chromatography; (B) SDS polyacrylamide gel electrophoresis. 1, aldolase (158,000); 2, hexokinase (100,000); 3, bovine serum albumin (68,000); 4, α_2-macroglobulin, reduced (170,000); 5, phosphorylase *b* (97,400); glutamate dehydrogenase (55,400); F, fucoidanase.

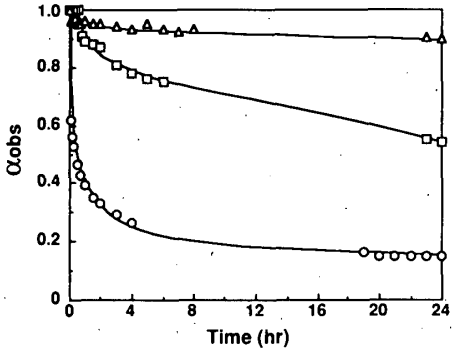

Fig. 5. Enzymic Hydrolysis of Fucoidan.

Enzyme activities for the hydrolysis of fucoidan were followed by measuring viscosity reduction. The reaction mixture consisted of 2 ml of 0.5% fucoidan solution, 2.95 ml of 50 mM acetate buffer (pH 5.5), and 0.05 ml of enzyme solution. After incubation at 30°C in an Ostwald-type viscometer, the flow time was measured. The value of viscosity reduction, α_{obs} was calculated according to the following formula; $\alpha_{obs}=(t_r-t_w)/(t_0-t_w)$, where t_0 was the flow time of the reaction mixture at 0 time, t_r was the flow time of the reaction mixture after incubation, and t_w was the flow time of water. Symbols: —○—, fucoidanase; —□—, α-L-fucosidase I; —△—, α-L-fucosidase II.

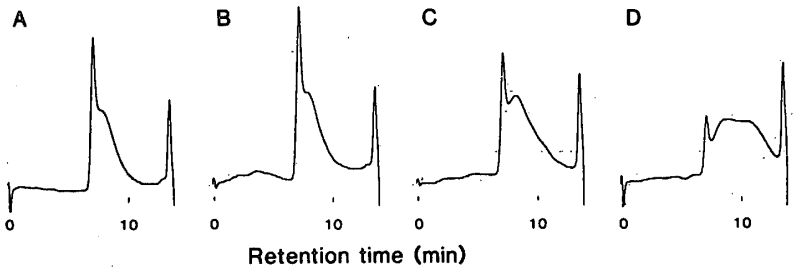

Fig. 6. HPLC of the Enzymic Hydrolysis of Fucoidan.

TSKgel G4000SW with 0.2 M sodium chloride as eluent was used at 40°C, and at a flow rate of 0.8 ml/min. Injection volume was 80 μl. (A) substrate fucoidan; (B) α-L-fucosidase II; (C) α-L-fucosidase I; (D) fucoidanase.

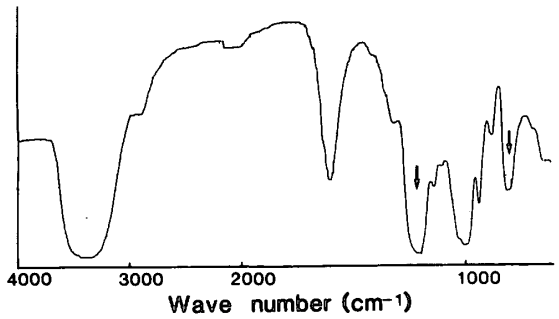

Fig. 7. IR Spectrum.

The absorptions of 1250 cm^{-1} and 850 cm^{-1} are indicated by arrows. JASCO IRA-1, NaCl tablet.

case of α-L-fucosidase II, the fucoidan peak did not change, since α-L-fucosidase II could not attack fucoidan to produce oligosaccharides (Fig. 6-B). In the case of α-L-fucosidase I, however, the fucoidan peak changed slightly, indicating that α-L-fucosidase I could partially hydrolyze fucoidan (Fig. 6-C).

The HPLC pattern of the hydrolysis products from fucoidan by fucoidanase is shown in Fig. 6-D. The pattern would indicate that oligosaccharides of various sizes were formed by the enzymic hydrolysis of fucoidan. The release of fucose and other neutral sugars could not be detected in the reaction products (data not shown). The oligosaccharide fractions (retention time 9.0—11.0 min fractions) were collected and desalted. They yield of the above oligosaccharide was 29.9% (w/w). The approximate molecular weight of the oligosaccharide was estimated to be 50,000. The oligosaccharide was electrophoretically homogeneous on a cellulose acetate membrane with 1.0 M acetic acid–pyridine (pH 3.5) and 0.1 N HCl. The backbone sugar of the oligosaccharide was only fucose. The molar ratio of sulfate to sugar residues was calculated to be 2 : 1. In the IR spectrum (Fig. 7), strong absorptions were observed at 1250 cm^{-1} and 850 cm^{-1}. This observation seemed to indicate that the sulfate residues of the oligosaccharide was attached to fucose residues.[21,22] The anti-thrombin activity of the sulfated oligosaccharide was 860 units/mg, as compared with a commercial heparin (140 units/mg) as a standard. This value of the activity was similar to the activity of the native fucoidan.

Discussion

Enzymes capable of degrading fucoidan have already been investigated, but most of the data were obtained with partially purified enzyme preparations.[7-12] The enzyme from the hepatopancreas of abalone hydrolyzed fucoidan of *Fucus gardneri*, but it contained sulfatase activity.[8] α-L-Fucosidase from abalone liver liberated monosulfated L-fucose from fucoidan of *Ecklonia cava*.[9,10] α-L-Fucosidase from *Fusarium oxysporum* liberated L-fucose from fucoidan of *Fucus vesiculosus*.[13] Fucoidanase, α-L-fucosidase, and arylsulfatase activities were detected in the homogenate of the hepatopancreas of *Patinopecten yessoensis*. These enzymes were separated from one another during our enzyme purification process. The fucoidanase obtained was electrophoretically homogeneous. The purified fucoidanase showed a noteworthy substate specificity, since the enzyme hydrolyzed fucoidan but not an artificial substrate such as *p*-nitrophenyl α-L-fucoside. From chemical and physical analysis, it was ascertained that one of the hydrolysis products by the purified fucoidanase was a sulfated oligosaccharide. The purified fucoidanase had a specific property for fucoidan, which degraded fucoidan to give sulfated oligosaccharides.

The fucoidanase also had an activity causing rapid viscosity reduction of fucoidan. The enzyme did not release L-fucose from fucoidan. So far as examined, the purified fucoidanase from the hepatopancreas of *Patinopecten yessoensis* seemed to be quite different from the enzymes of other origins.[7-13] Thus, we would like to refer to this enzyme entity as an endotype fucoidanase. But the enzyme did not completely degrade fucoidan. This might be attributed to the intricate structure of fucoidan.

In connection with the structure and the anticoagulant activity of sulfated polysaccharides, it has been reported that the anticoagulant activity of heparin is dependent on molecular weight.[23] It has been also reported that the anticoagulant activities of other sulfated polysaccharides such as dextran sulfate (MW 3500—200,000)[24] and sulfated xylan (MW 3000—56,000)[25] are dependent on the molecular weight. On the relationship of the molecular weight of fucoidan and its anticoagulant activity, Nishino *et al.* stated that the lower limit of molecular weight of fucoidan of *Ecklonia kurome* was 30,000.[4] The sulfated oligosaccharide formed by the fucoidanase had anticoagulant activity, and its value was similar to that of unhydrolyzed fucoidan of *Nemacystus decipieus*. We obtained that the approximate molecular weight of the sulfated oligosaccharide was 50,000. In comparing our results with other reports, the molecular weight of the sulfated oligosaccharide for anticoagulant activity was

similar to those of other sulfated oligosaccharides. On the study of the relationships between structure and anticoagulant activity, fucoidanase would be useful for the preparation of various sulfated oligosaccharides. Further investigations on the relationships between the structure and the anticoagulant activity will be reported elsewhere in detail. This fucoidanase would also greatly assist further studies on the structure of fucoidan.

Acknowledgments. We thank Towa Chemical Industry Co. for the generous gift of the fucoidan of *Nemacystus decipieus* and Shoji Store Co. for the generous gift of hepatopancreas of *Patinopecten yessoensis*.

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