A new diagnostic tool for neurocysticercosis is a member of a cestode specific hydrophobic ligand binding protein family[1]

Nahid Saghir[2], Phillip J. Conde, Peter M. Brophy*, John Barrett

Institute of Biological Sciences, University of Wales, Aberystwyth, Ceredigion SY23 3DA, UK

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Abstract A protein of unknown function has been identified as a key serological tool for diagnosis of human tapeworm neurocysticercosis, a major worldwide neurological disease. Our own sequence analysis predicts that this protein is a member of a newly identified cestode specific oligomeric hydrophobic ligand binding protein family. In this report, using a rat cestode model, we confirm that homologues of this protein can bind fatty acids and their derivatives, and thus suggest a biological function for this key diagnostic tool.

Key words: Hydrophobic ligand binding protein; Cestoda; Diagnosis; *Hymenolepis diminuta*

1. Introduction

Recent investigations identified a new protein as a key serological tool for diagnosis of human tapeworm neurocysticercosis, a major worldwide neurological disease [1]. Our own sequence analysis predicts that this protein is a member of the oligomeric hydrophobic ligand binding protein (HLBP) family. In this report, using a rat tapeworm model, we propose a biological function for this key diagnostic tool.

It is appropriate that a diagnostic tool is associated with a novel parasite biochemical pathway. Natural parasitic helminth infections are characteristically chronic with adult parasites often surviving 10 years or more in micro-oxygenated and potentially toxic host environments [2]. Adult helminths have evolved adaptations in cellular metabolism in order to survive under low oxygen tension. One unique classical physiological feature is the down regulation of enzymes responsible for the synthesis of hydrophobic metabolites (usually requiring oxygen at some stage in their synthetic pathway). Adult worms do not synthesise long chain fatty acids, cholesterol or related sterols and rely on uptake from the host, nor can parasites desaturate preformed fatty acids [3]. Thus HLBPs are widely distributed in helminths and can be divided into two groups: the classical 14 kDa β-barrel fatty acid binding proteins [4] and a second group of new helix-rich proteins, specific to helminths. Nematode polyprotein allergens (NPAs) are expressed as dimers and are the most intensively investigated member of this newly discovered group [5]. We have also previously reported an oligomeric helix-rich lipid binding protein in the sheep cestode *Moniezia expansa* [6].

2. Materials and methods

2.1. Purification of cestode HLBP

Adult *Hymenolepis diminuta* were recovered from the small intestine of male Wistar rats previously infected, per os, with 10 cysticercoids each [7]. The HLBP was isolated by a combination of ammonium sulphate fractionation, gel filtration and anion exchange chromatography essentially as described previously [6]. The homogeneity of the preparation was monitored by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; Phast System, Pharmacia) and the protein concentration determined by Coomassie Blue G-250 binding assay, using bovine serum albumin as the standard [7]. Chromatofocusing in the range pH 6–4 was carried out according to the manufacturer's instructions (Pharmacia).

Following blotting onto a PVDF membrane, N-terminal sequencing was carried out by Dr K. Bailey, Queens Medical Centre, Nottingham, UK using an Applied Biosystems Model 473A automatic analyser. Molecular weight determinations by electrospray mass spectrometry were carried out using a Micromass LCT (TOF) instrument with a Z-spray inlet.

2.2. Immunological analysis

Polyclonal antibodies to purified *Hymenolepis* HLBP (*H*-HLBP) were raised by the intramuscular injection into a rabbit of a suspension of 250 μg of *H*-HLBP in 250 μl of phosphate buffered saline (PBS) and 250 μl of Titremax. The IgG was purified in a two step procedure using octanoic acid and $(NH_4)_2SO_4$ [8] and the titre was estimated by standard dot blot enzyme-linked immunosorbent assay. In-house tissue samples from *M. expansa*, *H. diminuta*, *Schistocephalus solidus*, *Fasciola hepatica*, *Panagrellus redivivus*, rat liver and rat small intestine were homogenised in PBS containing 0.1% (v/v) Triton X100 and centrifuged for 15 min at $14\,000 \times g$ at 4°C. The protein samples were separated on 15% SDS–polyacrylamide gels and transferred electrophoretically onto a nitrocellulose membrane (Amersham Pharmacia Biotech). Following transfer, the membrane was blocked for 3 h using 3% (w/v) casein in Tris-buffered saline pH 7.5 (TBS), washed 3×5 min in TBS before being incubated with the primary IgG (1:2000 dilution) for 90 min. After a second identical wash step the secondary IgG (1:30 000 dilution), goat anti-rabbit with conjugated alkaline phosphatase (Sigma), was incubated for 1 h before washing in TBS and developing with the BCIP/NBT liquid substrate system (Sigma).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) isolation of HLBP cDNA

RNA was isolated from *H. diminuta* using guanidinium thiocyanate and *N*-sarcosine extraction followed by oligo-d(T)-cellulose chromatography. First-strand cDNA was prepared by a synthesis kit (Amer-

*Corresponding author. Fax: (44)-1970-622350.
E-mail: pmb@aber.ac.uk

[1] The nucleotide sequence for *Hymenolepis diminuta* hydrophobic ligand binding protein has been submitted to GenBank nucleotide sequence databases under accession number AF249884.

[2] Present address: CRC Beatson Laboratories, Glasgow G61 1BD, UK.

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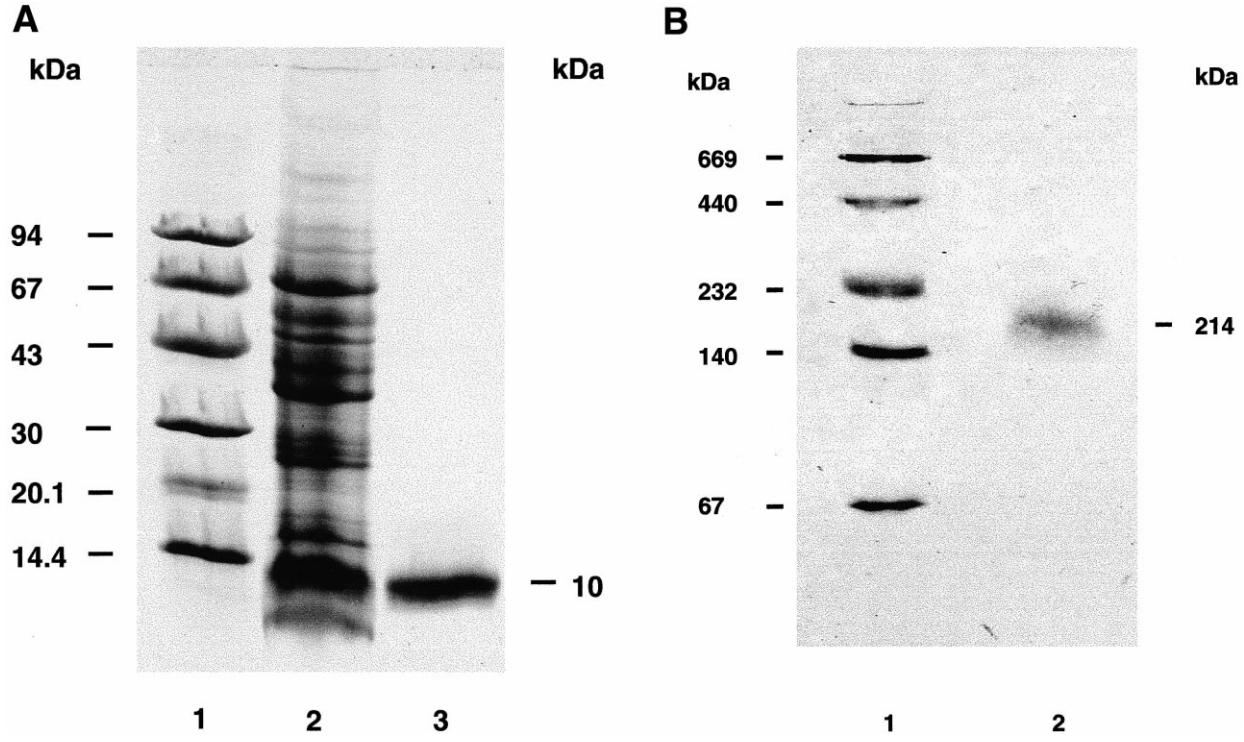

Fig. 1. A: PhastGel 8–25 gradient gel under denaturing conditions (SDS). Lane 1, low molecular mass markers; lane 2, *H. diminuta* somatic extract (2 μg); lane 3, purified *H. diminuta* HLBP (1.4 μg). B: Native Phastgel 8–25. Lane 1, native high molecular mass standards; lane 2, purified *H. diminuta* HLBP (1.38 μg).

Table 1
Binding of fatty acids and their acyl-CoA derivatives by *H. diminuta* HLBP

Ligand	Apparent K_D (nm)	Tryptophan quenching
Caproic acid (6:0)	160 ± 11	+
Caprylic acid (8:0)	180 ± 10	+
Capric acid (10:0)	143 ± 7.4	+
Lauric acid (12:0)	128 ± 7.1	+
Myristic acid (14:0)	60 ± 3.1	+
Palmitic acid (16:0)	66 ± 3.7	+
Stearic acid (18:0)	30 ± 2.3	+
Arachidic acid (20:0)	60 ± 4.8	+
Oleic acid (18:1)	17 ± 8	+
Arachidonic acid (20:4)	44 ± 3.4	+
Linoleic acid (18.2)	38 ± 1.8	+
Linolenic acid (18.3)	68 ± 3.5	+
Palmitoleic acid (16:1)	44 ± 3.4	+
Docosahexaenoic acid (22:6)	29 ± 2.7	+
Petroselenic acid (18:1)	18 ± 1.2	+
Elaidic acid (18:1)	40 ± 3	+
Nervonic acid (24:1)	34 ± 2.7	+
Erucic acid (22:1)	16 ± 1	+
n-Octanoyl CoA (8:0)	–	+
Palmitoleoyl CoA (16:0)	115 ± 5.7	+
Palmitoleoyl CoA (16:1)	177 ± 5.3	+
Oleoyl CoA (18:1)	95 ± 6	+
Stearoyl CoA (18:0)	188 ± 9	+
Linoleoyl CoA (18:2)	109 ± 6.3	+
Behenoyl CoA (22:0)	–	–
CoA lithium salt	–	–

Apparent K_D values were obtained by the displacement of bound DAUDA, as described in Section 2, and are means ± S.E.M. of three experiments. +, tryptophan quenching detected; –, no binding or tryptophan quenching detected.

sham Pharmacia Biotech). The cDNA synthesis was completed using an oligo-d(T) based primer which also served as the downstream primer, and the upstream primer was a degenerate primer directed against the N-terminal HLBP protein sequence (*H*-HLBP was not N-terminally blocked and the amino acids at the first 25 positions were sequenced).

PCR products were excised from 1.5% agarose gels by Sephaglas (Amersham Pharmacia Biotech) and after treatment with Klenow fragment and T4 polynucleotide kinase, blunt-end ligated into the *Sma*1 site of pUC18, and sequenced on a LI-COR system.

2.4. Spectrofluorimetry

Fluorescent measurements were made at 20°C in a Shimadzu RF-530/PC spectrofluorimeter using 2 ml samples in a quartz cuvette. Raman scattering by the solvent was corrected for, where necessary, by using appropriate blank solutions. All spectra are uncorrected unless otherwise stated.

Fluorescent ligands were stored as stock solutions (10 mM) in ethanol in the dark at −20°C and freshly diluted to 0.1 mM with 50 mM potassium phosphate buffer, pH 7.4 prior to use. Stock solutions of oleate (10 mM) in ethanol were also stored at −20°C in the dark and diluted to 50 μM with ethanol prior to fluorescent competition assays. Fluorescent titrations were carried out as previously described [6]. The fluorescent ligand was added in small aliquots (2–5 μl) to 2 ml of protein in 50 mM potassium phosphate buffer, pH 7.4. Fluorescent measurements were corrected for dilution and solvent effects and for the fluorescent contribution of the unbound ligand. Fluorescent data (bound ligand versus unbound ligand) were fitted by standard non-linear regression techniques to a single non-competitive site model with non-specific binding to give estimates of the apparent dissociation constant (K_D) and number of binding sites (n) per cestode HLBP monomer.

The binding of non-fluorescent ligands to *H*-HLBP was evaluated by measuring the displacement of bound DAUDA (11-[5-dimethylaminonaphthalene-1-sulphonyl amino] undecanoic acid) and the K_D calculated by standard analysis [9]. Alternatively the binding of non-fluorescent ligands was followed by measuring the decrease in endogenous tryptophan fluorescence at 326 nm (Ex_{max} 295 nm). Since differences in protein tryptophan fluorescence caused by ligand binding are,

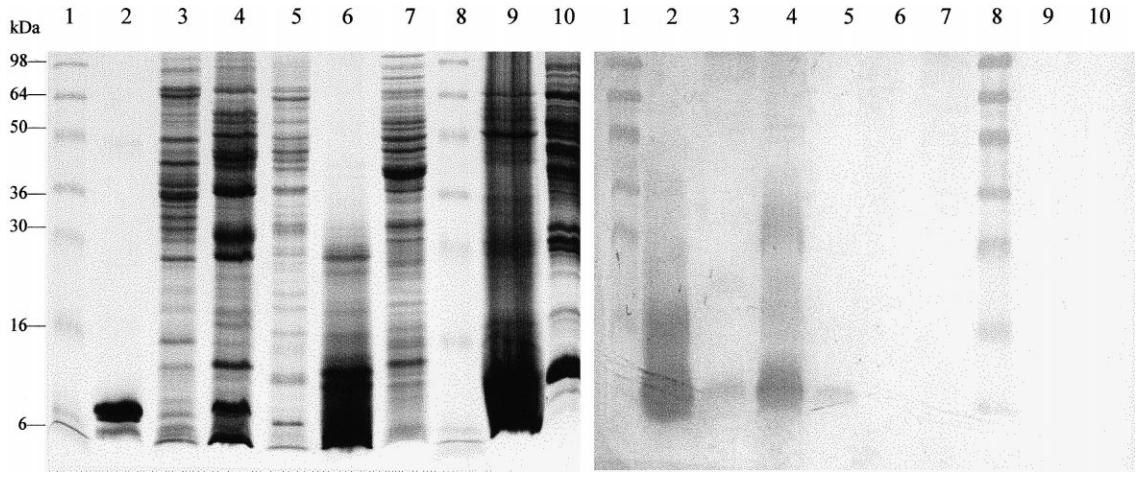

Fig. 2. Western blot analysis of cestode, digenean, nematode and mammalian tissue somatic extracts incubated with polyclonal *H*-HLBP antibody. Coomassie stained gel is shown on the left, Western blot on the right (alkaline phosphatase). Lanes 1 and 8, prestained markers; lane 2, purified *H*-HLBP; lane 3, *M. expansa* (cestode) somatic extract; lane 4, *H. diminuta* somatic extract; lane 5, *S. solidus* (cestode) somatic extract; lane 6, *F. hepatica* (digenean) somatic extract; lane 7, *P. redivivus* (nematode) somatic extract; lane 9, rat intestine somatic extract; lane 10, rat liver somatic extract. For details see Section 2.

in general, not proportional to the amount of bound ligand, no attempt was made to estimate K_D or n using this method.

3. Results and discussion

3.1. Purification

H-HLBP constitutes approximately 9.5% of the soluble cytosolic protein and was purified with a yield of approximately 60%. The purified protein showed both a single band under reducing (Fig. 1A, 11 kDa) and native conditions (Fig. 1B). The native molecular mass was estimated to be 115 kDa under our gel filtration conditions (FPLC Superose 12 and 150 mM NaCl, 20 mM Tris–HCl, pH 7.4). The p*I* of the native protein determined on PhastGel IEF gels was approximately 5.2 and this was confirmed by chromatofocusing (p*I* 5.0).

3.2. Immunological analysis

Western blot analysis (Fig. 2) showed that the anti-*H*-HLBP polyclonal antibody had relatively high specificity for the native *H. diminuta* HLBP protein (lanes 2 and 4) and there was cross reactivity with a protein of similar size in the two other cestode extracts (*M. expansa* and *S. solidus*). There was no cross-reactivity with proteins from digeneans (*F. hepatica*), nematodes (*P. redivivus*) or from mammals (rat liver and intestine). Pre-immune sera did not recognise *H. diminuta* HLBP (results not shown).

3.3. Sequence analysis

The amino acid sequence predicted from cDNA (GenBank accession number AF249884) confirmed the Edman degradation of the native protein. The predicted molecular mass of *H*-HLBP of 8423 Da was in reasonable agreement with the mass of the native protein determined by electrospray mass spectroscopy of 8469 Da.

Only five database proteins (all cestode proteins) were found which showed significant similarities with the *H. diminuta* HLBP (Fig. 3): a *M. expansa* lipid binding protein [6] and four common diagnostic antigens of unknown function, antigen B from *Echinococcus granulosus* and *Echinococcus multilocularis* and the immunodiagnostic antigens from *Taenia crassiceps* and *Taenia solium*. The cestode immunodiagnostic antigens have a hydrophobic-rich N-terminal extension absent in the two soluble cestode HLBPs (Fig. 3). Thus it appears

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HdHLBP                                EQ--ETNPIRRIKTKAKEYFAARERFYDEDPLGKQIAAHLSKW    41
MeHLBP                                EQ--ETNPIRAIKKRITSYLKSREEFYDKDPLGQKIAKFYGEW    41
EgHLBP          MLLALALVSFVVVTQA-----DD-GLTSTSRSVMKMIGE----RKYFFERDPLGQKVVDLLKEL    54
EmHLBP          ----------------------DD-GLTSTSRSMMKMLGE----MKYFFERDPLGQKLVDLLKEL    38
TsHLBP          MRASIFLAVAILVITVVAAPDDDKGQEDLNMTVMLQLGE----VRRFFTEDPLGRNVTLQLKEM    60
TcHLBP          MRASIFLALAILVITVVAAPTDDKGPEDLKKKMMKQLGE----VRRFFREDPLGQKIIDHFQET    60

HdHLBP          REIIRDVRARLRGYLRKYLNDLQKEypka                                    70
MeHLBP          KELVAEVRKRVRARIAAYVKKLQEE----                                    66
EgHLBP          EEVFQLLRKKLRTALKSHLRELVAEGK--                                    81
EmHLBP          EEVFQMLRKKLRTALKSHLRELVAEGK--                                    65
TsHLBP          IAIAKVIPHRIRKCLGEYLKGLENE----                                    85
TcHLBP          VSICKAIRERIRKRLGEYLKGLENE----                                    85
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Fig. 3. Amino acid sequence alignment of *H. diminuta* HLBP (GenBank accession number AF249884) with *M. expansa* putative lipid binding protein (sequence from [6]), and the new diagnostic antigens from *E. granulosus* [Z26336], *E. multilocularis* (S41924 and [11]), *T. crassiceps* (U07150 and [12]) and *T. solium* (AF076609 and [1]).

that cytosolic/membrane bound or secreted members of the HLBP family are expressed. A potential signal peptide and cleavage site (between positions 19 and 20: VAA-PD) in the N-terminal extension of the cestode immunodiagnostic antigens supported this prediction [10].

3.4. Substrate analysis

Purified *H*-HLBP bound both saturated and unsaturated fatty acids with K_D values in the nM range (Table 1). Long chain fatty acids (C_{14}–C_{24}) had the highest affinity, whilst short and medium chain fatty acids (C_6–C_{12}) were bound less strongly. Mono-unsaturated fatty acids had lower K_D values than their saturated equivalents, but the insertion of additional double bonds (e.g. $C_{18:1}$ to $C_{18:3}$) reduced the affinity for the protein. The *H*-HLBP also bound the CoA derivatives of fatty acids, but with a much lower affinity. All ligands that bound to *H*-HLBP, displacing DAUDA, also quenched intrinsic protein tryptophan fluorescence.

In conclusion, this report further indicates that the new diagnostic tool for tapeworm infection is a member of a relatively highly expressed and cestode specific HLBP family.

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References

[1] Chung, J.Y., Bahk, Y.Y., Huh, S., Kang, S.S., Kong, Y. and Cho, S.-Y. (1999) J. Infect. Dis. 180, 1307–1315.
[2] Behnke, J.M., Barnard, C. and Wakelin, D. (1992) Int. J. Parasitol. 22, 861–907.
[3] Cox, G.E. (1985) in: Modern Parasitology, 2nd edn, Blackwell Scientific, Oxford.
[4] Becker, M.M., Kalinna, B., Waine, G.L. and McManus, D.P. (1994) Gene 148, 321–325.
[5] Kennedy, M.W. (2000) Biochim. Biophys. Acta 1476, 149–164.
[6] Barrett, J., Saghir, N., Timanova, A., Clarke, K. and Brophy, P.M. (1998) Eur. J. Biochem. 250, 269–275.
[7] Brophy, P.M. and Barrett, J. (1990) Parasitol. 100, 137–141.
[8] McKinney, M.M. and Parkinson, A. (1987) J. Immunol. Methods 96, 271–278.
[9] Bagshaw, C.R. and Harris, D.A. (1987) in: Spectrophotometry and Spectrofluorimetry: a Practical Approach (Harris, D.A. and Bashford, C.L., Eds.). IRL Press, Oxford.
[10] Nielsen, H., Engelbrecht, J., Brunak, S. and Von Heijne, G. (1997) Prot. Eng. 10, 1–6.
[11] Frosch, P., Hartmann, M., Mühlschlegel, F. and Frosch, M. (1994) Mol. Biochem. Parasitol. 64, 171.
[12] Zarlenga, D.S., Rhoads, M.L. and Al-Yaman, F.M. (1994) Mol. Biochem. Parasitol. 67, 215–223.