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**2001 12**

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. .....	3
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1. ....	7
가. ....	7
. ....	7
. ....	7
2. ....	8
가. ....	8
. ....	8
. cDNA expression library .....	9
(1) RNA mRNA .....	9
(2) cDNA library .....	9
. ....	10
. In vivo excision .....	11
. DNA .....	11
. ....	12
. ....	14
. ....	15
1. ....	15
2. cDNA expression library .....	15

3.	.....	19
4.	.....	25
IV.	.....	29
V.	.....	33
	.....	34
	.....	38

1. Mse	. . . . .	13
2. SDS-PAGE	. . . . .	16
3.	. . . . .	17
4. cDNA library 1-3	. . . . .	18
5. Alkyl-hydroperoxide reductase cDNA	. . . . .	20
6. Alkyl-hydroperoxide reductase cDNA	. . . . .	21
7. Mse 4 GST-fusion (A) anti-GST, (B)	. . . . .	22
8. GST (lane 1, 3) AHR1F AHR1R(lane 2, 5) PCR GST fusion protein AHR2F and AHR2R(lane 3, 6) PCR GST fusion protein anti-GST (lane 1-3) (lane 4-6)	. . . . .	23
9. Computer simulation alkyl-hydroperoxide reductase 3	. . . . .	24

10. (A) alkyl-  
hydroperoxide reductase(B)

ELISA . . . . . 27

11. , ,  
. . . . . 28



ion exchange (DEAE-Sepharose Resource Q) gel filtration (Superdex 200), 31, 62, 97 kDa

BALB/c 3 polyclonal antibody cDNA library (6 X 10<sup>5</sup> plaque)

, DNA sequencing, GenBank, 31 kDa

alkyl-hydroperoxidase reductase, 62 kDa

alcohol dehydrogenase 3 97 kDa

12 9 (75%) coding alkyl-hydroperoxidase reductase cDNA (*Mse* I) PCR

mapping . 14- 51  
 가 .  
 , ELISA  
 . ,  
 , 가  
 100% 92% . alkyl-hydroperoxidase reductase

---

: , , , , ,  
 , alkyl-hydroperoxidase reductase

< >

.

(*Entamoeba histolytica*) 5 가  
, 4 가 ,  
(amoebiasis) 4 10  
가 ,  
가

,  
가 가 가 .  
, ,

. 가  
가 *E.*  
*histolytica* *E. dispar*가

<sup>2</sup>. 1987 Sargeant<sup>3</sup> starch gel isoenzyme ,  
 , hexokinase, phosphoglucomutase, glucose phosphate isomerase  
 가 E.  
*histolytica* *E. dispar* 가  
 . 가 20  
 type , type II, XIV  
 (*E. histolytica*) . <sup>4</sup>,  
 PCR <sup>5,6</sup>.  
 가 가  
 , galactose  
 lectin 가 170 kDa adhesin <sup>7,8</sup>. galactose  
 가  
 가 <sup>9</sup>.  
 cysteine proteinase <sup>10,11</sup>. Tannich  
 6 cysteine protease가 ,  
 (ACP5),  
 (ACP3) , <sup>12,13</sup>. Petri  
 Tannich transfection vector  
 ,  
 가 <sup>14-16</sup>. 가  
 , .  
 가 , 260 kDa  
 galactose-inhibitable lectin<sup>17</sup>, 29 kDa <sup>18</sup>, pore-forming peptide<sup>19</sup>,

superoxide dismutase<sup>20</sup> .

,  
,  
가<sup>21</sup>,  
YS-27<sup>22</sup>, cytokine  
(IL-8)<sup>23</sup>, PCR,  
가<sup>24</sup>,  
IgG  
가<sup>25</sup>,  
(indirect fluorescent antibody test)

ELISA

가  
, 가 , ELISA  
, ELISA가 .

kDa

90%

98%

<sup>26</sup>, SREHP

가 82%,

가 98%

<sup>27</sup>.

29 kDa

가

87%

94%

<sup>28</sup>.

가

가

가

YS-27 (strain) 가

, *E. coli*

(recombinant antigen)

1.

가.

1969

가 1995 (axenic cultivation)  
YS-27 TYI-S-33<sup>29</sup> 4

(31, 62 97kDa )  
100 µg/ml 6 BALB/c Freund's complete  
adjuvant , 3 Freund's  
incomplete adjuvant 2 boosting .  
2 adjuvant 1

20

(1:2560 )

(1:64 1:256)

(1:16 )

2.

가.

$10^8$

E-64 leupeptin 50  $\mu$ M

가 50 mM Tris-Cl pH 7.4, 0.5 mM EDTA, 1 mM 2-mercaptoethanol

10 5 , 12,000g

45, 65, 85% ammonium sulfate .

ammonium sulfate 50 mM Tris-Cl pH 7.5, 0.5 mM EDTA, 1 mM

2-mercaptoethanol 4 3

DEAE-Sepharose, Superdex 200 FPLC, Resource Q FPLC

ELISA

가 .

DEAE-Sepharose

FPLC

. 50 mM Tris-Cl, pH 7.5, 0.5 mM EDTA, 1 mM

2-mercaptoethanol

(DEAE-Sepharose) ammonium sulfate , 0.0 M

1.0 M NaCl linear gradient . 20 mM

Tris-HCl, pH 7.4 filtration (Superdex 200)

. 50 mM Tris-Cl, pH 8.0, 0.5 mM EDTA, 1

mM 2-mercaptoethanol Resource Q

, 0.0 M - 1.0 M



NaCl linear gradient .

### . cDNA expression library

(1) RNA mRNA

denaturing (5M guanidium thiocyanate, 10 mM EDTA, 2% SDS, 25 mM Tris-HCl, pH 7.6) , phenol/chloroform , isopropyl alcohol 가 ResinTack RNA , DEPC 1 . RNA oligo-dT 가 cellulose resin mRNA DEPC -70 .

(2) cDNA library

ZAP Express<sup>TM</sup> cDNA Synthesis kit (Stratagene) ,

mRNA first strand . 5 $\mu$ l 10X first-strand , 3 $\mu$ l first-strand methyl nucleotide mixture, 2 $\mu$ l (1.4 $\mu$ g/ $\mu$ l) linker-primer, 1 $\mu$ l (40U/ $\mu$ l) RNase block , poly A mRNA 5  $\mu$ g . M-MuLV reverse transcriptase 1.5 $\mu$ l (50U/ $\mu$ l) 37 1 . first strand 20 $\mu$ l 10X second strand , 6 $\mu$ l second strand nucleotide mixture, 2 $\mu$ l dNTP, 2 $\mu$ l (1.5U/ $\mu$ l) RNase H, 11  $\mu$ l (9U/ $\mu$ l) DNA polymerase 16 2.5 second strand . Phenol:chloroform 가 double stranded DNA alcohol . Klenow fragment dNTP mix , cDNA , EcoR I adaptors .

T4 polynucleotide kinase      rATP      *EcoR* I ends ,  
*Xho* I      .      cDNA      ZAP Express<sup>TM</sup> vector      T4  
DNA ligase      . Gigapack II Gold packaging extract  
in vitro packaging      . XL1-Blue MRF'  
plating      titring      .      vector      LacZ promoter      가  
X-gal      blue-white selection      가  
insert      가      가      가      .      plate  
white      pBK-CMV double stranded phagemid      cDNA insert

0.2% maltose, 10 mM MgSO<sub>4</sub>      가      LB  
XL1-blue MRF'      37 , 200 rpm      16      , OD 600      0.5  
. 2 × 10<sup>4</sup> phage      200 μℓ      37      15  
가      , 50      3 Mℓ top agarose (10 g NaCl, 10  
g tryptone, 5 g yeast extract, pH 7.0 per liter)

NZY plate (5 g NaCl, 2 g MgSO<sub>4</sub> • 7 H<sub>2</sub>O, 5 g yeast extract, 10 g NZ  
amine, 15 g agar, pH 7.5 per liter)      . 42      plaque가  
(      4      )      ,      10      mM      IPTG(isopropyl      -D-  
thiogalactopyranoside)      nitrocellulose membrane(Bio-Rad Lab. Inc,  
Hercules, CA, USA)      3.5      37

. Membrane      TBST      (10 mM Tris-HCl, pH 7.5, 150 mM  
NaCl, 0.05% Tween 20)      3% skim milk      TBST  
. 1  
1: 500      1      , membrane  
TBST      10      3      . 2      alkaline phosphatase

conjugated goat anti-mouse IgG 1: 2,000 1 ,  
 membrane BCIP/NBT .

**. In vivo excision**

ZAP Express vector pBK-CMV phagemid  
 XL1-Blue MRF', XLOLR ExAssist helper phage in vivo  
 excision . 3 phage ( $1 \times 10^5$   
 $10^5$  pfu/Ml) 250  $\mu$ l XL1-Blue MRF' (O.D.  $_{600} = 1.0$ ) 200  $\mu$ l 1 $\mu$ l  
 ExAssist helper phage ( $1 \times 10^6$  pfu/Ml) 15 37 .  
 3 Ml NZY broth 가 3 single excision  
 65 20 (1,000 g,  
 15 ) 1  $\mu$ l 100  $\mu$ l XLOLR (O.D.  $_{600} = 1.0$ )  
 15 37 . 100  $\mu$ l NZY broth 가  
 37 45 100  $\mu$ l LB/kanamycin (50  $\mu$ g/Ml)  
 agar plate . 16 colony plasmid  
 cDNA ,

**. DNA**

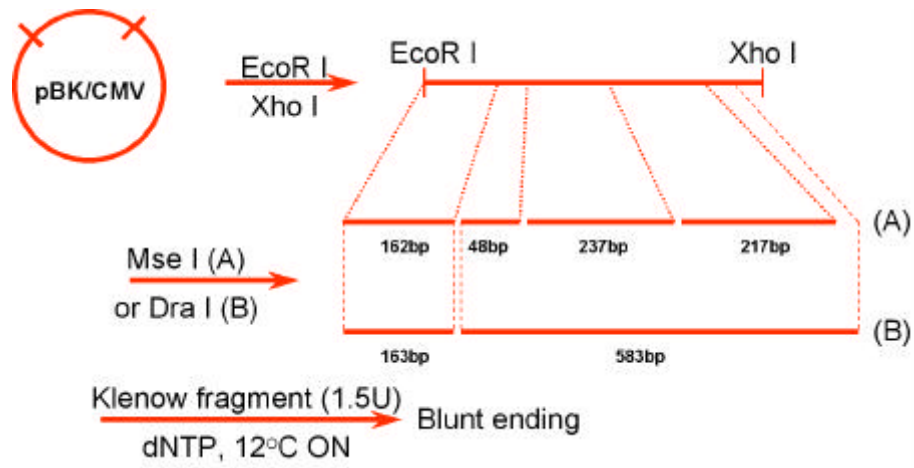
plasmid QIAprep Spin Miniprep Kit (QIAGEN,  
 Valencia, CA, USA) , *EcoR* *Xho* 3  
 7 1 cDNA insert 1% agarose gel  
 Sanger <sup>30</sup> dideoxy chain termination  
 T7 Sequenase version 2.0 DNA sequencing kit (Amersham Life

Science, Cleveland, USA) insert 5' 3'  
 primer T3 (5'-AATTAACCCTCACTAAAG  
 GG-3') T7 (3'-CGGGATACCACTCAGCATAATG-5') . 5 µg  
 plasmid DNA ssDNA primer DNA  
 5 [<sup>35</sup>S]dATP dideoxy  
 NTP가 37 5  
 6% polyacrylamide X-ray film  
 DNA BLAST (Basic Local Alignment Search Tool)  
 GenBank, EMBL database

가 , 가 transcript alkyl-  
 hydroperoxidase reductase coding cDNA 가  
 cDNA가 pBK-CMV *EcoR* I *Xho* I  
 insert cDNA , *Mse* 4 nucleotide  
 , nucleotide 1.5U Klenow fragment 0.5 mM dNTPs 16  
 ( 1), pGEX4T 1 mM IPTG  
 12% SDS-PAGE ,

2 set primer (AHR1F: ATGTCTTGCAATCAAC  
 AHR1R: GCTTTTATTCTTGGACAACA, AHR 2F: TGTCAAGAGAAAG  
 AATG AHR2R: TTAAATTCTGGTGCTTC) , denaturing 94  
 1 , annealing 50 1 , extension 72 2 35 cycle . PCR  
 pGEM T-Easy vector , *EcoR* I insert  
 , pGEX4T-3 . pGEX4T-3 1 mM

IPTG



1. *Mse*

1 mM IPTG

3

10

10

3

, 12,000g

glutathion-S-transferase(GST) fusion

GST

ELISA

(

, )

1.

E-64 leupeptin 50 μM 가 50 mM Tris-Cl (pH 7.4)

. 가 , 170, 97, 62, 42, 40, 31, 28, 14 kDa ( 2). ammonium sulfate 45%, 65% 85% DEAE-Sepharose, Superdex 200 FPLC, Resource Q FPLC

45% ammonium sulfate 170 kDa, 65% 62 kDa, 85% 31, 97 kDa ( 3).

2. cDNA expression library

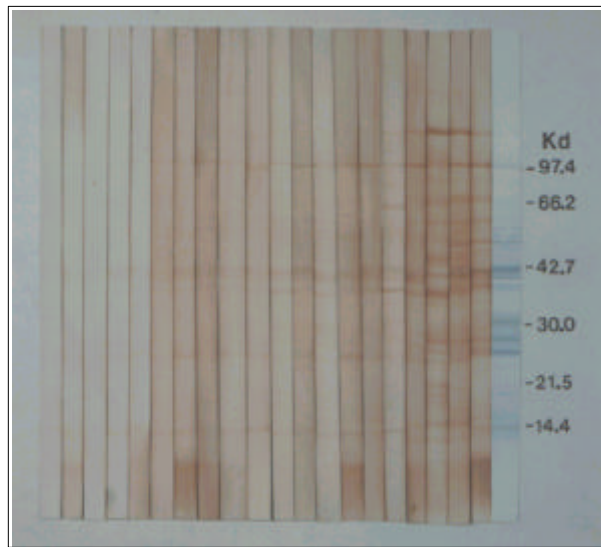
cDNA library titer 6 X 10<sup>5</sup> , recombinant plaque 97% . cDNA library (31, 62, 97 kDa)

3 ( 4). , (31, 62, 97 kDa)

cDNA , 12 , 31 kDa

alkyl-hydroperoxidase reductase , 62 kDa alcohol

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

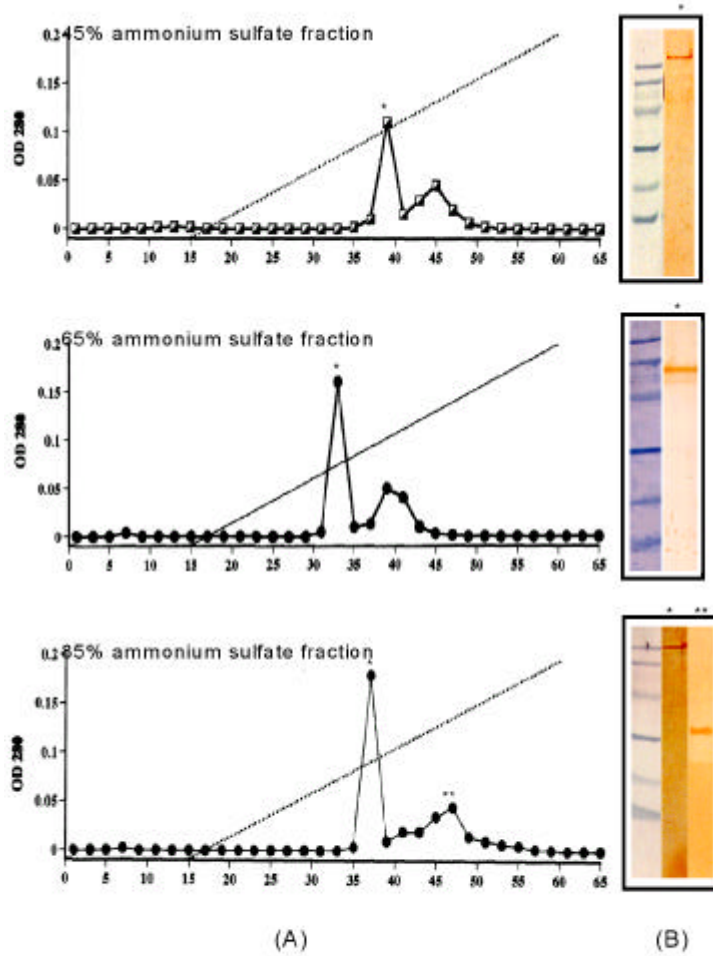


2.

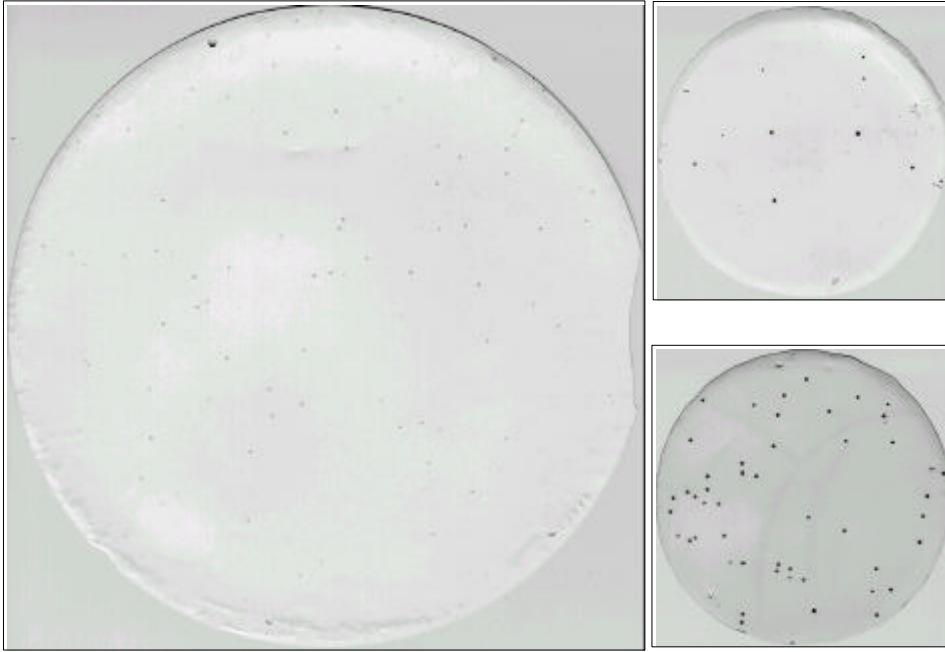
### SDS - PAGE

. 1 5, ; 6 20,  
; 21, Coomassie Blue .





3. . (A) ammonium sulfate FPLC Resource Q , (B) peak analysis. 45% ammonium sulfate - 170 kDa , 65% ammonium sulfate - 62 kDa , 85% ammonium sulfate - 97 kDa 31 kDa .



1

2 ( ) 3 ( )

4. cDNA library 1-3 nitrocellulose membrane. 1  
 500 1, 2 1:  
 conjugated goat anti-mouse IgG 1: 2,000 alkaline phosphatase  
 BCIP/NBT 1 ,

dehydrogenase 3 . 97 kDa

12

9 (75%) alkyl-hydroperoxide reductase

, peripheral membrane antigen (2 ), unknown (1 )

가 alkyl-hydroperoxide reductase

coding cDNA .

Alkyl-hydroperoxide reductase coding cDNA 766 bp

, 233 ( 5).

### 3.

alkyl-

hydroperoxide reductase coding cDNA 가

*Mse* I 2 set primer, AHR1F AHR1R (Sa,

1-30), AHR2F AHR2R (Sb, 14-54) PCR

( 6). *Mse* I 4 pGEX4T

, 1mM IPTG ,

, ( 1-54) (

7). 2 set primer PCR PCR

pGEM T-Easy vector , *EcoR* I insert

, pGEX4T-3 . pGEX4T-3 1 mM

IPTG ,

. , Sb (AHR2F AHR2R primer set PCR

, 14-54) ( 8, 9). , 가

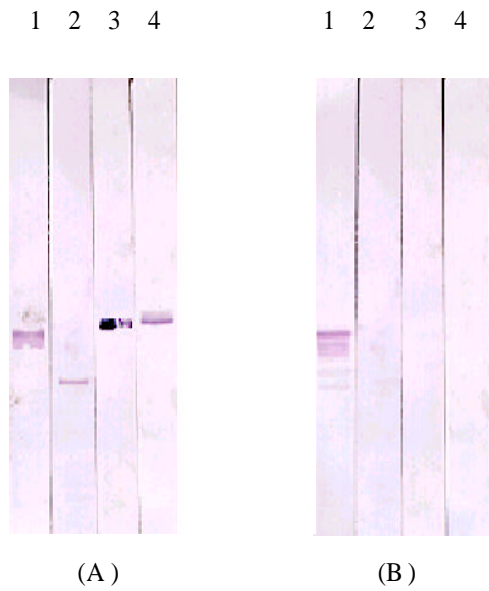
Sb .

' GCCGAGGTCAAATGTCCTTCCAATCAACAAAAAGAGTGTGTGTAATAAAGAATGTCAAGAG  
           M S C N Q Q K E C C K K E C Q E  
 AAAGAATGTTGTAAGAATGTTGTTGTCGAAGAATAAAAGCATTTAAGAAATTTATAAAC  
   K E C C K E C C C P R I K A F K K F I N  
 ACATTTGAAAAAGCACAAATTGGAAAAGAACACCAGAATTTAAACCACCACATATTGT  
   T F E K A Q I G K E A P E F K A P A Y C  
 CCATGTGGTCAATCAAGAGATTGATATTAATGAATATAGAGGAAAATATGTTGATTG  
   P C G S I K E I D I N E Y R G K Y V V L  
 TTGTTTTATCCATTGGATTGGACATTTGTTTGTCCAACAGAAATGATTGGATATAGTGAA  
   L F Y P L D W T F V C P T E M I G Y S E  
 CTTCCAGGACAATTGAAGAAATCAATTGTGAAGTATTGGAGTGTAGATTGAGTT  
   L A G Q L K E I N C E V I G V S V D S V  
 TATTGTCATCAAGCATGGTGTGAAGCAGATAAAAGTAAACGAGGAGTACGAAAGTTGACA  
   Y C H Q A W C E A D K S K G G V G K L T  
 TTCCATTAGTATCAGATATTAAGAGATCCATTTCTATCAAATATCGAATGTTAAATGTC  
   F P L V S D I K R C I S I K Y G M L N V  
 GAACCAGGAATTGCAAGAAGAGGATATGTCATCATTGACGATAAACGAAAAGTAAAGATAC  
   E A G I A R R G Y V I I D D K G K V R Y  
 ATTCAAATGAATGATGATGGAATTGGAAGATCAACCGAAGAACAATCAGAATAGTGAAA  
   I Q M N D D G I G R S T E E T I R I V K  
 GCAATTCATTCAGTGTGAACATGGACAGTTTGTCCACTCAATTGGAACCAGCCAAA  
   A I Q F S D E H G A V C P L N W K P G K  
 GACACCATTGAACCAACACCAGATGGAATTAAGAATATTTAACACCACATTAACAACAA  
   D T I E P T P D G I K K Y L T A H \*  
 CAAGATAATTTAATACAAATTATTTTAAAAAAAAAAAAAAAAAAAAA 3'

5. Alkyl-hydroperoxide reductase cDNA

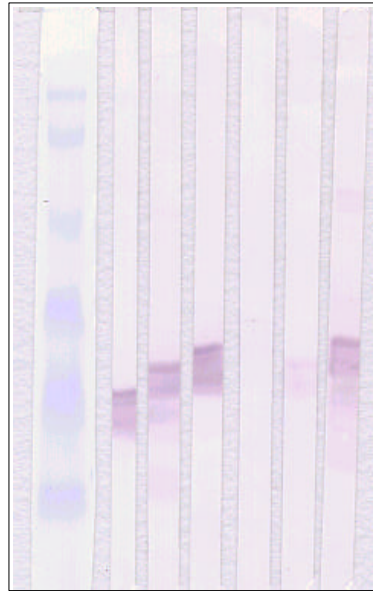
.                           766 bp                           , DNAsis  
 ,                           233 , 26,250 Da                           , pI 7.44  
 .





7. *Mse* 4 GST - fusion  
 (A) anti-GST , (B)  
 (A), (B) lane 1: 162bp (1-162), 2: 48bp (163-211)  
 3: 237bp (232-469), 4: 217bp (495-712)

1 2 3 4 5 6



8. GST (lane 1, 3) AHR1F AHR1R(lane 2, 5)  
PCR GST fusion protein AHR2F and  
AHR2R(lane 3, 6) PCR GST fusion  
anti-GST antibody (lane 1-3)  
(lane 4-6) .



9. Computer simulation alkyl-hydroperoxide  
 reductase 3 . (☞)  
 ( 14-51 ) 가 .



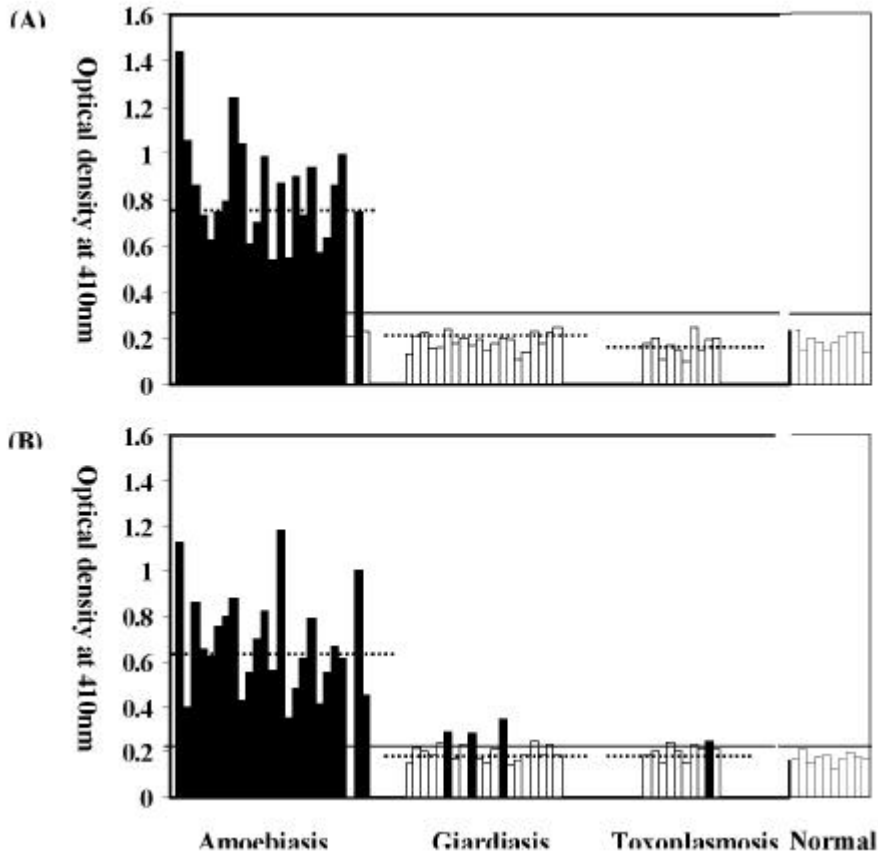
4.

*Mse* I PCR alkyl-hydroperoxide reductase coding  
cDNA , GST  
alkyl-hydroperoxide reductase  
GST

ELISA 가  
ELISA , 10 ,  
3 cut-off value  
Cut-off value 0.24,  
alkyl-hydroperoxide reductase 0.21 . ELISA  
25 20  
10 ,  
ELISA titer 0.2- 1.47 ( titer  
0.73) (25 23 , 92%),  
( 100%).  
alkyl-hydroperoxide reductase , 25 24  
, 30 4 (  
86.6%) ( 10).

ELISA OD value가  
( 11). alkyl-hydroperoxide

reductase coding cDNA



10. alkyl-hydroperoxide reductase(B)

3

alkyl- hydroperoxide reductase  
cut- off value , ( - - )

(A)

ELISA . 10  
cut- off value  
0.24,  
0.21 . ( - )

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



11.

1  $\mu\text{g/well}$  SDS-PAGE, Nitrocellulose membrane  
 1: 1,000 1  
 1: 5,000 alkaline phosphatase가  
 2 1, NBT/BCIP . 1- 8,  
 ; 9- 11, ; 12- 14,  
 ; 15- 19, .

#### IV.

가 가

가

가

, ELISA , ELISA가

ELISA , 170 kDa adhesin, Serine-rich *E. histolytica* protein (SREHP), 96 kDa

<sup>7,26,31</sup> 가

ELISA

31, 62, 97 kDa

( 3.),  
cDNA , 31 kDa

alkyl-hydroperoxidase reductase ,  
cDNA library

( 12  
9 coding ). 62 kDa  
alcohol dehydrogenase 3 .  
97 kDa . 97 kDa

3' cDNA library 가

가

alkyl-hydroperoxide reductase coding cDNA 766

bp , DNA sis , 233

, pI 7.44, 26,250 Da ( 5).

alkyl-hydroperoxide reductase

*E. dispar* <sup>32</sup>

, *E. dispar* 10 (KEYCCKEYCK) 20

30 , 35 가 I M, 54 P Q

가

alkyl-

hydroperoxide reductase coding cDNA 가

*Mse* I cysteine rich(CR, 1-54)

cysteine poor(CP) , CP CP 4

CR ( 7.).

Velaquez <sup>7</sup> 170 kDa adhesin 가

cysteine rich (649- 1202)

가

2 set primer PCR 2 (Sa; 1-30, Sb;14-54)  
, Sb ( 8).  
alkyl-hydroperoxide reductase 가 14-54  
, .  
*E. dispar* 10 35 가 I  
M, 54 P Q .  
가 .  
. .  
alkyl-hydroperoxide reductase  
ELISA  
( 10 11).  
ELISA  
, , .  
(1:64 1:256) (1:2560 )  
, .  
(1:16  
) 25 ,  
20 , 10  
10 .  
ELISA titer 0.2- 1.47  
( titer 0.73) (25 23 , 92%),  
( 100%).  
alkyl-hydroperoxide reductase 25 24

, 30 4 (
   
 86.6%)( 11). cathepsin-L1
   
 cathepsin-L1 가 ELISA
   
 98.9% 100% ,
   
 100% 94.6% 가
   
 Cornelissen JBWJ <sup>33</sup> .
   
 alkyl-hydroperoxide reductase 29 kDa 가
   
 87, 94%
   
 28
   
 , .
   
 가 alkyl-hydroperoxide
   
 reductase immunodominant ,
   
 ELISA 100%
   
 . alkyl-hydroperoxidase reductase
   
 .



V.

가

가

exchange (DEAE-Sephacel)  
 Resource Q) filtration (Superdex 200)  
 31, 62, 97 kDa  
 BALB/c 3  
 cDNA library  
 , 31 kDa alkyl-hydroperoxidase reductase 3  
 , 62 kDa alcohol dehydrogenase  
 97 kDa  
 가 31 kDa  
 alkyl-hydroperoxidase reductase cDNA  
 , 14-51 가  
 alkyl-hydroperoxidase reductase  
 ELISA  
 가 100% 92%  
 alkyl-hydroperoxidase reductase

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## Abstract

### **Cloning of major antigen of *Entamoeba histolytica* and serodiagnosis of amebiasis using a recombinant protein**

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*Entamoeba histolytica* is a major cause of morbidity and mortality worldwide. The causative agent, the intestinal protozoan parasite *E. histolytica*, probably infects more than 500 million people, resulting in an estimated 50 million cases of diarrhea and liver abscess and 40,000 deaths annually. To develop an improved serodiagnostic test for amoebiasis, we performed a detailed analysis of a major antigen for *E. histolytica* Korean strain YS-27 and analysed the immunodominant protein fragment containing *E. histolytica* specific epitopes. We performed a detailed analysis of the immunodominant epitopes of a major antigen, alkyl-hydroperoxide reductase, and evaluated its sensitivity and specificity. ELISA based on the fragment containing the immunodominant epitope was evaluated further and compared with full-length recombinant alkyl-hydroperoxide reductase. Specificity and sensitivity of the two ELISAs were assessed using 55 human sera of parasitic protozoa infection cases (25 amebiasis, 20 giardiasis

and 10 toxoplasmosis sera) and 10 healthy control sera. The immunodominant epitope of the alkyl-hydroperoxide reductase is localised only in the N-terminus 14 to 54 amino acid residues. The sensitivities of the two ELISAs were very high, 92% and 96%, respectively. The specificity of the fragment was 100%, whereas the specificity of the full-length alkyl-hydroperoxide reductase was 86.6%. These results indicate that the fragment containing the immunodominant epitope of the alkyl-hydroperoxide reductase can be used to accurately serodiagnose amebiasis without cross-reactivity from other parasites.

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**Key Words:** *Entamoeba histolytica*, amoebiasis, alkyl-hydroperoxide reductase, epitope, serodiagnosis