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Molecular Characterization of a 70 kDa Heat Shock Protein (HSP70) Gene in *Entamoeba dispar*

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Abstract

Amebiasis caused by *Entamoeba histolytica* is still mentioned as one of the major health problems in tropical and subtropical areas. *E. histolytica* has recently been redescribed as two distinct species; *E. histolytica* and *E. dispar*. In the present study, we characterized the 70 kDa Heat Shock Protein (HSP70) of *E. dispar* at molecular level and compared it with that of *E. histolytica*. With these findings, we were able to distinguishe *E. dispar* from the infectious *E. histolytica*. Pairs of 21 nucleotide primers were designed from highly conserved regions of the same gene in other eukaryotic cells. Mentioned primers were utilized in PCR by using isolated genomic DNA template of *E. dispar* and the PCR fragments were then sequenced. By the time, 1020 nucleotides have been sequenced and characterized within open reading frame of this new gene which encode a polypeptide with 337 amino acids. Nucleotide sequence comparison in gene data banks (NCBI, NIH) for both the partial DNA and its deduced amino acid sequence revealed significant homology with members of the eukaryotic 70 kDa HSP family. Small parts of the mentioned sequences from *E. dispar* were about 100% identical to the sequences of 70 kDa HSP from *E. histolytica* other eukaryotic cells. The new partial gene fragment and its encoded protein have been submitted to the gene data banks (NCBI, NIH) and registered under the accession number of AY763790.

Keywords: *Amebiasis, HSP70,* Entamoeba dispar*, DNA*

Introduction

Amebiasis, caused by *Entamoeba histolytica* is still regarded as one of the major health problems in tropical and subtropical areas. It is estimated that amebiasis causes up to 110 000 deaths per yr (1). This infection is usually characterized by low socioeconomic status and poor hygienic situations that favor the indirect fecal-oral transmission of the infection (2). Previously two morphologically identical species of *Entamoeba* had been found, and revealed that only one of them was able to cause infection in kittens or human volunteers (3). However, *E. histolytica* has recently been redescribed as two distinct species; the pathogenic species *E. histolytica* and the nonpathogenic *E. dispar* (4-13). Exposure of cells to stress such as heat shock, oxidant injury, heavy metals (14) and ultraviolet radiation (15) results in the activation of heat-shock genes and the synthesis of HSPs (16). Based on both size and function, several families of HSPs can be distinguished (17). The stress-inducible HSP70 constitute one of the most highly conserved protein and gene families (18). As molecular chaperones they play essential roles in protein metabolism and protein translocation under both stress and non-stress conditions (19, 20). In the field of parasitology, the HSP70 multigene family of *E. histolytica* has recently been investigated intensively (21), whereas little information is available on HSPs of the *E. dispar*.

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In this study, we have explained the molecular characterization and analysis of a gene encoding a 70 kDa HSP of this amoeba. The molecular characterization of this gene as well as definition of its possible role in the physiological function of *E. dispar* is still under investigation.

Materials and Methods

Isolation of nucleic acids Total RNA from *E. dispar* was isolated by a method described previously (14). The poly $(A)^+$ RNA was obtained from total RNA and used for cDNA synthesis.
High-molecular-weight DNA from *E. dispar* was isolated by a modification of the method of Rezaie et al (22). Briefly, the harvested amoeba cells were suspended to DNA extraction buffer containing: 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 3% SDS, and 50 µl of proteinase-K (20 mg/ml). The supension was then incubated at 65 °C for 1 h and the cellular debris was removed by centrifugation at 2500×g for 15 min. After addition of 25 µl RNase-H (10 mg/ml), the supension was incubated at 37 °C for 30 min, extracted once with phenol-chloroform-isoamyl alcohol (25: 24: 1) and once with chloroform-isoamyl alcohol (24:1). The DNA was precipitated by addition of an equal volume of isopropanol, followed by centrifugation at 15000×g for 30 min. The DNA pellet was rinsed with 70% ethanol and resuspended in distilled water.

PCR analysis PCR and reverse transcriptase PCR (RT-PCR) analysis of genomic DNA as well as final double-stranded cDNA obtained from *E. Dispar* were performed according to a standard protocol (10) by using synthetic oligonucleotide primers including: B1; 5'CACCAGGCAAGACAGACCGAC3' as sense and B2; 5'GTCAAGTCTCAACCGAGCCGT3' as reverse primers. Briefly, 20 ρM of PCR analysis of genomic DNA was performed according to standard protocol (22) using synthetic oligonucleotide primers. Briefly, 0.5µM of each primer was added in a volume of 50 µl containing: 20 mM $(NH4)_2$ SO_4, 75 mM Tris-HCl (pH. 8.8), 1 mM $MgCl_2$, 0.2 mM dNTP mix, 1.2 Units of thermostable DNA polymerase (Advance Biotechnologies, UK), and 1 µl of template (genomic or complementary DNA).The PCR cycle employed was 95 °C for 60s, 58 °C for 150s, and 72 °C for 180s, with a total of 35 cycles. PCR products were analysed by eletrophoresis through a 1% agarose gel.

Sequencing of the PCR fragments
Sequencing of the amplified cDNA fragment was performed with the Dye Terminator Cycle Sequencing Kit (MWG, Germany), by using the amplified double stranded cDNA as template and synthetic 21-meric primers designed according to the obtained DNA sequence fragments from other eukaryotic cells. Sequencing of each part was repeated at least three times for both strands. The nucleotide sequence of DNA was compared with the sequences in gene data banks in National Centre for Biotechnology Information (NCBI, NIH).

Results

After amplification of different parts of gene by PCR and RT-PCR, it has been identified as a DNA with the approximate molecular weight of 1.3 kbp (Fig.1). However; the molecular weight of amplified cDNA was approximately 1.1 kbp (Fig. 1). This revealed the presence of intron in the genomic DNA of the amplified gene. The nucleotide sequence of the partial cDNA is presented (Fig. 2). The amount of 1020 bp of the cDNA has been sequenced. The sequenced cDNA contains an ORF encoding a 337 amino acids protein. Nucleotide sequence comparison in gene data banks (NCBI, NIH) for the DNA and its deduced amino acid sequence revealed significant homology with members of the eukaryotic 70 kDa HSP family. The analysis of this new encoded protein, which will hereafter be referred to as EdHSP70, revealed that it was rich in glycine (9.8%), alanine (9.5%), and glutamic acid (5.9%). In contrast, the amounts of tryptophan (0.6%) and cysteine (0.9%) were poor.

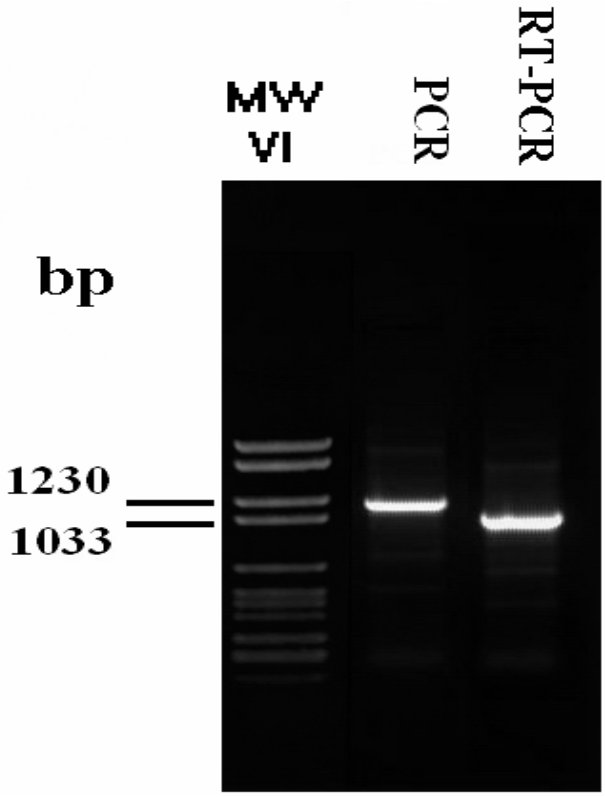

Fig. 1: PCR products of the EdHSP70 gene

Fig. 2: Partial nucleotide sequence of the EdHSP70 DNA (Genbank accession Nr. AY763790)

1 tttgagtcca tgagtaaata cacaggacca gccgttggta ttgatttagg tactacatat
61 gatgacacaa tggaactcat ggccaacgac cagggtaacc ggaccacacc ctcgttcgtt
121 gcctgcacag acacccagcg tctgatcgcg gatgccgcca gaaatcaagt cgcgatgaac
181 ccgtccaacc cagtcgttga cgccaagggt ctcattcgcc gcaagttcgc tggtcccgag
241 gtccaatctg acatgaagca cttggccttc aaggccattg acaaggcgcg aaggccagcc
301 agaagagtca gtgatccagc aatccaaaat ggtatgaacc attggtcatt cagaggtatc
361 gtcctgacga agatgagaga aagtgcggaa ccctaccttg gaggcactgt taacaaggct
421 gtcggtactg tccgtgccta tttcaacgat tcccagcgtc aagctaccaa ggatgctggt
481 gttggaaaag aagtaaagaa tgcagttatt acatgtccag catatttcaa tgattctcaa
541 tatggtcttg acaagaaggc cgagggtgaa cgcaacgttc tcatcttcga cttgggtggt
601 gaaccaactg cagcagctat tgcatatgga cttgataaaa agagtgacag agaaaagaac
661 gctggtgacc ctcatcttgg tggtgaggat ttcgataacc gcctcgtgaa ccatttcgtc
721 ggagtatttg aagttaaagc aagtaatgga gatacccatc ttggaggaga agattttgat
781 ctccgcagtg cctgcgaacg tggcatgcgc accgtctccc ctgccggaca gacatccacc
841 gaaatcgaat ccctatacgt gggcattgat ttctatacct ccatcaccgg tgcccgttcc
901 gaggagcctt gggaggacct cttcggctcc accatggacc cagttgagcg cgtgctcggc
961 gatgcaaaga tcgataaatc tagcgtcgat gagatagtcc tggtgggtgg atccacccgt

Fig. 3: Comparison of partial amino acid sequence of HSP70 from *E. dispar* (Genbank accession Nr. AY763790) with that from *E. histolytica* (Genbank accession Nr. AAA29102), *Caenorhabditis elegans* (Genbank accession Nr. AAA28078) *Leishmania major* (Genbank accession Nr. AAA29251), and *Toxoplasma gondi* (Genbank accession Nr. AAD09230)

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A-dispar: 1 MSKYTGPAVGIDLGTTY---------DDTMELMANDQGNRTTPSFVACTDTQRLIADAARNQ 53
A-histol :  1  MSKYTGPAVGIDLGTTYSCVGIWQNDRVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ 60
C-elegans:3   MSKHN----AVGIDLGTTYSCVGVFMHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ 58
L-major: 8          -------AIGIDLGTTYSCVGVWQNERLDIIANDQGNRTTPSYVAFTDSERLIGDAAKNQ 60
T-gondi: 7         ------PAVGIDLGTTYSCVGVWKNDAVEIIANDQGNRTTPSYVAFTDTERLVGDAAKNQ 60

Aa-dispar: 54  VAMNPSNPVVDAKGLIRRKFAGPEVQSDMKHLAFKAIDKARRPARRVSDPAIQNGMNHWS 113
A-histol:   61  IAMNVKNTVFDAKRLIGRRF--------------------------SDPAIQNDMKHWS 93
C-elegans:59 VAMNPHNTVFDAKRLIGRKFDDPAVQSDMKHWPFKVISAEGAKPKVQVEYKGENKIFTPE 118
L-major : 61 VAMNPHNTVFDAKRLIGRKFNDSVVQSDMKHWPFKVTTKGDDKPVISVQYRGEEKTFTPE 120
T-gondi : 61 VARNPENTIFDAKRLIGRKFDDPSVQSDMKHWPFKVIAGPGDKPLIEVTYQGEKKTFHPE 121

A-dispar: 114 FRGI-VLTKMRESAEPYLGGTVNKAVGTVRAYFNDSQRQATKDAGVGKEVKNAVITCPAY 172
A-histol: 94       FKVIDDGHDKPLIEVEYK-GEVKKFTPEEISSMVLTKMKETAESFVGKEVKNAVITCPAY 152
C-elegans:119 EISSMVLLKMKKTAEAFLEPTVKDAVVTVPTYFNDSQRQATKDAGAIAGLNVLRIINEPT 178
L-major : 121 EISSMVLLKMKETAEAYLGKQVKKAVVTVPAYFNDSQRQATKDAGTIAGLEVVRII---- 176
T-gondi : 122  EVSAMVLGKMKEIAEAYLGKEVKEAVITVPAYFNDSQRQATKDAGTIAGLSVLRIINEPT 181

A-dispar: 173 FNDSQYGLDKKAEGERNVLIFDLGGEPTAAAIAYGLDKKSDREKNAGDPHLGGEDFDNRLV 233
A-histol: 153   FNDSQRQATKDAGTIAGMNVMRIINEPTAAAIAYGLDKKSDREKNVLIFDLGGGTFDVSLL 213
C-elegans:179 AAAIAYGLDKKGHGERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNRMV 239
L-major : 177                 ----------------------NEPTAAAIAYGLDKGDDGKERNVLIFDLGGGTFDVTLL 214
T-gondi : 182 AAAIAYGLDKKGCGEMNVLIFDMGGGTFDVSLLTIEDGIFEVKATAGDTHLGGEDFDNRLV 242

A-dispar: 234 NHFVGVFEVKASNGDTHLGGEDFD-----------------------------LRSACERG 265
A-histol:   214 AIDDGVFEVKASNGDTHLGGEDFDNRLVNHFIAEFKRKYK-KDISGNARAVRRLRTACERA 273
C-elegans:240 NHFCAEFKRKHKK-DLASNPRALRRLRTACERANETLSSSCQASIEIDSLFEGIDFYTNIT 299
L-major : 215 TIDRGIFEVKATNGDTHLGGEDFDNRLVTFFTEEFKRKNKGKNLASSHRALRRLRTACERA 275
T-gondi : 243 DFCVQDFKRKNRGKDISTNSRALRRLRTQCERTKRTLSSSTQATIEIDSLFEGIDYSVSIS 303

A-dispar: 266 MRTVSPAGQTSTEIESLYVGIDFYTSITGARSEEPWEDLFGSTMDPVERVLGDAKIDKSSV 326
A-histol:  274 KRTLSSAATANIEVDQLFDGIDFYTSITRARFEELNIDLFKSTIGPVERVLQDAKLDKGSI 334
C-elegans:300 RARFEELC----------------------A-------DLFRSTMDPVEKSLRDAKMDKSQV 332
L-major: 276 KRTLSSATQATIEIDALFENIDFQATITRARFEELCGDLFRSTIQPVERVLQDAKMDKRSV 336
T-gondi  30                    RARFEELCMDYFRN-SL----------------------L------PVEKVLKDSGIDKRSV 336

A-dispar: 327    DEIVLVGGSTR 337
A-histol: 335    DDVVLIGGSTR 345
C-elegans:333 HDIVLVGGSTR 343
L-major : 337  HDVVLVGGSTR 347
T-gondi : 337    SEVVLVGGSTR 347
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Discussion

In the present study, we report the identification and molecular characterization of a *E. dispar* gene encoding a protein with a molecular mass of 70.729 kDa which belongs to the HSP70 family and will be referred to as EdHSP70. The amino acid sequence of the encoded protein was about 51-60% identical to the sequence of 70 kDa HSP from *E. histolytica,* 49-57% with that from *Leishmania major,* 53-62% with that from *Caenorhabditis elegans*, and 49-57% with the *Toxoplasma gondi* HSP70 gene. The analysis of EdHSP70 DNA sequence, together with the information deduced from the alignment

with other HSP70s, indicated that it encompassed the partial length gene coding sequence. To our knowledge, EdHSP70 is the first HSP gene and proteins of this *Entamoeba* characterized so far. The molecular characterization of this *E. dispar* gene described here may open the way to the disclosure of the functional characteristics of EdHSP70 and to the assessment of its possible role in the physiology of *E. dispar*. Characterization of EdHSP70 may also guide us to develop new ways in differentiation of *E. dispar* form the pathogenic amoeba *E. histolytica*.

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