

## Molecular Characterization of Aldolase from *Heterodera glycines* and *Globodera rostochiensis*

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**Abstract:** Fructose-bisphosphate aldolase (EC 4.1.2.13) is a key enzyme in glycolysis. We have characterized full-length coding sequences for aldolase genes from the cyst nematodes *Heterodera glycines* and *Globodera rostochiensis*, the first for any plant-parasitic nematode. Nucleotide homology is high (83% identity), and the respective sequences encode 40 kDa proteins with 89% amino acid identity. Genomic sequences contain six introns located at identical positions in both genes. Intron 4 in the *H. glycines* gene is >500 bp. Partial genomic sequences determined for seven other cyst nematode species reveal that the large fourth intron is characteristic of *Heterodera* but not *Globodera* aldolase genes. Total aldolase-like specific activity in homogenates from *H. glycines* was 2-fold lower than in either *Caenorhabditis elegans* or *Panagrellus redivivus* ( $P = 0.001$ ). Activity in *H. glycines* samples was higher in juvenile stages than in adults ( $P = 0.003$ ). *Heterodera glycines* aldolase has  $K_m = 41 \mu\text{M}$  and is inhibited by treatment with carboxypeptidase A or sodium borohydride.

**Key words:** aldolase, cyst nematode, enzyme, gene, *Globodera*, *Heterodera*, intron.

Plant-parasitic nematodes are dependent primarily upon carbohydrate metabolism for several critical life processes, and investigation of the glycolytic pathway may offer insight into development of control strategies based upon energy production. Glycolytic enzymes have been examined extensively in animal-parasitic nematodes (Komuniecki and Harris, 1995) and to a lesser extent in plant-parasitic nematodes (Barrett and Wright, 1998). A key glycolytic step responsible for the reversible cleavage of fructose 1,6-bisphosphate to the triose phosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate is catalyzed by fructose-1,6-bisphosphate aldolase (EC 4.1.2.13). Aldolase has been biochemically characterized in free-living (Ells, 1969; Inoue et al., 1997; Reznick and Gershon, 1977) and animal-parasitic (Kochman and Kwiatkowska, 1982; Rhodes, 1972) nematodes but not in plant-parasitic nematodes.

Although glycolysis is one of the most extensively studied metabolic pathways, and its component enzymes have been conserved during evolution (Marsh and Lebherz, 1992), among the invertebrates fewer than 10 aldolase genes have been characterized thus far. Of these, three of the genes are from nematodes (*Caenorhabditis elegans*, Inoue et al., 1997; *Onchocerca volvulus*, McCarthy et al., 2002). No aldolase gene has been described for any plant-parasitic nematode. We report here characterization of the aldolase gene from the cyst nematodes *Globodera rostochiensis* and *Heterodera glycines*, partial genomic sequences from seven other cyst nematode species, and aldolase-like activity in homogenates prepared from different developmental stages of *H. glycines*.

### MATERIALS AND METHODS

**Nematodes:** *Heterodera glycines* (population NL1-RHp) was reared on soybean plants according to Sardanelli and Kenworthy (1997). *Globodera rostochiensis* cysts were kindly provided by V. Dzhavakhiya, Laboratory of Molecular Biology, All Russian Research Institute of Phytopathology, Golitsino. The microbivorous species *Caenorhabditis elegans* (strain N2) and *Panagrellus redivivus* (population LKC26) were reared as described previously (Chitwood et al., 1995).

**Aldolase assay:** Nematodes were homogenized for enzyme assay in sterile water using a motor-driven ground glass homogenizer. The 20,000 × g supernatants were vacuum dried and dissolved in assay buffer (TEA; triethanolamine, 100 μM, pH 7.6; Sigma, St. Louis, MO), and protein was estimated with the microBCA assay (Pierce Chemical Co., Rockford, IL). Aldolase-like activity was detected using a spectrophotometric assay modified from Rajkumar et al. (1966) and Misset and Opperdoes (1984). The reaction was started by addition of substrate (fructose 1,6-bisphosphate, Sigma, St. Louis, MO), and progress was monitored by decreasing absorbance at 340 nm. Reactions were performed at 37 °C, thereby providing adequate absorbance signals for measuring aldolase activity in crude nematode homogenates. One aldolase unit is defined as the amount of enzyme causing loss of 1 nMol NADH/minute, and data are expressed as units per mg protein ± SE. Inhibition of aldolase activity was tested either by digestion with carboxypeptidase A (Sigma, St. Louis, MO) or treatment with sodium borohydride (Sigma, St. Louis, MO) dissolved in assay buffer.

**DNA preparation:** A *H. glycines* cDNA library constructed in bacteriophage λ vector Uni-ZAP XR (Stratagene, La Jolla, CA) was provided by E. L. Davis, North Carolina State University. Total cellular RNA was isolated from frozen *G. rostochiensis* cysts by a standard guanidinium thiocyanate method followed by cDNA preparation with M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) for polymerase chain reaction (PCR) amplification. Alternatively, total RNA was con-

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verted to 5'- and 3'-elongated cDNA for rapid amplification of cDNA ends (RACE), with the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA). Genomic DNA was extracted from 100,000 fresh juveniles of *H. glycines* and from *G. rostochiensis* cysts with the MasterPure DNA purification kit (Epicentre, Madison, WI).

Genomic DNA was also prepared from four other *Heterodera* species: *H. schachtii* (Belgium), *H. avenae* (France, Villasavary), *H. ripae* (Belgium), and *H. litoralis* (New Zealand); and from three other *Globodera* species: *G. pallida* (Germany), *Globodera* sp. (Peru, Huamachuco), and *Globodera* sp. (New Zealand). For each species, one to four cysts were used for DNA extraction as described previously (Subbotin et al., 2000).

**Identification of aldolase cDNA:** The PCR and sequencing procedures have been described (Kovaleva et al., 2002). The PCR primers were chosen on the basis of multiple alignment of nucleotide sequences of several known aldolase genes. Positive PCR products amplified from *H. glycines* cDNA were then sequenced to confirm homology to known aldolase genes, and the sequences of overlapping fragments were combined. The 5'-end and the 3'-end regions of the *H. glycines* aldolase gene were amplified from a cDNA library using nested PCR with M13REV and T3 sequencing oligonucleotides as sense PCR primers for the 5'-end, and M13-21 and T7 sequencing oligonucleotides as antisense PCR primers for the 3'-end, along with corresponding specific primers derived from the obtained informative sequence. Similar techniques were applied for identification of aldolase sequence from *G. rostochiensis*, but the 5'-end and the 3'-end regions were detected by the RACE method.

**Identification of introns:** Genomic fragments spanning the introns of aldolase from *H. glycines* and *G. rostochiensis* were amplified by PCR and sequenced. The precise splicing sites of the introns were identified by the alignment of cDNA and genomic DNA sequences, and by using GENSCANW (Burge and Karlin, 1998). Other gene-prediction programs used were GENWISE (Birney et al., 2004) and HMMgene (Krogh, 2000). Based on the established genomic sequences of *H. glycines* and *G. rostochiensis* aldolase genes, we designed primers to amplify fragments with predicted lengths from 850 to 1350 bp, spanning four introns. Using primers Ald-F: 5'-ATGGCAGAGGTCGGAAC-3' and Ald-R1: 5'-GCTTTGTAGGTGTAGGC-3', we obtained fragments of aldolase genes from other Heteroderidae. Two to three clones for each species were then sequenced using primers Ald-F and Ald-R2 (5'-CACGATGGGCA-C AAGTC-3'), and positions of introns were established.

## RESULTS

Aldolase activity was detected in tissue samples prepared from immature and mature stages of *H. glycines*,

including eggs and second-stage juveniles (J2), and females and males. The amount of activity was highest in preparations of J2 and eggs, and lowest in adults ( $P = 0.003$ ). Specific activities at 100  $\mu\text{M}$  substrate were  $86 \pm 6$  units/mg protein for J2 and  $69 \pm 6$  for eggs. Extracts of males had 50% to 60% of those activities ( $40 \pm 17$  units/mg protein), and females had  $<5$  units/mg. Aldolase levels in *C. elegans* and *P. redivivus* were each 2-fold higher than in *H. glycines* ( $P = 0.001$ ;  $213 \pm 29$  and  $195 \pm 6$  units/mg protein, respectively).

In samples prepared from J2, activity was tested with substrate concentrations varying from 12 to 200  $\mu\text{M}$ , and  $K_m$  was estimated at  $41 \pm 4$   $\mu\text{M}$ . Inhibition experiments at 100  $\mu\text{M}$  substrate showed 50.8% loss of activity following carboxypeptidase A digestion, and 48.8% and 90.0% losses in the presence of sodium borohydride (5 mM and 10 mM, respectively).

Full cDNA and genomic aldolase sequences were characterized for *H. glycines* and *G. rostochiensis*. The mRNA sequences corresponding to complete coding sequence (CDS) of aldolase were deposited into GenBank under the accession numbers AF318604 (*H. glycines*) and AY160988 (*G. rostochiensis*). The predicted cyst nematode aldolases are proteins of 366 (*H. glycines*) and 365 (*G. rostochiensis*) amino acids with molecular weight  $\sim 40$  kDa. The glycolytic enzyme domain in both species occupies almost the entire sequence, from residue Glu-19 to the carboxy terminus. Functionally important residues were established by similarity. Arg-60 and Lys-150 bind the C-1-phosphate group of the substrate, and a catalytically important C-terminal tyrosine is essential for enhanced activity of the enzyme toward fructose biphosphate as compared with fructose-1-phosphate. Lysine-233, involved in Schiff base formation with dihydroxyacetone phosphate, is located in the aldolase signature (Ile-225 and Asn-235). Overall homology of the two amino acid sequences is 89%, and nucleotide sequence identity of the coding regions is 83%.

Using primers Ald-F and Ald-R2 (Fig. 1), partial genomic sequences were determined for *H. avenae*, *H. litoralis*, *H. ripae*, *H. schachtii* (accession numbers AY494940-3), and *G. pallida*, *Globodera* sp. Peru, *Globodera* sp. New Zealand (accession numbers AY497332-4). The obtained fragments corresponded to residues 14 to 177 of aldolase sequences from *H. glycines* and *G. rostochiensis* (Fig. 2). Multiple alignment of the sequences shows a high degree of homology among aldolase genes from all species (Fig. 2). Within the *Heterodera* group, similarity was 92% to 100% (100% identity between *H. glycines* and *H. schachtii*). Similarity within the *Globodera* group is 90% to 94% (highest between *G. rostochiensis* and *G. pallida*). Nucleotide sequence homologies were also very high: 86% to 98% within the *Heterodera* spp. (highest between *H. glycines* and *H. schachtii*) and 88% to 91% within the *Globodera* species (highest between *G. rostochiensis* and *G. pallida*).

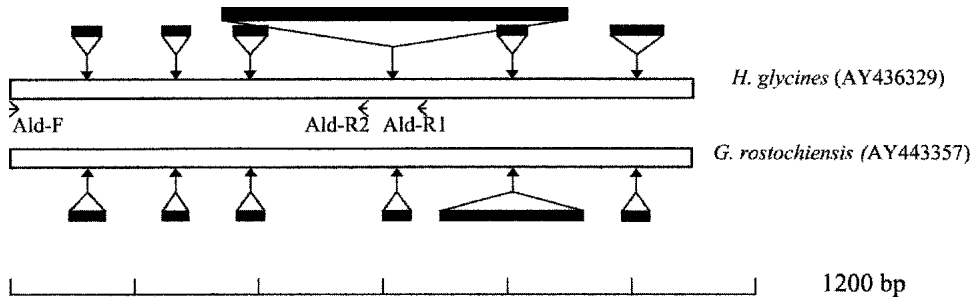


FIG. 1. Schematic diagram of exon/intron composition of aldolase gene from *Heterodera glycines* and *Globodera rostochiensis*. The length of the geometrical figures is a representation of the sequence length. Location of primer binding positions are labeled F (forward) and R1, R2 (reverse).

Two overlapping fragments that together span the entire CDS were amplified from genomic DNA of *H. glycines* and *G. rostochiensis* and used to determine the contiguous sequences of the aldolase genes. Comparative analysis of genomic and cDNA sequence data showed that, for both cyst nematodes, the CDS was interrupted by six introns at identical amino acid positions: 42, 88, 130, 208, 270, and 337 (Fig. 1). The fourth intron in *H. glycines* is exceptionally large (554 bp), unlike the corresponding intron in *G. rostochiensis*. We examined other *Heterodera* and *Globodera* species for the

presence of this large intron by using primers Ald-F and Ald-R1 (Fig. 1). The PCR results showed that all *Heterodera* products were 1250 to 1350 bp long whereas all *Globodera* products were 850 to 900 bp long (Fig. 3). Based on this difference in size we conclude that the aldolase gene in *Heterodera* spp. contains the large fourth intron and the *Globodera* spp. gene does not. All nine species have introns 1 through 4 in the same position. Introns 1 through 3 were sequenced for all species and were short (mostly 40 to 60 bp). Intron 3 was most variable in size (46 to 138 bp) and sequence.

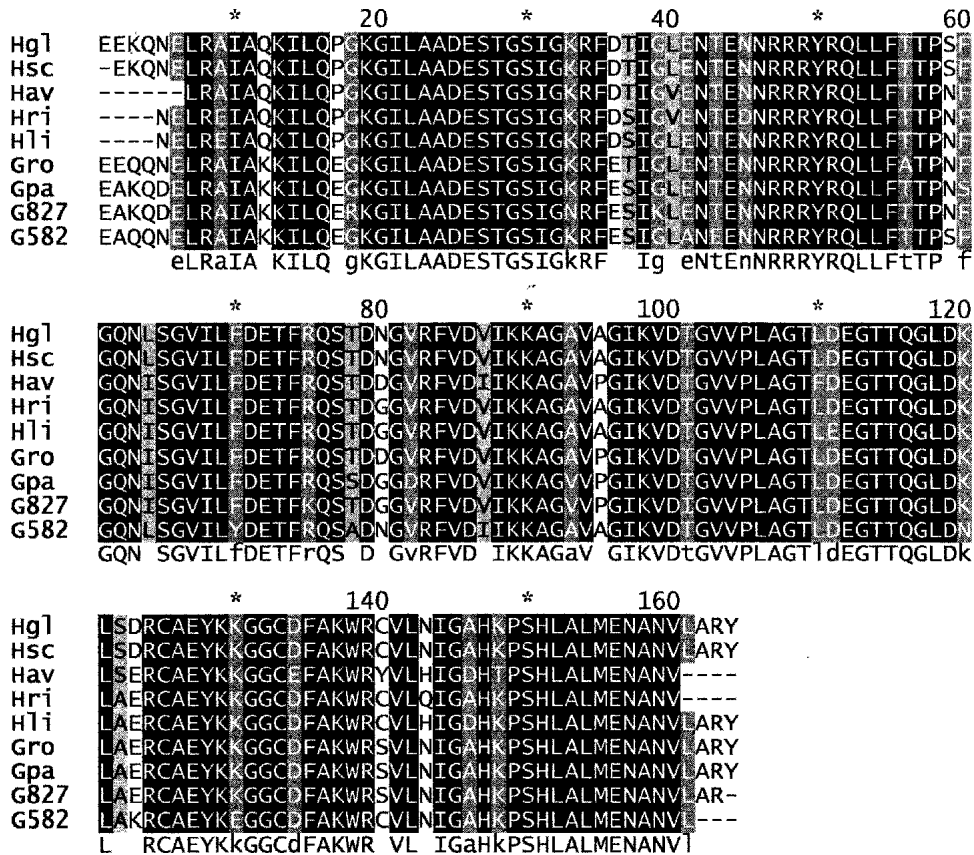


FIG. 2. Alignment of aldolase amino acid sequences from *Heterodera* and *Globodera* species. Partial sequences were aligned starting with residue position 14 of *H. glycines* (Hg1) and *G. rostochiensis* (Gro) aldolase sequences. (Hsc, *H. schachtii*; Hav, *H. avenae*; Hri, *H. ripae*; Hli, *H. litoralis*; Gpa, *G. pallida*; G827, *Globodera* sp. Peru; G582, *Globodera* sp. New Zealand). The alignment is viewed using GeneDoc (Nicholas et al., 1997). Positions with identical residues are labeled with white letters on a black background, positions with a consensus are white on gray, and positions without a consensus but with conserved substitutions are black on gray.

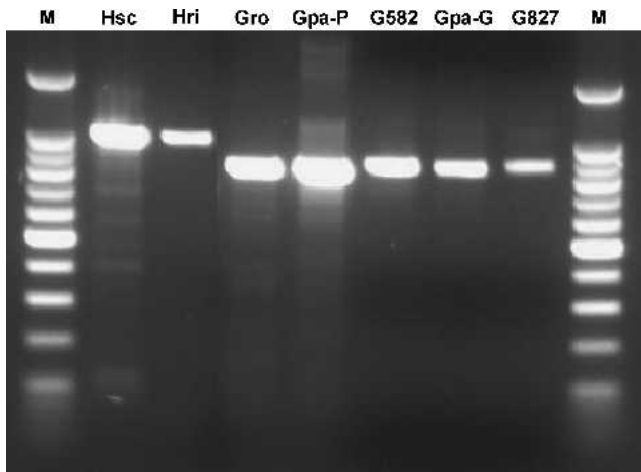


FIG. 3. PCR products from genomic DNA of cyst nematodes. Primers Ald-F and Ald-R1 used. Lanes 2 to 8: Hsc = *Heterodera schachtii* Belgium, Hri = *H. ripae*, Gro = *Globodera rostochiensis* Belgium, Gpa-P = *G. pallida* Peru, G582 = *Globodera* sp. New Zealand, Gpa-G = *G. pallida* Germany, G827 = *Globodera* sp. Peru. M = 100 bp ladder (Promega, Madison, WI).

## DISCUSSION

Despite its importance in energy metabolism and abundant information for vertebrates, an understanding of aldolase biochemistry in plant-parasitic nematodes is lacking and will benefit from a genetic approach. Difficulties presented when attempting to work with obligate parasites in the laboratory have also limited the molecular characterization of genes from plant-parasitic nematodes. Recently, using the expressed sequence tag (EST) approach, a number of sequences have become publicly available, including those from several parasitic species. The EST database contains fragments of aldolase-like sequences from *H. glycines* (Gao et al., 2001), *H. schachtii* (CF101130), *G. rostochiensis* (Popeijus et al., 2000), and *Meloidogyne incognita* (McCarter et al., 2003). Nevertheless, aldolase genes have not been described for any plant-parasitic nematode thus far, and phylogenetic analysis of this gene across the phylum Nematoda has not yet been attempted.

We have detected and characterized aldolase genes in nine plant-parasitic nematodes, providing the first molecular analysis of this enzyme for cyst nematodes. Aldolase-like activity was detected in both immature and mature stages with substrate affinity similar to that reported for free-living and animal-parasitic nematodes (Inoue et al., 1997; Reznick and Gershon, 1977; Rhodes, 1972; Zeelon et al., 1973). Inhibition of *H. glycines* aldolase activity by carboxypeptidase A and sodium borohydride is consistent with the behavior of aldolases from other organisms (Blonski et al., 1997; Choi et al., 2001; Gefflaut et al., 1995). Despite similar substrate affinities, the amount of aldolase on a unit protein basis is greater in whole body homogenates prepared from free-living species than in *H. glycines*.

Whether this is relevant to the energy requirements of these nematodes is not known.

Comparative studies of aldolase activity in samples prepared from different developmental stages of *H. glycines* showed similar activity in eggs and J2, and lower activity in adults. Aldolase activity in eggs is mostly attributable to the presence of pre-infective juveniles, which explains the similarity of aldolase activity in eggs and J2. The lower activity detected in adults compared with immature stages, although warranting further examination, is not surprising. In *Turbatrix aceti*, for example, age-synchronized cultures sampled periodically over a 7-week period showed a continuous decline in aldolase specific activity to 50% of initial levels (Zeelon et al., 1973).

Deduced amino acid sequences for *H. glycines* and *G. rostochiensis* aldolases have notably high homology, and each contains primary structure features conserved among aldolases (Blonski et al., 1997; Choi et al., 2001). Molecular weights of 40 kDa are expected for subunits of the aldolase homotetramer. Partial genomic sequences from seven additional cyst nematode species revealed nucleotide and amino acid similarity across all species. Thus, the minimum sequence similarity among all the *Heterodera* and *Globodera* spp. is 85% (amino acid identity) and 80% (nucleotide identity). The extensive sequence conservation suggests a close phylogenetic association among cyst nematodes. The same conclusion can be reached by analysis of intron location. All six introns in the aldolase gene from *H. glycines* and *G. rostochiensis* have identical positions; the positions of the first three introns were confirmed for seven more cyst nematodes. The *C. elegans* aldolase genes have three (*ce-2*) and two (*ce-1*) introns. No intron information is available for the aldolase gene from *O. volvulus*. The position of intron 1 from *H. glycines* and *G. rostochiensis* seems most conserved, coinciding with the position of intron 2 in *C. elegans ce-2*. All other introns are located in different places in the cyst nematodes and *C. elegans*. Based upon intron characteristics, it appears that *ce-2* is more similar to the cyst nematode aldolase genes than is *ce-1*. In addition, *C. elegans Ce-2* aldolase has higher amino acid similarity to aldolase from the cyst nematodes (72%) than does *Ce-1* (60%).

Noting that *H. glycines* and *G. rostochiensis* aldolases are more similar (sequence identity 89%) than the two *C. elegans* aldolases, *Ce-1* and *Ce-2*, are to each other (66%), we suggest that the aldolases predicted from genes described from cyst nematodes are homologous to *Ce-2* aldolase from *C. elegans*, whereas the *O. volvulus* enzyme is more closely related to *Ce-1* (74%) than to *Ce-2* (63%). Two genes reported for *C. briggsae* code for the hypothetical proteins CBG09060 and CBG15316, which have high amino acid sequence similarity to *Ce-2* and *Ce-1* aldolases, respectively. The cyst nematode amino acid sequences reported here have the same high homology to CBG09060 that they have to *Ce-2*,

and lower homology to CBG15316, such as they exhibit to Ce-1. Also, intron location in the gene encoding CBG09060 is the same as in *ce-2*, with the same similarity to cyst nematode aldolase introns. Lack of additional information on nematode aldolases does not allow further phylogenetic analysis.

The presence of the large fourth intron in *Heterodera* spp. and its absence from *Globodera* spp. provides a potentially useful molecular diagnostic marker within the cyst nematodes. High nucleotide homologies will allow construction of specific PCR primers that may be tested over a broad range of cyst nematode species for differentiation on the basis of intron sizes. Large numbers of cyst nematode species could be screened using this PCR-based assay, thus avoiding any need for DNA sequencing.

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