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# Expression of the Alcohol Dehydrogenase (ADH) Domain of *Entamoeba histolytica* EhADH2 Enzyme[1]

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## Introduction

*Entamoeba histolytica* is the agent of amebic dysentery and amebic liver abscess, both of which are significant causes of morbidity and mortality worldwide. Amebiasis is primarily treated by the drug metronidazole; however, serious side effects, such as neurologic complications have been associated with this treatment. An additional concern arises from the possible development of resistance to metronidazole which has been found in other protozoan parasites, such as *Trichomona vaginalis* and *Giardia lamblia*. Therefore, the search for alternative agents to treat amebiasis is of great importance.

*Entamoeba histolytica* is an eukaryotic anaerobe with no mitochondria, which obtains energy from the fermentation of glucose to carbon dioxide, acetate, and ethanol. This pathway constitutes a potential target for new antiamebic drugs, due to its importance for the survival of the parasite and to the requirement of several enzymes which are absent from other eukaryotes. In previous studies, we described the cloning and the sequencing of the gene encoding EhADH2, a bifunctional $NAD^+$-linked enzyme with both an alcohol dehydrogenase (ADH), as well as an acetaldehyde dehydrogenase (ALDH) activities (1). EhADH2 is a critical enzyme in the glycolytic pathway, catalyzing two reactions in the process (2). Homologues of EhADH2 are the AdhE enzyme from *Escherichia coli* and other enzymes from anaerobic and facultative anaerobic bacteria (3,4). We have recently described the expression of a functional recombinant EhADH2 in *E. coli* (5). We have developed a mutant *E. coli* which requires EhADH2 for anaerobic growth; this strain can be used as a tool for identifying compounds with anti-EhADH2 activity (5). Here, we report the cloning and expression of cDNA encoding a region of the EhADH2 molecule predicted to contain the ADH domain. The isolated recombinant ADH domain is approximately 49 kDa and reacts with a polyclonal antibody generated against EhADH2. By expressing ADH by itself, we now have a tool that will enable us to dissect the structure/function relationships of the EhADH2 molecule.

## Materials and Methods

***Escherichia coli* Strains.** Strains DH5α, BL21(DE3), and SHH31 (DE3 Δ*adh*E *zch*::Tn10 *fad*R met *tyr*T) (5) were used for transformation and the expression of ADH. Cultures were grown in LB broth medium with agitation at 37°C and 1.5% Bacto Agar (Difco) was added for solid media.

**Construction of the ADH Expression Vectors.** The T7 promoter-based vector pET23a (Novagen) was used for *E. coli* expression of ADH. The putative ADH domain was obtained by PCR from the pET3a/EhADH2 construct (5). The sequences flanking the putative ADH domain were modified by the incorporation of a *Bam*HI site next to the termination codon TAA at the 3' end of EhADH2 and a *Nhe*I site with an initiating codon at position 1,251 of EhADH2 DNA sequence (Figure 1A). The fragment containing the ADH sequence was then ligated into the *Nhe*I and *Bam*HI digested pET23a vector.

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1A

EHADH2 AMINOACID SEQUENCE

ALDH DOMAIN

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1   M S T Q Q T M T V D E H I N Q L V R K A Q V A L K E Y L K P E Y T Q E K I D Y I V K K A S V A 47
48  A L D Q H C A L A A A A V E E T G R G I F E D K A T K N I F A C E H V T H E M R H A K T V G I 94
95  I N V D P L Y G I T E I A E P V G V V C G V T P V T N P T S T A I F K S L I S I K T R N P I V 142
143 F S F H P S A L K C S I M A A K I V R D A A I A A G A P E N C I Q W I E F G G I E A S N K L M 188
189 N H P G V A T I L A T G G N A M V K A A Y S S G K P A L G V G A G N V P T Y I E K T C N I K Q 235
236 A A N D V V M S K S F D N G M I C A S E Q A A I I D K E I Y D Q V V E E M K T L G A Y F I N E 282
283 E E K A K L E K F M F G V N A Y S A D V N N A R L N P K C P G M S P Q W F A E Q V G I K V P E 329
330 D C N I I C A V C K E V G P N E P L T R E K L S P V L A I L K A E N T Q D G I D K A E A M V E 376
377 F N G R G H S A A I H S N D K A V V E K Y A L T M K A C R I L H N T P S S Q G G I G S I Y N Y 423
                                                                                  |
                                                                              PCR start

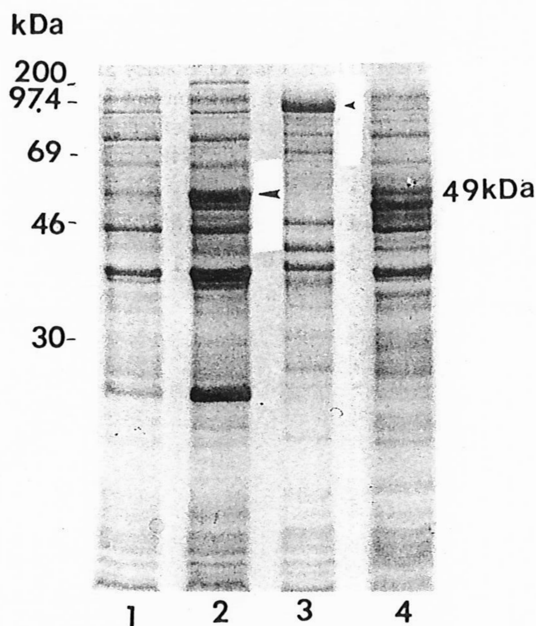
424 I W P S F T L G C G S Y G G N S V S A N V T Y H N L L N I K R L A D R R N N L Q W F R V P P K 470
NAD BINDING FINGERPRINT G X G X X G

471 I F F E P H S I R Y L A E L K E L S K I F I V S D R M M Y K L G Y V D R V M D V L K R R S N E 517

                                                   ADH DOMAIN
518 V E I E I F I D V E P D P S I Q T V Q K G L A V|M N T F G P D N I I A I G G G S A M D A A K I 564
565 M W L L Y E H P E A D F F A M K Q K F I D L R K R A F K F P T M G K K A R L I C I P T T S G T 611
612 G S E V T P F A V I S D H E T G K K Y P L A D Y S L T P S V A I V D P M F T M S L P K R A I A 658
659 D T G L D V L V H A T E A Y V S V M A N E Y T D G L A R E A V K L V F E N L L K S Y N G D L E 705
706 A R E K M H N A A T I A G M A F A S A F L G M D H S M A H K V G A A F H L P H G R C V A V L L 752
                                            G X X H X X A H X X G X X X X X P H G  FE BINDING DOMAIN

753 P H V I R Y N G Q K P R K L A M W P K Y N F Y K A D Q R Y M E L A Q M V G L K C N T P A E G V 799
800 E A F A K A C E E L M K A T E T I T G F K K A N I D E A A W M S K V P E M A L L A F E D Q C S 846
847 P A N P K V F M V K D M E K I L K A A Y Y F I A * 870
                                                |
                                             PCR stop
```

1B




1C

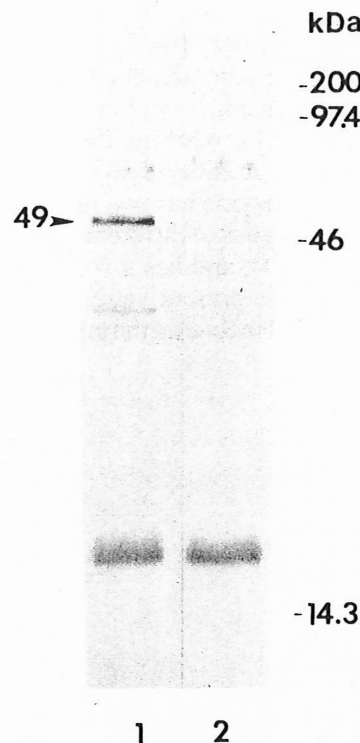



**Figure 1.** EhADH2 domains and expression of the putative ADH domain. A). EhADH2 amino acid sequence with the ALDH domian, $NAD^{+}$ binding fingerprint, ADH domain and $Fe^{2+}$ binding region is shown (3). Arrows indicate the positions at which the corresponding DNA oligonucleotides were designed for construction of the ADH domian. B). Expression of the ADH protein in *E. coli*. Coomassie staining of SDS-PAGE separated lysates expressing the expected 49 kDa ADH protein (arrow), before induction (lane 1) and after induction (lane 2). The Eh ADH2 97 kDa protein can be seen in lane 3. Lane 4 shows pET23a alone. C). Western blot of lysates of *E. coli* expressing the ADH protein (lane 1) and the vector alone (lane 2) using rabbit antiserum aganist the 6His-EhADH2 recombinant protein. A band at 49 kDa (arrow) corresponding to the ADH protein is evident in lane 1.

**Expression of Recombinant ADH in *E. coli* BL21 (DE3) and SHH31 (DE3Δ*adh*E).** ADH was expressed in BL21(DE3) using the pET23a/ADH vector by standard procedures. SDS/PAGE analysis of bacterial lysates for expression of ADH was done as described (5). Western blot analysis was performed using a 1:100,000 dilution of rabbit antiserum raised to a recombinant 6His-EhADH2 fusion protein as described previously (5). The pET23a/ADH vector was then expressed in SHH31 (DE3 Δ*adh*3) to test ADH activity.

## Results and Discussion

**Expression of ADH in *E. coli.*** Nucleotides 1,251-2,615, which represent the entire putative ADH coding region, including the $NAD^+$ and $Fe^{2+}$ binding domains, were expressed in BL21(DE3) using the pET23a construct as described. As shown in Figure 1B, a band at 49 kDa (the predicted size of the isolated ADH domain) is seen in lysates from BL21(DE3) transformed with the ADH encoding plasmid (lane 1), while *E. coli* BL21 (DE3) transformed with pET3a/EhADH2 plasmid expresses the full length 96 kDa EhADH2 molecule (lane 2). To confirm that the species at 49 kDa was ADH, Western blot analysis of the bacterial lysates separated by SDS/PAGE was performed using antiserum against the 6His-EhADH2 recombinant protein. As shown in Figure 1C, the antiserum reacted with the 49kDa protein specifically (lane 1), whereas the strain transformed with the vector alone showed no reactivity (lane 2).

The EhADH2 enzyme has two major domains. Each domain is homologous to different prokaryotic ADH or ALDH enzymes (4), and has a $NAD^+$ binding domain between them. The present study has focused on the expression of ADH including the putative $NAD^+$ binding domain (Figure 1A). Our results demonstrate that ADH can be expressed independently from ALDH and is specifically recognized by the anti-EhADh2 antiserum. Preliminary data suggests that the isolated domain retains ADH activity.

In conclusion, we have achieved successful expression of the isolated ADH domain of the EhADH2 molecule, a critical bifunctional enzyme in the amebic fermentation pathway. The independent expression of active ADH and ALDH proteins will allow us to further characterize the structure/function relationships of EhADH2, determine the relative importance of each enzyme activity in the metabolic pathway, and determine whether $NAD^+$ and iron binding domains are required for each enzyme's activity.

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**KEY WORDS:** *Entamoeba histolytica;* Alcohol dehydrogenase; Fermentation pathway.