A Novel Aldo-Keto Reductase, HdRed, from the Pacific Abalone *Haliotis discus hannai*, Which Reduces Alginate-derived 4-Deoxy-\(\alpha\)-L-erythro-5-hexoseulose Uronic Acid to 2-Keto-3-deoxy-\(\alpha\)-glucoconate*

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Abalone feeds on brown seaweeds and digests seaweeds’ alginate with alginate lyases (EC 4.2.2.3). However, it has been unclear whether the end product of alginate lyases (i.e. unsaturated monouronate-derived 4-deoxy-\(\alpha\)-L-erythro-5-hexoseulose uronic acid (DEH)) is assimilated by abalone itself, because DEH cannot be metabolized via the Embden-Meyerhof pathway of animals. Under these circumstances, we recently noticed the occurrence of an NADPH-dependent reductase, which reduced DEH to 2-keto-3-deoxy-\(\alpha\)-gluconate, in hepatopancreas extracts of the pacific abalone *Haliotis discus hannai*. In the present study, we characterized this enzyme to some extent. The DEH reductase, named HdRed in the present study, could be purified from the acetone-dried powder of hepatopancreas by ammonium sulfate fractionation followed by conventional column chromatographies. HdRed showed a single band of ~40 kDa on SDS-PAGE and reduced DEH to 2-keto-3-deoxy-\(\alpha\)-gluconate with an optimal temperature and pH at around 50 °C and 7.0, respectively. HdRed exhibited no appreciable activity toward 28 authentic compounds, including aldehyde, aldose, ketose, \(\alpha\)-keto-acid, uronic acid, deoxy sugar, sugar alcohol, carboxylic acid, ketone, and ester. The amino acid sequence of 371 residues of HdRed deduced from the cDNA showed 18–60% identities to those of aldo-keto reductase (AKR) superfamily enzymes, such as human aldose reductase, halophilic bacterium reductase, and sea hare norsorinic acid (a polyketide derivative) reductase-like protein. Catalytic residues and cofactor binding residues known in AKR superfamily enzymes were fairly well conserved in HdRed. Phylogenetic analysis for HdRed and AKR superfamily enzymes indicated that HdRed is an AKR belonging to a novel family.

**Alginic acid is a structural polysaccharide of brown seaweeds and certain bacteria, comprising \(\beta\)-\(\alpha\)-mannuronate (M) and \(\alpha\)-\(\alpha\)-guluronate (G), which form poly(M), poly(G), and random(MG) blocks in alginate polymer (1–3). Alginate from brown seaweeds has been widely used as a viscosifier and gelling agent in the food and pharmaceutical industries because sodium alginate solution exhibits high viscosity and forms an elastic gel upon forming calcium salt (1, 4). Alginate oligosaccharides are also recognized as functional materials that exhibit various biological functions, such as promotion of root growth of higher plants (5, 6), acceleration of growth rate of *Bifidobacterium* sp. (7), and stimulation of proliferation of endothelial cells (8). Further, 4-deoxy-\(\eta\)-erythro-hexoseulose uronic acid (DEH), the end product of alginate lyases (EC 4.2.2.3 and EC 4.2.2.11) (see Fig. 1), was recently proven to be available for ethanol fermentation with genetically modified microorganisms (9–11). These findings have increased the practical potentiality of both alginate and alginate-producing brown seaweeds.**

Besides such practical aspects, alginate is an important food source for herbivorous gastropods like abalone (12–16). Namely, abalone feeds brown seaweeds as a daily diet and digests poly(M) block of alginate to DEH with alginate lyases (poly(M) lyases) in the digestive tract (14–16). Although certain intestinal bacteria of abalone are also known to digest and ferment alginate, providing volatile short chain fatty acids to the host animal (17, 18), it has still been unclear whether abalone itself can completely metabolize poly(M) block by its own metabolic system because DEH does not flow into the Embden-Meyerhof pathway. In the case of alginate-assimilating bacteria, alginate-derived DEH is first reduced to 2-keto-3-deoxy-gluconate (KDG) by DEH reductase (Fig. 1) and then metabolized to glyceraldehyde-3-phosphate and pyruvate via the Entner-Doudoroff pathway (19, 20). Recently, DEH-specific reductases, A1-R (21), A1-R’ (22), and FlRed (23), were identified in algino-lytic bacteria *Sphingomonas* sp. strain A1 and *Flavobacterium* sp. UMI-01, respectively.

These bacterial DEH reductases were identified as the members of the short chain dehydrogenase/reductase superfamily (24). Because many bacteria can assimilate KDG, reduction of DEH to KDG is regarded as the key reaction of alginate metabolism in bacteria (19–23). On the other hand, DEH reductase has not been identified in herbivorous (alginate-feeding) gastropods like abalone. Further, there appears to be no informa-

**reductase; contig, group of overlapping clones; Ni-NTA, nickel-nitri
triacetic acid; rHdRed, recombinant HdRed.**
tion about the enzymes acting on DEH in eukaryotes. Therefore, investigation of the DEH-acting enzymes in abalone may provide important information about the alginate metabolism in many alginate-assimilating organisms.

Under these circumstances, we recently investigated the occurrence of DEH-reducing enzyme in various tissue extracts from the pacific abalone *Haliotis discus hannai* and noticed that the hepatopancreas extract showed high activity. This enzyme(s) may become a clue for the elucidation of the alginate-metabolic pathway in abalone. Therefore, in the present study, we isolated this enzyme and characterized it. Interestingly, the abalone DEH reductase, named HdRed in the present study, was not a member of the short chain dehydrogenase/reductase superfamily (21–23) but a member of the aldo-keto reductase (AKR) superfamily (25–27).

**Experimental Procedures**

**Materials**—Living abalones (*H. discus hannai*, average shell size 8 × 6 cm) were obtained from a local market in Hakodate, Hokkaido Prefecture, Japan. Sodium alginate (*Macrocystis pyrifera* origin) was purchased from Sigma-Aldrich. DEH was prepared by the digestion of sodium alginate with crude enzyme from *Flavobacterium* sp. UMI-01 as reported previously (23). TOYOPEARL Butyl-650M, TOYOPEARL DEAE-650M, and TOYOPEARL QAE-550C were purchased from Toyo Soda Mfg. Co. (Tokyo, Japan). MonoQ 5/50 GL and Superdex 75 10/300 GL were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The Oligotex-dT30 mRNA purification kit, TaKaRa premix *Taq* DNA polymerase, and restriction endonucleases were purchased from TaKaRa (Tokyo, Japan). Dyna Express TA PCR cloning kit, including pTAC-1 vector, was purchased from BioDynamics Laboratory Inc. (Tokyo, Japan). Silica gel TLC-60 plates were purchased from Merck. Matrices for MALDI-TOF/MS (i.e. 2,5-dihydorxybenzoic acid and α-cyano-4-hydroxycinnamic acid) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). NADH, NADPH, α-keto-glutaric acid, pyruvic acid, sodium phenylpyruvate, d-glucose, d-mannose, d-galactose, d-ribose, l-arabinose, d-xylose, d-fructose, l-fucose, d-deoxyribose, d-glucuronic acid, mannitol, sorbitol, maleic acid, citric acid, 2-furoic acid, dL-glyceraldehyde, glutaraldehyde, benzaldehyde, p-nitrobenzaldehyde, p-phenylaldehyde, 4-methyl-2-pentanone, 2,3,5-hexanedione, 3-chloropropiophenone, ethyl pyruvate, ethyl benzoylacetate, and other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) or prepared by the digestion of sodium alginate with crude enzyme from *Flavobacterium* sp. UMI-01 as reported previously (23).

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FIGURE 1. Production of KDG from alginate by the actions of alginate lyases and DEH reductase. Alginate lyases (poly(M) and poly(G) lyases) convert poly(M) and poly(G) blocks of alginate to a C4-C5 unsaturated monouronate. The monouronate spontaneously changes to a single uronate derivative, DEH. The DEH is reduced by DEH reductase to KDG in the presence of NADPH or NADH. Cofactor specificity of DEH reductase is dependent on the enzymes.

3 S. Mochizuki, R. Nishiyama, A. Inoue, and T. Ojima, unpublished observations.
Sigma-Aldrich Japan Inc. 1,2-Diamino-4,5-methylenedioxybenzene (DMB) was purchased from Dojindo (Tokyo, Japan).

Preparation of Crude Enzyme from Abalone Hepatopancreas—Crude enzyme was extracted from acetone-dried powder of abalone hepatopancreas as follows. Abalone was anesthetized by cooling on ice for 1 h, and hepatopancreas was collected after dissection. The hepatopancreas (total 90 g from seven individuals) was cut into pieces (≈5 × 5 mm) and rinsed three times with 300 ml of ice-cold sterilized phosphate-buffered saline to remove body fluid and possible contaminants. The rinsed hepatopancreas was homogenized with 270 ml of 10 mM sodium phosphate buffer (pH 7.0) by a Polytron homogenizer (Central Scientific Commerce, Inc., Tokyo, Japan) for 30 s on ice. Then it was again homogenized with a Potter-type Teflon homogenizer (100-ml size), which was attached to a LABOSTIRRER (LS-15, Yamato, Tokyo, Japan), at 800 rpm for 3 min on ice. After the homogenization, 2 volumes of −40 °C acetone were quickly added to the homogenate and mixed vigorously. The precipitates formed were collected by centrifugation at 10,000 × g for 10 min, suspended with 2 volumes of −20 °C acetone, and centrifuged. The acetone treatment was repeated twice more, and the final precipitates were collected by vacuum filtration using number 2 filter paper (ADVANTEC, Tokyo, Japan) and air-dried at 20 °C. The acetone-dried power (17.6 g) was then suspended in 352 ml of 10 mM sodium phosphate buffer (pH 7.0) and extracted at 0 °C for 30 min with occasional stirring. The suspension was centrifuged at 10,000 × g for 20 min, and the supernatant (crude enzyme) was used for purification of DEH reductase.

Purification of DEH Reductase—The crude enzyme was subjected to ammonium sulfate fractionation. Relatively high DEH-reducing activity was detected in the fraction precipitated between 30 and 40% saturation of ammonium sulfate. This fraction was collected by centrifugation at 10,000 × g for 10 min, dissolved in and dialyzed against 20% saturation of ammonium sulfate in 10 mM sodium phosphate buffer (pH 7.0), and subjected to a TOYOPEARL Butyl-650 M column (2.5 × 30 cm) pre-equilibrated with the same buffer. Proteins adsorbed to the column were eluted by the linear gradient of ammonium sulfate from 20 to 0% saturation in 10 mM sodium phosphate buffer (pH 7.0). This chromatography, the DEH-reducing activity was detected in the fractions eluted at around 3% saturation of ammonium sulfate. The active fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and concentrated to 3 ml with a VIVASPIN20 centrifugal concentrator (Sartorius AG, Goettingen, Germany). The concentrate was then subjected to AKTA-FPLC (GE Healthcare) on a Mono-Q 5/50 GL column pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.0). Proteins adsorbed to the column were eluted by the linear gradient from 0 to 0.3 M NaCl. The DEH-reducing activity was detected in the fractions eluted at around 0.2 M NaCl (Fig. 2A). The active fractions were pooled, dialyzed against 0.3 M NaCl in 10 mM sodium phosphate (pH 7.0), and concentrated to 1 ml with a VIVASPIN20 centrifugal concentrator. The concentrate was then subjected to a Superdex 75 10/300 GL column pre-equilibrated with the same buffer. In this chromatography, a protein with the DEH-reducing activity was eluted as a single peak (Fig. 2B). The molecular mass of this protein was estimated to be 40 kDa on SDS-PAGE, whereas it was estimated to be >80 kDa by gel filtration through Superdex 75 10/300 GL or Superdex 200 10/300 GL, suggesting oligomer formation under non-denaturation conditions. We named this enzyme HdRed and used it for the characterization.

Assay for DEH-reducing Activity—DEH-reducing activity was determined at 30 °C in a standard reaction mixture containing 1 mM DEH, 1 mM NADPH, 10 mM sodium phosphate (pH 7.0), and an appropriate amount of enzyme. The reaction was initiated by the addition of enzyme to the substrate solution, and the progress of the reaction was monitored by measuring the decrease in absorbance at 340 nm due to the oxidation of NADPH to NADP+. One unit of DEH-reducing activity was defined as the amount of enzyme that consumes 1 μmol of NADPH/min. Temperature dependence of HdRed was determined at 15–75 °C in the standard reaction mixture. Heat stability of HdRed was assessed by measuring the activity remaining after the heat treatment at 4–75 °C for 20 min. pH dependence of HdRed was determined in reaction mixtures adjusted to pH 4.0–6.0 with 20 mM sodium acetate buffer, pH 5.6–8.2 with 10 mM sodium phosphate buffer, and pH 7.2–10.0 with 20 mM glycine-NaOH buffer. In the above activity measurements, NADPH caused autoxidation at temperatures >30°C and pH <5. In such cases, the enzyme activity was determined by subtracting the absorbance value in the absence of enzyme from the value in the presence of enzyme. The average values with S.D. for triplicate assays are indicated in each figure. Substrate and cofactor specificities of HdRed were determined with reaction mixtures containing 1 mM substrate (i.e. carboxylic acids, aldoses, ketones, aldehydes, ketones, and esters) and 1 mM NADPH or NADH.

Thin Layer Chromatography (TLC)—Reaction products produced by HdRed were analyzed by thin layer chromatography using silica gel TLC-60 plates. One hundred μl of reaction mixture containing 10 mM DEH, 10 mM NADPH, 10 mM sodium phosphate buffer (pH 7.0), and 0.08 units/ml HdRed was incubated at 30 °C for 10–180 min. At appropriate time intervals, 20 μl of reaction mixture was taken out and heated at 100 °C for 1 min to inactivate enzyme. Then 3 μl of each reaction mixture was applied to the TLC-60 plate and developed with a solvent comprising 1-butanol, acetic acid, and water (2:1:1, v/v/v). The reaction products developed on the plate were visualized by either sulfuric acid staining (28) or thiobarbituric acid staining (29).

Mass Spectrometry—Mass spectrometry for the reaction products of HdRed was carried out with a Proteomics 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). The reaction products (1 μl) prepared as described under “Thin Layer Chromatography” were mixed with 1 μl of 10 mg/ml 2,5-dihydroxybenzoic acid in acetonitrile and applied to a sample plate. Molecular masses of reaction products were determined in a negative ion mode.

Chemical Modification of KDG-like Product with 1,2-Diamino-4,5-methylene-dioxybenzene—α-Keto-acid structure of the reaction product of HdRed (i.e. KDG-like product) was confirmed by chemical modification with DMB (30). Approxi-
mately 10 mg of KDG-like product produced by HdRed was dissolved in distilled water and subjected to TOYOPEARL QAE-550C column (1.0 × 20 cm) pre-equilibrated with distilled water. The KDG-like product adsorbed to the column was eluted by a linear gradient of NaCl from 0 to 0.2 M. The KDG-like product eluted at around 0.1 M NaCl was collected and lyophilized. The product was dissolved in 0.7 M HCl to make the final concentration 0.8 mg/ml, and an aliquot of the solution (200 μl) was mixed with an equal volume of DMB solution containing 7 mM DMB, 2 M 2-mercaptoethanol, and 56 mM sodium dithionite. After the incubation at 100 °C for 60 min, the reaction mixture was diluted with 2 ml of 0.35 M HCl. The fluorescence spectrum of the DMB derivative was measured with a JASCO FP-6200 spectrofluorometer (Tokyo, Japan) with the excitation wavelength at 370 nm.

**Amino Acid Sequence Analysis**—Partial amino acid sequences of HdRed were analyzed by using tryptic fragments. After the SDS-PAGE, HdRed was in-gel digested with 10 μg/ml trypsin, reduced with 10 mM dithiothreitol, and carboxymethylated with 55 mM monoiodoacetic acid. The peptide fragments were extracted from the gel and desalted with a ZipTip pipette tip (Applied Biosystems), and 1 μl of the peptide solution was applied to the sample plate. One μl of 10 mg/ml α-cyano-4-hydroxycinnamic acid was added and subjected to a MALDI-TOF/TOF MS (Proteomics 4700). The amino acid sequences were predicted by an MS/MS mode and DeNovo Explorer software (Applied Biosystems).

**Protein Concentration**—Protein concentration was determined by the biuret method (31) and the method of Lowry et al. (32) using bovine serum albumin as a standard protein.

**SDS-PAGE**—SDS-PAGE was carried out by the method of Porzio and Pearson (33) using 0.1% SDS, 10% polyacrylamide gel. After the electrophoresis, the gel was stained with 0.02% Coomassie Brilliant Blue R-250 in 50% methanol, 10% acetic acid, and destained with 7% acetic acid, 5% methanol. Protein Marker, Broad Range (2–212 kDa) (New England Biolabs) or PageRuler Unstained Protein Ladder (ThermoFisher) was used as a size marker.

**Transcriptome Analysis for Abalone Hepatopancreas**—Hepatopancreas was dissected from an abalone and rinsed with phosphate-buffered saline (i.e. 0.9% (w/v) NaCl, 10 mM sodium phosphate (pH 7.4)). Total RNA was extracted from 1.4 g of the hepatopancreas with the NucleoSpin RNA kit (TaKaRa/Clontech, Tokyo, Japan) according to the manufacturer’s protocol. Selection of poly(A) T RNA, synthesis of cDNA, ligation of Illumina adapter to cDNA, and amplification of cDNA were performed with TruSeq Sample Prep Kits (Illumina, Inc., San Diego, CA). The PCR products were purified with the AMPure XP kit. Nucleotide sequences of the cDNAs were analyzed with an Illumina HiSeq sequencer by the paired-end method with 100-bp reading from Hokkaido System Science (Sapporo, Japan). By the de novo assembling of nucleotide sequences with the Trinity server, 111,139 contigs comprising 99 Mbp of nucleotide sequences were identified.

**Amplification of HdRed cDNA**—Total RNA was extracted from 1 g of abalone hepatopancreas with RNAiso Plus (TaKaRa/Clontech). mRNA was selected with the Oligotex dT-30 mRNA purification kit (TaKaRa). cDNA was synthesized with the PrimeScript first strand cDNA synthesis kit (TaKaRa).

cDNAs encoding HdRed were amplified by PCR using the hepatopancreas cDNA as a template and degenerated primers designed on the basis of partial amino acid sequences of HdRed and specific primers designed on the basis of transcriptome analysis for abalone hepatopancreas. PCR was carried out with 30 cycles of reaction comprising incubations at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s in 20 μl of a reaction mixture containing 0.5 units of TaKaRa Taq DNA polymerase. The amplified cDNAs were cloned with a DynaExpress TA PCR cloning kit using pTAC-1 vector (BioDynamics Laboratory Inc.), and the nucleotide sequences of the cDNAs were analyzed with an ABI 3130xl genetic analyzer (Applied Biosystems). A homology search for the deduced amino acid sequences was performed with the BLAST tools provided by the National Center for Biotechnology Information. cDNAs encoding HdRed in the transcriptome data were identified by local BLAST analysis.

**Bacterial Expression of Recombinant HdRed**—HdRed DNA was amplified by PCR using pTAC-1-HdRed cDNA as a template and forward and reverse primers (i.e. HdRed-NdeF, 5′-GAAGGAGATATACATATGGCGGCGGTACCAGAAGA-3′; HdRed-inf8G8H-R, 5′-CACCCTCACGGATCTCCCCCGGAACTGTTCTGTACGG-3′, respectively) with PrimeStarMax DNA polymerase (TaKaRa). Amplified DNA was subcloned into the NdeI-XbaI cloning site of pT7-7 plasmid, which had been modified for adding the His tag to the C-terminus of recombinant proteins as in our previous report (23). The pT7-7-HdRed recombinant plasmid was transfected to E. coli BL21(DE3) and cultured at 37 °C for 16 h in 2 liters of 2 × YT medium and further incubated at 20 °C for 1 h. Then expression of recombinant HdRed (rHdRed) was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to make the final concentration 0.1 mM. After the incubation at 20°C for 12 h, cells were harvested by centrifugation 8,000 × g for 15 min, suspended in 60 ml of 10 mM imidazole-HCl (pH 7.4), 0.5 mM NaCl, and 0.5% Triton X-100, and sonicated with an ULTRASONIC homogenizer VP-050 (TAITEC, Saitama, Japan) at 20 kHz and 25 watts for 15 s 10 times on ice. The cell lysate was centrifuged at 10,000 × g for 15 min, and the supernatant was mixed with 250 μl of Ni-NTA resin (Qiagen, Hilden, Germany). The resin was suspended at 4 °C for 30 min and collected by centrifugation at 800 × g for 10 min. The resin was set to a disposable plastic column (1 × 5 cm); washed with 10 ml of 30 mM imidazole-HCl (pH 7.4), 0.5 mM NaCl; and eluted with 5 ml of 150 mM imidazole-HCl (pH 7.4), 0.5 mM NaCl. The rHdRed eluted was dialyzed against 10 mM sodium phosphate buffer (pH 7.4), 0.1 mM NaCl and stored on ice until use.

**Phylogenetic Analysis for HdRed and AKR Enzymes**—Phylogenetic analysis for the amino acid sequences of HdRed and AKRs was carried out using sequence data enrolled in GenBank™ and the AKR Superfamily. The amino acid sequences retrieved from these data bases were first aligned with the sequence of HdRed by the ClustalW program and then manually adjusted in reference to conservative functional residues of the AKR superfamily enzymes (e.g. human aldehyde reductase (AKR1A1, GenBank™ accession number NP_006057). The aligned sequences were trimmed with GBLOCKS, and a phylo-
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...mantic tree was drawn by the maximum likelihood algorithm on the basis of the LG model implemented in the Molecular Evolutionary Genetics Analysis version 6.0 (MEGA 6) software. The bootstrap values were calculated from 1,000 replicates. Classification of HdRed into an AKR family was conducted according to the nomenclature provided by Jez et al. (26) and Penning (27).

**Results**

**Isolation and Characterization of HdRed**—HdRed was successfully purified from the hepatopancreas crude enzyme at a yield of 0.5% with the specific activity 13.75 units/mg by ammonium sulfate fractionation followed by the conventional column chromatographies (Table 1 and Fig. 2, A and B). The specific activity of HdRed 13.75 units/mg was comparable with those of A1-R (56.9 units/mg) from Sphingomonas sp. A1 (21), A1-R/H11032 (42.8 units/mg) from the same strain, and FlRed (4.0 units/mg) from Flavobacterium sp. UMI-01 (23). The molecular mass of HdRed was estimated to be 40 kDa by SDS-PAGE (Fig. 2), whereas it was estimated to be >80 kDa by gel filtration through Superdex 75 10/300 GL. Namely, HdRed eluted between blue dextran 2,000 (2,000 kDa) and conalbumin (75 kDa) in this chromatography (Fig. 2B). This estimation was supported by Superdex 200 10/300 GL gel filtration. These results suggested that HdRed was in an oligomeric form, probably dimer, under non-denaturation conditions. AKR superfamily enzymes are generally known as monomeric enzymes; however, some AKRs, such as rat liver aflatoxin dialdehyde reductase (34) and Candida tenuis xylose reductase (35), are known as dimeric enzymes. The physiological significance of the oligomer formation of AKRs has remained unclear.

HdRed showed an optimal temperature and pH at around 50°C and 7 and withstood the incubation at 30°C for 20 min (Fig. 3). HdRed showed high preference to NADPH (specific activity in the presence of NADH was 2% of the activity in the presence of NADPH) (Table 2). HdRed showed no appreciable activity (0.01 units/mg) toward 28 authentic compounds, including α-keto-acid (α-keto-glutaric acid, pyruvic acid, and sodium phenylpyruvate), aldose (D-glucose, D-mannose, D-galactose, D-ribose, L-arabinose, and D-xylose), ketose (D-fructose), sugar alcohol (mannitol and sorbitol), deoxy sugar (D-deoxyribose and L-fucose), uronic acid (D-glucuronic acid), carboxylic acid (maleic acid, citric acid, and 2-furoic acid), aldehyde (DL-glyceraldehyde, glutaraldehyde, benzaldehyde, p-nitrobenzaldehyde, and p-phthalaldehyde), ketones (4-methyl-2-pentanone, 2,5-hexanedione, and 3-chloropropiophenone), and ester (ethyl pyruvate and ethyl benzoylecetate) (Table 2). These results indicate that HdRed is the enzyme highly specific to DEH and NADPH.

**TABLE 1**

| Preparation   | Total protein | Total activity | Specific activity | Purification | Yield % |
|---------------|---------------|----------------|-------------------|--------------|---------|
| Crude         | 4,828 mg      | 223.2 units    | 0.05 units/mg      | 1 -fold      | 100     |
| AS            | 173.4 mg      | 114.7 units    | 0.66 units/mg      | 13.2 -fold   | 51.4    |
| Butyl         | 16.1 mg       | 15.4 units     | 0.96 units/mg      | 19.2 -fold   | 6.9     |
| MonoQ         | 0.79 mg       | 4.9 units      | 6.20 units/mg      | 124.0 -fold  | 2.2     |
| Superdex      | 0.08 mg       | 1.1 units      | 13.75 units/mg     | 275.0 -fold  | 0.5     |

*One unit of HdRed was defined as the amount of enzyme that consumes 1 μmol of NADPH/min.

**FIGURE 2.** Purification of HdRed by column chromatographies on Mono-Q and Superdex 75. A, proteins partially purified by TOYOPEARL Butyl-650M column chromatography were dialyzed against 10 mM sodium phosphate (pH 7.0) and subjected to Mono-Q 5/50 GL column chromatography. Each fraction contained 0.5 ml. The active fractions indicated by a solid bar were pooled. B, the active fraction obtained in the Mono-Q 5/50 GL column chromatography was subjected to gel filtration through Superdex 75 10/300 GL. Each fraction contained 0.5 ml. Elution positions of blue dextran 2,000 (2,000 kDa) and conalbumin (75 kDa) are indicated with arrows with their molecular masses in the chromatogram. The active fractions indicated by a solid bar were pooled and used for characterization of HdRed. SDS-PAGEs for the active fractions are shown in the inset of each panel. Mk, molecular mass markers; mAUI, milliabsorbance units.
Then the reaction products of HdRed were analyzed by thin layer chromatography (Fig. 4, A and B). Before the reaction, the substrate DEH on the TLC plate was hardly detected by sulfuric acid staining; however, a new band (KDG-like product) appeared after the reaction (Fig. 4A). Conversion of DEH to KDG-like product was more clearly shown by thiobarbituric acid staining. Specifically, the substrate DEH gradually decreased by the extension of reaction time, and the KDG-like band concomitantly increased (Fig. 4B). It should be noted that the color intensity of the KDG-like band was considerably higher than that of DEH (Fig. 4B). This may be ascribable to the difference in liability to periodic acid degradation between DEH and KDG-like product. Because thiobarbituric acid staining (29) is based on the color-developing reaction between thiobarbituric acid and malondialdehyde produced from the deoxy-sugars like DEH and KDG by periodic acid pretreatment, staining intensity generally correlates with the amount of malondialdehyde produced. Then the KDG-like product was subjected to MS to examine whether it was indeed KDG (Fig. 5).

**TABLE 2**

| Substrate/cofactor | Relative activity |
|--------------------|------------------|
| Cofactor (electron donor) |  |
| NADPH | 100.0 |
| NADH | 2.2 |
| Substrate (electron acceptor) |  |
| DEH | 100.0 |
| α-Keto acid: α-ketoglutaric acid, pyruvic acid, sodium phenylpyruvate | ND |
| Aldose: D-glucose, D-mannose, D-galactose, D-ribose, L-arabinose, D-xylene | ND |
| Ketone: D-fructose | ND |
| Sugar alcohol: mannitol, sorbitol | ND |
| Deoxy sugar: d-deoxyribose, l-fucose | ND |
| Uronic acid: D-glucuronic acid | ND |
| Carboxyl acid: maleic acid, citric acid, 2-furoic acid | ND |
| Aldehyde: D-glyceraldehyde, glutaraldehyde, benzaldehyde, p-nitrobenzaldehyde, p-phthalaldehyde | ND |
| Ketone: 4-methyl-2-pentanone, 2,5-hexanedione, 3-chloropropiophenone, Ester: ethyl pyruvate, ethyl benzoylacetate | ND |

**FIGURE 3. Effects of temperature and pH on the activity of HdRed.** A, temperature-dependent activity of HdRed was measured at 15–75 °C in a reaction mixture containing 1 mM DEH, 1 mM NADPH, 10 mM sodium phosphate (pH 7.0), and 0.005 mg/ml HdRed. B, thermal stability of HdRed was assessed by measuring the activity remaining after the heat treatment at 5–75 °C for 20 min. C, pH dependence of HdRed was determined with reaction mixtures of 20 mM sodium acetate buffer (filled triangles), 10 mM sodium phosphate buffer (filled circles), and 20 mM glycine-NaOH buffer (filled squares). Error bars, S.D.

**FIGURE 4. Thin layer chromatography of the reaction products produced by HdRed.** The reaction was carried out in a reaction mixture containing 10 mM DEH, 10 mM NADPH, 10 mM sodium phosphate buffer (pH 7.0), and 0.08 units/ml HdRed at 30 °C for 10–180 min. The reaction products were developed on a TLC-60 plate. A, materials were stained by the sulfuric acid method (28). B, materials were stained by the thiobarbituric acid method (29). RP, reaction product. NADPH in the reaction mixture was detected only by the sulfuric acid method.

*ND, not detected (<1.0%).
COOH) was detected as a 175 m/z peak (Fig. 5A), whereas a new peak with 177 m/z corresponding to KDG (CH₂OH-CHOH-CHOH-CH₂-C(=O)-COOH) appeared by the reaction and increased along with the extension of reaction time (Fig. 5, B–E). Essentially the same results have been obtained in the reduction of DEH to KDG by FlRed (23). To confirm the formation of KDG by HdRed, chemical modification analysis with DMB (35) was performed. The result indicated that the KDG-like reaction product exhibited a fluorescence peak at around 490 nm as did DEH (data not shown). This indicated that the α-keto group of DEH was not reduced by HdRed. Accordingly, we concluded that HdRed reduced the aldehyde group of DEH, producing KDG.

Primary Structure Analysis for HdRed—To obtain information about the primary structure of HdRed, we first attempted to determine the N-terminal amino acid sequence with a protein sequencer. However, it was unsuccessful, probably due to the blocking of N terminus by a certain modification. Therefore, we determined the internal amino acid sequences of HdRed by MS/MS using tryptic fragments. As a result, amino acid sequences of eight fragments (P1 (Y(L/I)GASNM(L/I)-GWQMQR), P2 (D(L/I)VPVSV(L/I)L/I)GTTK), P3 (L/I)PYPYEFNTR), P4 (SES(L/I)L/GSW(L/I)K), P5 (VYYNF(L/I)GK), P6 (S(L/I)AQV5(L/I)R), P7 (FV(L/I)ATK), and P8 (WV(L/I)QK)) were predicted. By the BLAST search using these sequences, some proteins and hypothetical proteins were retrieved. They were oxidoreductases and aldo-keto reductases from bacteria, cyanobacteria, polychaetes, gastropods, etc. Among them, the most closely related to HdRed was considered to be the norsolorinic acid (a kind of polyketide) reductase-like protein (XP_005112670) annotated in the genome of Aplysia californica. Namely, all of the partial amino acid sequences determined with P1–P8 could be located in the deduced sequence of the Aplysia putative enzyme with 50–80% identities. Then we attempted to identify the cDNAs encoding HdRed in the abalone transcriptome data by the local BLAST (BLAST+ program (National Center for Biotechnology Information)) using the deduced amino acid sequence of the Aplysia putative enzyme as a reference sequence. As a result, a contig sequence (Comp34519_c0_seq1) comprising 2,212 bp including an entire translational region of 371 amino acids was retrieved as the candidate for HdRed cDNA. Then we designed specific forward and reverse primers (5′-AGTTTTTCTGACAGCTGGACGTGTGC-3′ and 5′-GCGATTTCATTGTCCCCGGTGAAG-3′, respectively) on the basis of nucleotide sequences of 5′- and 3′-untranslated regions of Comp34519_c0_seq1 and amplified the cDNA by the PCR from abalone hepatopancreas cDNAs. As a result, a cDNA, named HdRed cDNA, comprising 1,215 bp encoding the amino acid sequence of 371 residues and a stop codon, was obtained (Fig. 6). All of the partial amino acid sequences determined with tryptic fragments P1–P8 were seen in the deduced amino acid sequence.

FIGURE 5. Mass spectrogram of the reaction products produced by HdRed. The reaction was carried out as described in the legend to Fig. 4. Reaction products obtained at 0, 10, 30, 90, and 180 min were subjected to mass spectrometry. The peak at 175 m/z corresponds to DEH, whereas that at 177 m/z corresponds to KDG.
The nucleotide and deduced amino acid sequences of HdRed cDNA are available from the DNA Data Bank of Japan, GenBank™, and EMBL with the accession number LC069040. In the nucleotide sequence of HdRed cDNA, the translational initiation codon (ATG) was seen in the nucleotide positions from 70 to 72, whereas the termination codon (TGA) was located from 1,183 to 1,185. Putative polyadenylation signal sequences were found in the 3'/H11032- untranslated region; however, we could not identify the poly(A)/H11001 tail. No signal peptide region was predicted in the N terminus by the SignalP 4.0 server, suggesting that HdRed was a cytosolic enzyme. Then we verified that HdRed cDNA was indeed encoding HdRed protein by using rHdRed. rHdRed was expressed with an E. coli expression system described under “Experimental Procedures.” As shown in Fig. 7A, a major part of rHdRed was in the insoluble fraction; however, ~20 μg of rHdRed could be prepared from the soluble fraction by Ni-NTA affinity chromatography. The rHdRed was confirmed to exhibit DEH-reducing activity with a specific activity of 13 units/mg, which is comparable with that of native HdRed (see Table 1). TLC analysis also indicated that HdRed produced KDG from DEH (Fig. 7B), as did native HdRed (Fig. 4). From these results, we may conclude that the HdRed cDNA is actually encoding HdRed protein.
the conversion of DEH to KDG by rHdRed appeared not to be completed (Fig. 7B). We consider that this is due to the instability of rHdRed compared with native HdRed because we had frequently experienced such instability of recombinant molluscan enzymes.\(^3\) Specifically, the long reaction time for TLC analysis may have caused the inactivation of rHdRed.

Sequence Comparison between HdRed and Other Aldo-Keto Reductases—The BLAST search for the amino acid sequence of HdRed retrieved several invertebrate hypothetical enzymes and proteins annotated in genomes (e.g., the above described norsolorinic acid reductase-like isoform X2 of sea hare *A. californica* (GenBank\(^\text{TM}\) accession number XP_005112670), limpet *L. gigantea* hypothetical protein (LoAKR, accession number ESP03523.1), halophilic bacterium *H. volcanii* reductase (HaAKR, accession number AAB71807), and human aldehyde reductase (HoALR, accession number NP_006057). Identical residues between HdRed and other proteins are marked in yellow. Catalytic residues known in AKR superfamily enzymes are marked in red. \(\alpha\)-Helices (H1–H8, HA, and HB), \(\beta\)-sheets (SA, SB, and S1–S8), and loops (Loops A–C) identified in HoALR (36) are indicated with solid lines, double lines, and dotted lines, respectively. Cofactor binding residues identified in human aldehyde reductase (36) are boxed. Amino acid identities between HdRed and ApAKR, LoAKR, HaAKR, and HoALR are estimated to be 60, 58, 36, and 18%, respectively.

**FIGURE 8.** Alignment of the amino acid sequences of HdRed with other AKR and AKR-like proteins. The amino acid sequence of HdRed was aligned with those of sea hare *A. californica* norsolorinic acid reductase-like protein (ApAKR, accession number XP_005112670), limpet *L. gigantea* hypothetical protein (LoAKR, accession number ESP03523.1), halophilic bacterium *H. volcanii* reductase (HaAKR, accession number AAB71807), and human aldehyde reductase (HoALR, accession number NP_006057). Identical residues between HdRed and other proteins are marked in yellow. Catalytic residues known in AKR superfamily enzymes are marked in red. \(\alpha\)-Helices (H1–H8, HA, and HB), \(\beta\)-sheets (SA, SB, and S1–S8), and loops (Loops A–C) identified in HoALR (36) are indicated with solid lines, double lines, and dotted lines, respectively. Cofactor binding residues identified in human aldehyde reductase (36) are boxed. Amino acid identities between HdRed and ApAKR, LoAKR, HaAKR, and HoALR are estimated to be 60, 58, 36, and 18%, respectively.

sequences of the *Aplysia* and *Lottia* putative enzymes (amino acid identity \(\approx 60\%\) with HdRed) and those of well-characterized human aldehyde reductase AKR1A1 (25–27, 36) (identity 18% with HdRed) and a reductase from a halophilic bacterium *Haloferax volcanii* (AAB71807) (identity 36% with HdRed) for the comparison with HdRed. As shown in Fig. 8, the catalytic residues of AKR superfamily enzymes (25–27) were found to be well conserved in invertebrate enzymes (i.e., they are seen as Asp-64, Tyr-69, Lys-95, and His-137 in the HdRed sequence). Further, some cofactor binding regions identified in human aldehyde reductase (36) also appeared to be conserved in the invertebrate enzymes (i.e., they are seen as Asp-64, Tyr-69, Lys-95, and His-137 in the HdRed sequence). Further, some cofactor binding regions identified in human aldehyde reductase AKR1A1 (36) also appeared to be conserved in the invertebrate enzymes (e.g., Thr-29, Asp-64, Ser-222, Leu-224, and Asn-317 of HdRed). However, secondary structural motifs identified in AKR1A1 appeared not to be well conserved. In addition, many insertions and gaps are needed to obtain maximal homology between the invertebrate and human enzymes. Thus, invertebrate AKRs appear to be significantly deviated from the vertebrate AKRs. Enzymatic properties of the invertebrate enzymes other than HdRed have not yet been characterized. Thus, it should be examined whether these invertebrate enzymes also show DEH-reducing activity because these invertebrates are seaweeds feeding animals like abalone (37–39).
Phylogenetic Analysis for HdRed—The AKR superfamily includes more than 190 members that have been classified into 15–16 families on the basis of amino acid sequence similarity (26, 27) (see the AKR Superfamily Web page on the University of Pennsylvania Perelman School of Medicine Web site). To examine which family HdRed belongs to, a phylogenetic tree was drawn using 59 amino acid sequences of representative AKRs from 15 families (AKR1–AKR15) and seven sequences of marine invertebrate putative AKRs, including HdRed (Table 3). We could not include AKR16 in the analysis because no sequence data of this family were available. As shown in Fig. 9, the phylogenetic tree implied that HdRed along with the marine invertebrate putative enzymes are closely related enzymes that can be arranged as an independent cluster distinct from other AKRs. The invertebrate enzyme cluster appears to be branched from AKR9, suggesting that the invertebrate enzymes belong to AKR9. However, according to the nomenclature for the AKR family, the invertebrate enzymes could not be included in AKR9. Specifically, in the nomenclature for the AKR family (26, 27), delineation of families is defined by a 40% amino acid identity level, and members of the same AKR family should have >40% amino acid identity with any other family. The amino acid identity among invertebrate enzymes was at a level of 50–60%, whereas the closest family 9 member, AKR-9C1 (aryl-alcohol dehydrogenase from H. volcanii), showed 36% identity. Therefore, we should delineate the invertebrate enzymes as a new AKR family. Because 16 families are currently defined in the AKR superfamily (27), the new invertebrate family can be delineated as AKR17.

Discussion

The Pacific abalone H. discus hannai is a typical herbivorous marine gastropod that feeds on brown seaweeds containing alginate. This animal possesses endolytic and exolytic alginate lyases (i.e. HdAly (14) and HdAlex (16)) and degrades the seaweeds’ alginate to unsaturated oligosaccharides and a C4-C5 unsaturated monouronate (Fig. 1). The monouronate spontaneously changes to an open-chain form, DEH; however, DEH has yet to be proven to be used as a carbon and energy source in abalone. In the present study, we isolated HdRed as a DEH-reducing enzyme of abalone and identified it as an AKR superfamily enzyme.

HdRed is an NADPH-dependent enzyme with high specificity to DEH (Table 2). In alginolytic bacteria, both NADPH-dependent and NADH-dependent DEH reductases have been identified. Namely, A1-R from Sphingomonas sp. A1 is NADPH-dependent (21), and A1-R’ from Sphingomonas sp. A1 (22) and FlRed from Flavobacterium sp. UMI-01 (23) are NADH-dependent. The bacterial DEH reductases were classified into the short chain dehydrogenase/reductase superfamily, whereas abalone DEH reductase was classified into the AKR superfamily.

The amino acid sequence of HdRed comprised 371 amino acid residues showing 50–60% identity with those of other invertebrates’ putative AKRs (Fig. 8). The most closely related protein to HdRed was a norsolorinic acid reductase-like protein annotated in the A. californica genome (GenBank accession number XP_005094978.1, 60% identity with HdRed). Catalytically important amino acid residues of AKR superfamily enzymes (e.g. catalytic tetrad and cofactor–binding residues) are fairly well conserved in HdRed and other invertebrate putative enzymes (Fig. 8). Amino acid identities between HdRed and the invertebrate putative enzymes are in the range of 50–60%; however, it is only 18–25% between HdRed and mammalian AKRs. HdRed and other invertebrate AKRs form an independent cluster in the phylogenetic tree (Fig. 9). This cluster appears to have deviated from AKR9; however, the invertebrate enzymes could not be included in the AKR9 according to the nomenclature for the AKR superfamily (26, 27) because the most closely related enzyme, AKR9C1, showed 36% identity. Therefore, we propose that HdRed and other invertebrate putative enzymes should be delineated as a new AKR family (e.g. AKR17). Because HdRed is the first enzyme of AKR17, the identification code AKR17A1 should be given to HdRed. To clarify the characteristics of AKR17, properties of other invertebrate AKRs should be investigated at the protein level. In this context,

### Table 3

| Family | AKR ID (accession number) |
|--------|--------------------------|
| 1      | 1A1 (NP_006057), 1B1 (NP_006169), 1B7 (P21300), 1B12 (C4AC8011), 1C1 (NP_001344), 1C7 (P05980), 1C15 (2008147B), 1C12 (NP_008805), 1D1 (NP_005980), 1E1 (AAB37274), 1E2 (Q691D6), 1G1 (NP_509242) |
| 2      | 2A1 (P28475), 2A2 (AAB97617), 2B1 (P31867), 2C1 (CAA98021), 2D1 (AAB61912), 2E1 (CA4B4997), 2E3 (AAD38635) |
| 3      | 3A1 (P14065), 3B1 (AAAB17362), 3C1 (CA2A5107), 3C2 (T37996), 3D1 (AAK55762), 3E1 (CAD43580), 3F1 (F272218), 3F2 (A64745) |
| 4      | 4A1 (P26690), 4B1 (CAA11226), 4B2 (AA1F17339), 4B4 (AAB97005), 4C1 (P23901), 4C5 (CA532834) |
| 5      | 5A1 (P20405), 5B1 (AAAB3334), 5C1 (Q46657) |
| 6      | 6A1 (AA53131), 6A3 (S66503), 6B1 (AA464631), 6C1 (AA887294) |
| 7      | 7A1 (P38918) |
| 8      | 8A1 (T3N918) |
| 9      | 9A1 (Q07277), 9A3 (Q01752), 9B1 (NP_014068), 9C1 (AAB71807) |
| 10     | 10A1 (AAD28516) |
| 11     | 11A1 (P46336), 11B1 (P80874), 11B2 (C64937), 11C1 (C83776) |
| 12     | 12A1 (AAD41821), 12B1 (AA84068), 12C1 (BAA45499) |
| 13     | 13A1 (Q99923), 13B1 (AA864538), 13C1 (O22804) |
| 14     | 14A1 (AA59168) |
| 15     | 15A1 (BAC97800) |
| Invertebrates | |
| HdRed (LC069040) | (H. discus hannai), ApAKR (XP_005112670) (A. californica), LoAKR (ES03523.1) (L. gigantean), CrAKR (EKC20233.1) (C. gigas), CaAKR (ELT99608.1) (Cetiella teletea), BaAKR (XP_002594628.1) (Branchiostoma floridae), StAKR (XP_792510.2) (S. purpuratus) |

DEH Reductase from Abalone
we recently examined the occurrence of DEH-reducing activity in several seaweed-feeding gastropods. According to our preliminary TLC analyses, hepatopancreas extracts from \textit{Littorina brevicula}, \textit{Aplysia kurodai}, and \textit{Omphalius rusticus} were shown to exhibit obvious DEH-reducing activity. On the other hand, the extracts from bivalves, such as \textit{Mizuhopecten yessoensis} and \textit{Spisula sachalinensis}, did not show appreciable activity. The above gastropods are known to possess alginate lyase (37–39), whereas the bivalves have not been shown to possess alginate lyase. These facts imply that the alginate lyase and DEH reductase are intimately related enzymes and take part in the assimilation of alginate in gastropods. Thus, it is important to examine the distribution of DEH reductase in as many algino-lytic gastropods as possible and to investigate whether such enzymes are also classifiable in the AKR superfamily like \textit{HdRed}.

The pacific abalone \textit{H. discus hannai} feeds on various kinds of seaweeds and digests seaweeds’ polysaccharides with polysaccharide-degrading enzymes (e.g. cellulase (40), alginate lyase (14, 16), laminarinase (41), mannanase (42), and amylase (43)) in the digestive tract. The degradation products of seaweeds’ \(\alpha\)- and \(\beta\)-glucans and \(\beta\)-mannans (i.e. glucose and mannose, respectively) are easily assimilated by the Embden-Meyerhof glycolytic system of abalone; however, the end product of alginate (i.e. DEH) cannot be metabolized by this system. In algino-lytic bacteria, alginate-derived DEH is first reduced to KG and metabolized through the Entner-Doudoroff pathway (19, 20). In a pathway revealed in bacteria and archaea, KG is first phosphorylated to 2-keto-3-deoxy-6-phosphogluconate by KG kinase (2-keto-3-deoxygluconate kinase), and 2-keto-3-deoxy-6-phosphogluconate is split into glycer-aldehyde-3-phosphate and pyruvate by 2-keto-3-deoxy-6-phosphogluconate aldolase (19, 20, 44). In another pathway known in archaea, DEH is directly split into glyceraldehyde and pyruvate by KG aldolase (45). To date, the KG kinase gene has not been discovered in eukaryote genomes or in the transcriptome of abalone. According to our recent results, KG was not phosphorylated in the presence of ATP-Mg\(^{2+}\) by partially purified abalone enzyme. On the other hand, pyruvate was produced from KG by the abalone enzyme.\(^3\) These results suggested that alginate-derived DEH in abalone is first reduced to KG by \textit{HdRed} and then split into glyceraldehyde and pyruvate by a KG aldolase-like enzyme. To fully understand the metabolic pathway of alginate in...
abalone, we are now trying to identify the KDG aldolase-like enzyme in abalone.

Author Contributions—S. M. conducted the isolation and characterization of the DEH reductase HdRed. R. N. and A. I. conducted transcriptome analyses for abalone hepatopancreas and prepared recombinant HdRed. T. O. designed the work and wrote the manuscript.

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