further exon/intron analysis was performed with Spidey program at NCBI.

Expression and the purification of recombinant XI

Ciona intestinalis cDNA clone CIEM 826e01 (GenBank gi: 23589613), which contains the *XI* gene, was from Nori Satoh's lab. Using this cDNA clone as a template, PCR was performed with the primers xylP1 (tttcatatgtcctcgtttgctccagcatc) and xylP2 (aaactcgagctaaaaa tatcggtttaaaactgcttc). After digestion with *Xho*I and *Nde*I, this fragment was ligated into pET28-a(+) plasmid (Novagen, Madison, WI, USA) and then transformed into the *Escherichia coli* BL21(DE3) strain. The recombinant-expressed xylose isomerase will have the hex-His tag at the N-terminal in the resulted expression plasmid. The overnight culture of BL21(DE3) containing the expression plasmid was inoculated 1:100 into 200 ml LB medium with 25 μg/ml kanamycin and was cultured at 37°C for 30–60 min. When the value of OD_{600} reached ~0.8, 0.1 mM IPTG was added and the culture was shaken in the incubator at 20°C. After induction with 1 mM IPTG for 6 h, the bacteria was harvested by centrifugation at 6000 *g* and was re-suspended in 50 ml buffer A (50 mM Tris, 500 mM NaCl, and 20 mM $MgCl_2$, pH 7.5). After pulsed supersonic disruption for 10 min, the final cell extract was centrifuged at 12,000 *g* and the supernatant was collected. The enzyme was purified as described previously [11]. Briefly, supernatant was loaded onto a 10 ml Ni^{2+}-chelating column. The column was washed with 40 ml washing buffer (buffer A containing 50 mM immidazole) and then eluted with elution buffer (buffer A containing 200 mM immidazole). The eluted fractions were collected and were desalted with Sephedex G25 column for further analysis. SDS–PAGE was performed according to Laemmli [12]. The Bradford method was used to determine the protein concentration [13].

Enzyme assays

Xylose isomerase activity was assayed by the method of Dische and Borenfreund [14]. Reaction was performed in buffer consisted of 200 mM Na_2HPO_4/NaH_2HPO_4 and 25 mM Mg^{2+}, pH 6.0. D-glucose, D-fructose, D-xylose, and D-xylulose (Sigma-Aldrich, St Louis, MO, USA) were used as substrates or standards to determine the enzyme activity unit. One unit was defined as the amount of enzyme needed to transform 1 μmol D-xylose (or D-glucose) into D-xylulose (or D-fructose) in 1 min.

The properties of the recombinant enzyme were studied by determining the activities of isomerase at various temperatures from 25 to 85°C, or at different pH conditions from pH 2.0 to pH 11. The thermal stability of this enzyme was also explored by measuring the isomerase activities at 75°C and pH 7.0 after incubating the purified enzyme at various temperatures. And the different concentrations of Mg^{2+} were added to study the effect of Mg^{2+} on the recombinant XI's thermal stability. EDTA was added as the chelator of Mg^{2+}. The K_m (the Michaelis constant) values of this enzyme with substrates of D-glucose and D-xylose were determined by the double reciprocal plotting method.

Results

Bioinformatic analysis for the enzymes participating the D-xylose metabolism in animals

To get the clues for the possible pathway of animals for utilizing the D-xylose, the NCBI EST database was screened with Blast program. Genes of bacteria, fungi, and plant from the two different D-xylose metabolic pathways, the xylose isomerases (XI) or the xylose reductases (XR), were used as seeds. The search was restricted in only metazon species. During the search on EST database, many animals, whose genome had been sequenced, were found to have a conserved XI pathway. No conserved XR genes were found in the metazon species. The result was listed in **Table 1**. In this unexpected resultant list, it was shown that xylose isomerases distribute from plant-like marine animal (sea squirt) to highly evolved vertebrate animal (bony fish). To confirm whether these genes were produced from the pollution during the construction of EST library, several sequences of these genes were mapped in their original genome database. The genomic exon/intron arrangement of *C. intestinalis, Strongylocentrotus purpuratu*, and *Tetraodon nigroviridis* was shown in **Fig. 1**. The genomic structure of the plant xylose isomerase from *Arabidopsis thaliana* was also shown as a reference. Their complicated exon/intron structures can prove that these animal species have this enzyme undoubtedly.

Multi-sequences alignment and phylogenetic analysis of xylose isomerases

With the ClustalW program, we compared the animal xylose isomerase sequences from *C. intestinalis*, *S. purpuratu*, and *T. nigroviridis* together with bacterial xylose isomerase sequences from *Thermotoga neapolitana*, *Bacillus subtilis*, and *Pirellula* sp. (**Fig. 2**). This result indicated that xylose isomerases were highly conserved during the evolution. The phylogenetic analysis was

Table 1 Animal xylose isomerase—the species and GenBank accession numbers

Species	NCBI accession numbers
Ciona intestinalis (Sea squirt)	gi|23589613
Ciona savignyi (Sea squirt)	gi|51739659
Molgula tectiformis (Sea squirt)	gi|67750489, gi|67740500
Strongylocentrotus purpuratus (Urchin)	gi|72029353, gi|57581578, gi|62385241, 57954432
Carcinus maenas (Shore crab)	gi|77991721|, gi|85056494, gi|77991220, gi|80465239, gi|61018984
Eurydice pulchra (Isopod)	gi|51098886, gi|48911230, gi|51099029, gi|48911415, gi|48911455
Euprymna scolopes (Mollusc)	gi|84425950
Helix aspersa (Mollusc)	gi|66908236
Bombyx mori (Silk moth)	gi|6902029, gi|90672402
Tetraodon nigroviridis (Bony fish)	gi|56248221
Takifugu rubripes (Bony fish)	gi|21882201
Oncorhynchus mykiss (Bony fish)	gi|90065851, gi|24597935, gi|90114356, gi|56977367, gi|24596860
Gasterosteus aculeatus (Bony fish)	gi|61960499, gi|76117336, gi|62058202, gi|83760392, gi|76028410, gi|62058200
Salmo salar (Bony fish)	gi|85033289, gi|40541606, gi|45319917, gi|83640423, gi|84210798, gi|45304301
Hippoglossus hippoglossus (Bony fish)	gi|90598664, gi|90605369, gi|90597886

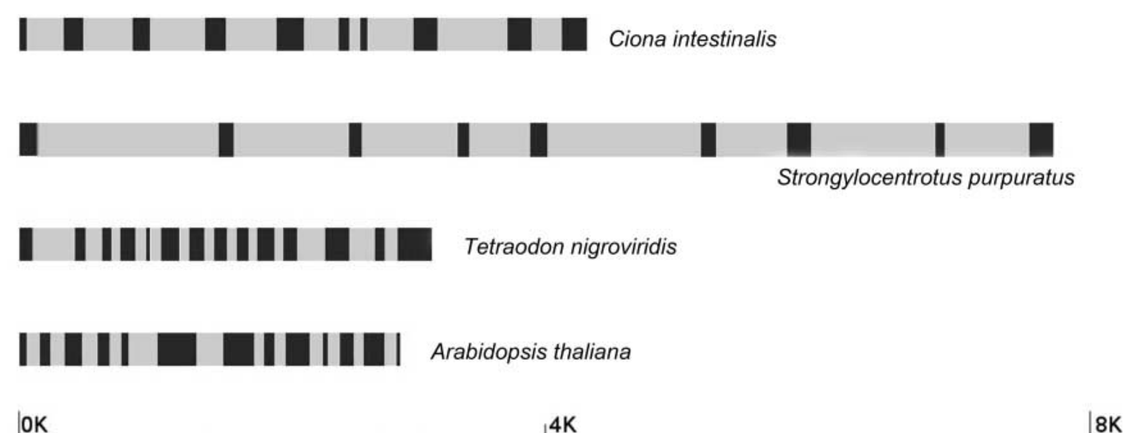

Fig. 1 Exon and intron structure mapped in the genome The exon was shown with black and the intron was shown with gray. Length of the nucleotides was shown at the bottom of this figure.

carried out with PHYLIP program, and the Neighbor Joining analysis was used. The final phylogenetic tree was shown in **Fig. 3**.

Expression and characterization of xylose isomerase from *C. intestinalis*

The xylose isomerase from *C. intestinalis* was selected and was heterogeneously expressed in *E. coli* to confirm its enzymatic function. With the pET expression system and induction at low temperature (20°C), we got enough amount of recombinant enzyme for further analysis. The SDS−PAGE of the 51 kDa *C. intestinalis* xylose isomerase purified from recombinant *E. coli* BL21(DE3) was shown in **Fig. 4**. At 30°C and pH 6.0, its specific activity on xylose substrate was 0.331 μmol/mg/min and the specific activity on glucose as substrate was 0.006 μmol/mg/min. This recombinant xylose isomerase was thermal stable. Its optimum reaction temperature is at 75°C as shown in **Fig. 5(A)**. The optimum pH of this enzyme was explored at this temperature. The result showed that at pH 7.0, this enzyme had a maximum activity on the substrate of D-xylose. All properties were summarized in **Table 2.** In the **Fig. 5(B)**, at pH 7.0 and the optimum temperature, this enzyme's activity on D-xylose can reach 2.172 μmol/mg/min. Its thermal stability was also affected by the concentration of Mg^{2+}, and

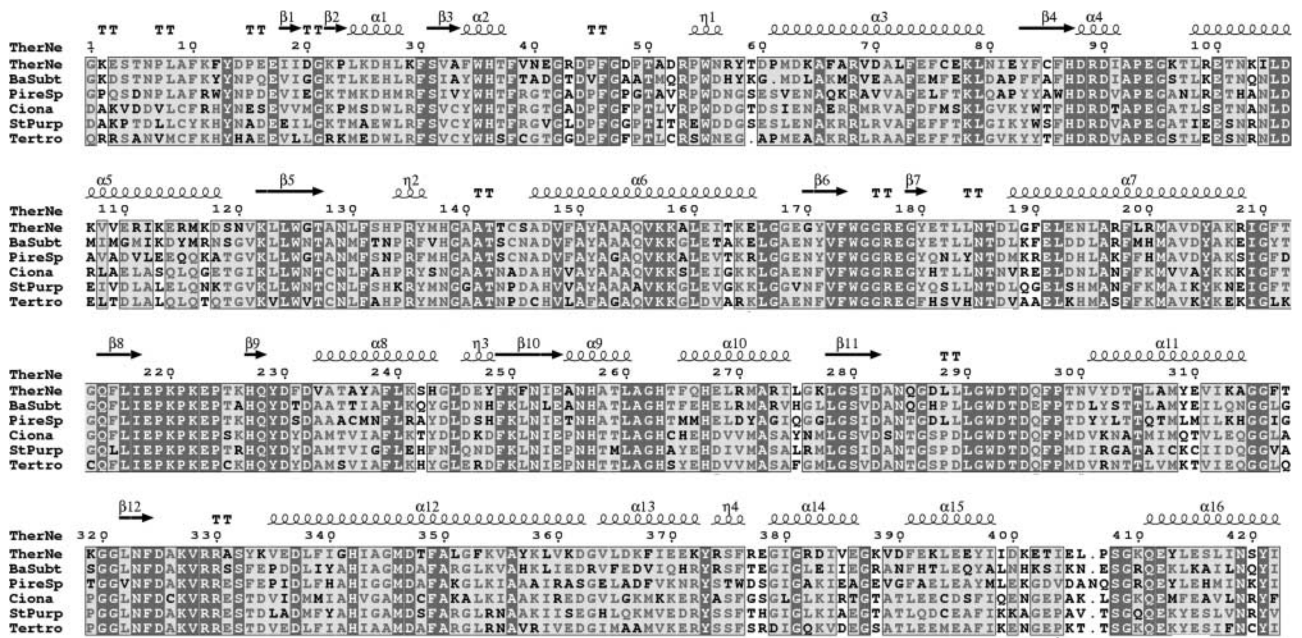

Fig. 2 Alignment of animal XIs with bacterial XIs TherNe, *Thermotoga neapolitana*; BaSubt, *Bacillus subtilist*; PireSp, *Piromyces* sp. E2; Ciona, *Ciona intestinalis*; StPurp, *Strongylocentrotus purpuratus*; Tertro: *Tetraodon nigroviridis*.

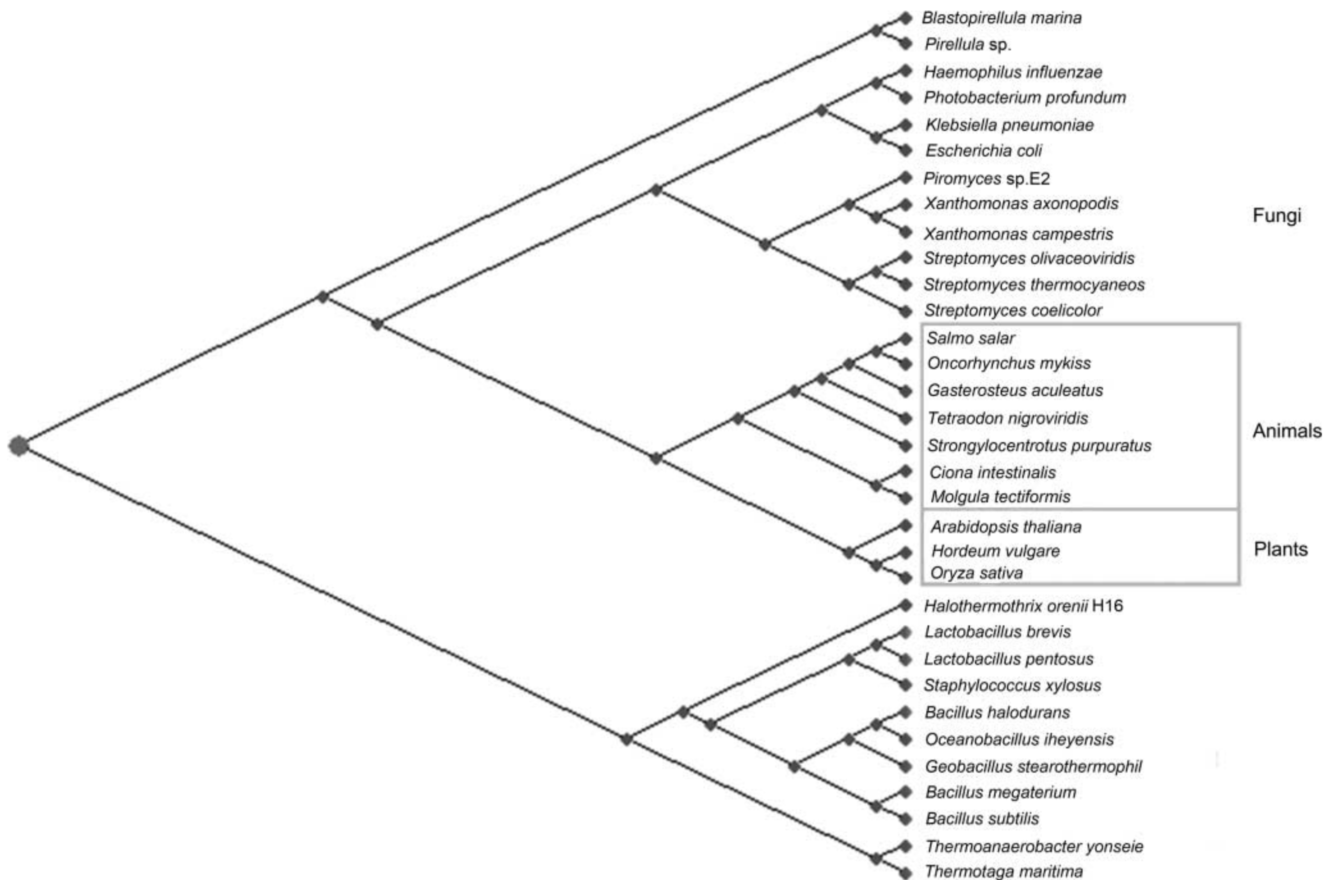

Fig. 3 Phylogenetic tree of the xylose isomerases from different species The fungi, animal, and plant species were marked specifically, the others were all bacterial. The XI of *Piremyces* sp. E2 was the only one case found in fungi species.

when EDTA was added to the enzyme, this enzyme became very unstable (**Fig. 6**).

Discussion

This report on the xylose isomerase of *C. intestinalis* was the first evidence that some animals can utilize the D-xylose directly with this enzyme. We got the enough amount of heterogeneously expressed enzyme from *E. coli* to explore its enzymatic characteristics. The xylose isomerase from *C. intestinalis* had good thermal stable characters with the Mg^{2+}. Its optimum temperature was at 75°C. This result was similar with the xylose isomerase from *T. neapolitana*, a thermophilic bacteria. And the xylose isomerase of *C. intestinalis* was more specific on the substrate of D-xylose than on the substrate of D-glucose. This can be explained by the different K_m values on these two different substrates. When EDTA, which can chelate the Mg^{2+}, was added into the reaction buffer, this recombinant xylose isomerase became very unstable. This character was the same as the bacteria xylose isomerases [1]. But the *C. intestinalis* xylose isomerase had higher activity against xylose ($IU_{xylose}/IU_{glucose} = 58$), and its activity against glucose was very low.

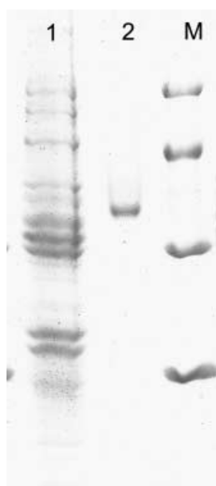

Fig. 4 SDS–PAGE of the recombinant *Ciona intestinalis* xylose isomerase purified from the *Escherichia coli* BL21(DE3) M, marker of molecular weight; lane 1, the whole cell lysate after IPTG induction; lane 2, purified xylose isomerase. The expression level of this XI in *E. coli* was very low, thus make it hardly be identified in lane 1.

The result of ClustalW indicated that xylose isomerases were highly conserved during the evolution. The consensus was very high, and the catalytic amino acids and divalent metal ions binding sites, as revealed in the crystal structures of bacterial xylose isomerases, were kept well in animal xylose isomerases. Considering that the XI is only a metabolism-related enzyme to utilize an unimportant (compared with glucose) pentose, such a case is not common.

There is only one case of xylose isomerase reported from fungi (*Piromyces* sp. *E2*) [15]. And when we performed the search in nucleotide databases, there were no other clues that can lead to the possible existence of xylose isomerase in other fungi species. But so many animal species kept this highly conserved bacterial xylose isomerase-like enzyme during the evolution. This result is very interesting and may be a strong support of some evolutionary theory [16,17]. By using PHYLIP program, we have drawn the phylogenetic inferences of the xylose isomerases from bacteria, plants, fungi, and animals. This phylogenetic tree (**Fig. 3**) shows clearly that animal xylose isomerases have an ancient origin and are the relatives of plant xylose isomerases.

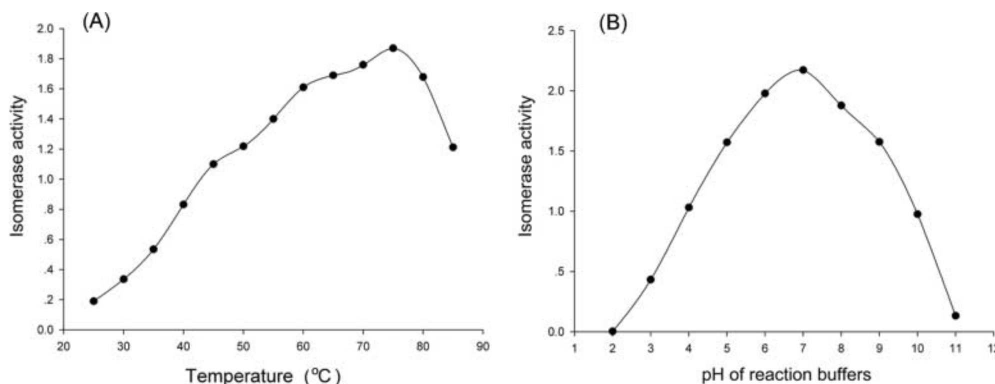

Fig. 5 The optimum temperature and the optimum pH of recombinant XI's enzyme activity (A) The isomerase activities were measured at different temperatures in the buffer of 200 mM Na_2HPO_4/NaH_2PO_4, with 25 mM Mg^{2+}, pH 6.0. (B) The activities were measured in various pH conditions at 75°C and with 25 mM Mg^{2+}.

Table 2 The enzymatic properties of the recombinant XI

Substrates	Specific activities[a] (μmol/mg/min)	K_m values[a] (mM)
D-xylose	0.331 ± 0.007	5.2 ± 0.7
D-glucose	0.006 ± 0.001	171.3 ± 10.1
L-arabinose	0.0	Not calculated
D-ribose	0.017 ± 0.003	Not calculated

[a]Averaged from the results of three parallel assays.

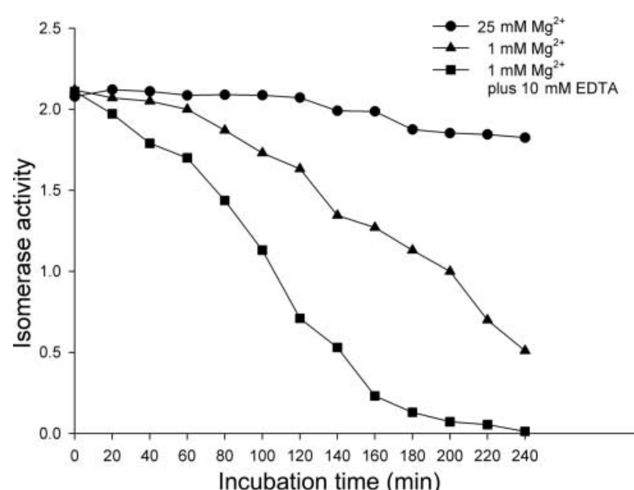

Fig. 6 Thermal stability of the recombinant XI at different concentrations of Mg^{2+} or Mg^{2+} plus EDTA Enzyme assays were performed at 75°C and pH 7.0. Before the reactions were performed, the enzyme was incubated without substrate at 75°C, and the Mg^{2+} or EDTA was added into the samples being incubated to control the concentration of Mg^{2+}.

About 20–40% of plant cell wall is composed with xylan, which becomes a vast resource of xylose. Starch and cellulose were also useful sugar resources, and they were consisted of glucose. There was a widespread belief that animals could not utilize cellulose directly until the first animal endogenous cellulase was reported in 1998 [18]. Compared with the cellulase, the endogenous xylanase we found in a mollusc, *A. crosseana* [4,19], was more puzzling because in biochemistry text book and previous studies, there was no knowledge about how animals transformed the D-xylose into the D-xylulose, which was a well-known intermediate in pentose metabolism cycle. The most common hypothesis was that animals utilize D-xylose cooperating with gut microorganism. Maybe the situation in mammalians is such, because during our database searching, it seems that they do not have xylose isomerases. But in widespread species from sea squirt to bony fish, the xylose isomerases were kept in a highly conserved form. At this point, the finding of animal xylose isomerases is not only important in the basic concept of metabolism but also can help us to understand the evolution in more detail.

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