Cloning, heterologous expression and antigenicity of a schistosome cercarial protease

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SUMMARY

A gene coding for the 30 kDa *Schistosoma mansoni* cercarial protease was amplified using the polymerase chain reaction (PCR) from genomic DNA templates. Cloning and sequencing of several independent PCR clones revealed the presence of an intron additional to the one described in the original cloning of the gene. The 3 exons were cloned into expression vectors so that they could be expressed as separate glutathione-S-transferase (GST) translational fusions. Recombinant bacteria carrying these expression plasmids expressed the fusion proteins at high levels. Western blotting of bacterial lysates with sera raised against the native *S. mansoni* cercarial protease showed that all 3 exons were recognized. Thus we have produced recombinant bacteria capable of providing large amounts of an *S. mansoni* antigen for immunological studies and evaluation as a candidate vaccine.

Key words: *Schistosoma mansoni*, cercarial-elastase, expression, recombinant-fusion-proteins.

INTRODUCTION

Transmission of schistosomes to humans occurs when cercariae penetrate skin and it has been suggested that entry is between skin cells, involving a mucosal secretion from the post-acetabular glands of the cercaria. This is followed by mechanical muscular probing and the secretion of serine proteases to digest skin macromolecules, allowing further migration of the larva (Lewert & Lee, 1956; Stirewalt & Fregeau, 1966; Campbell *et al.* 1976; Landsperger, Stirewalt & Dresden, 1982). Penetration of the skin is significantly inhibited in the presence of serine protease inhibitors (McKerrow & Doenhoff, 1988), suggesting that cercarial proteases have a vital role in aiding host invasion.

One protease in particular, of approximately 30 kDa has been identified as crucial for skin penetration by cercariae (McKerrow *et al.* 1985 *a*). The protease has been purified from *Schistosoma mansoni* cercarial extracts and is also present in secretions released from cercarial acetabular glands in the presence of skin lipids (McKerrow *et al.* 1983). The enzyme has a broad substrate specificity, having activity against elastin, Azocoll, gelatin, laminin, fibronectin, keratin and types IV and VIII collagen (McKerrow *et al.* 1985 *b*) and has become known as cercarial elastase. There is also evidence to suggest that the protease is able to cleave immunoglobulins (Doenhoff *et al.* 1990).

There is a close similarity between the cercarial elastase and some proteases found in other skin-invasive parasites, for example, the serine protease released by the 3rd-stage larvae of *Necator americanus*, which also shows activity against the Fc portion of IgG immunoglobulin (Kumar & Pritchard, 1992). Due to a possible role in transmission, these proteases could be used to provide novel targets for vaccine development.

Molecular studies on the schistosome cercarial elastase have given further insights into the structure of the protein and the gene encoding for it. Four cDNA clones have been isolated for the cercarial protease gene from an *S. mansoni* sporocyst cDNA library (Newport *et al.* 1988). The nucleotide sequence shows significant homology to other members of the trypsin family of serine proteases, particularly rat pancreatic elastases I and II. The protein is synthesized as a zymogen, with a 27 amino acid signal peptide in the amino terminus of the mature protein (Newport *et al.* 1988).

MATERIALS AND METHODS

Parasite

Schistosoma mansoni cercariae were produced from life-cycles maintained in *Biomphalaria glabrata* snails and random-bred mice. The isolates of the parasite from Puerto Rico, Brazil, Kenya and Egypt

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contributed to the pool of cercariae used for extraction of DNA and have been described in detail (Bickle & Doenhoff 1987).

Molecular biological reagents

Oligonucleotides were purchased from Genosys (Cambridge, UK). The sequences used were: Primer1 5′-d (CGTGTGTCAACCTGGTTG), Primer2 5′-d (TTGTTCAAATATTGGAGC), Primer3 5′-d (AACCGGATCCATACGTAG-TGGTGAA) *Bam*HI site underlined. Primer4 5′-d (ATCTCTAGACCGAATCACT) *Xba*I site underlined. Primer5 5′-d (GTCTGAATTCAGGTTT-CATTTCTCACA) *Eco*RI site underlined. Primer6 5′-d (ACCTTGAGCTCTTACTTCAATATT CCA) *Sac*I site underlined. Primer7 5′-d (GTTGCTCGAGGGTCGAGCGACTA) *Xho*I site underlined. Plasmid vector pT7T3α18 was purchased from Gibco BRL (Uxbridge, UK). Plasmid expression vector pGEX-KG was kindly donated by KunLiang Guan and Jack E. Dixon from Purdue University, West Lafayette, Indiana, USA (Guan & Dixon, 1991). All restriction enzymes were purchased from Promega Corp. (Madison, USA)

Antigens and antisera

Three polyclonal rabbit sera were used to probe immunoblots of expressed fusion proteins: Serum BR67 was raised by immunizing a rabbit with strips of nitrocellulose paper (NCP) carrying a 27 kDa *S. mansoni* larval protein which had been identified as the *S. mansoni* cercarial elastase (Curtis, 1988). The antigen was purified onto NCP by electroblotting after protein separation by SDS–PAGE. The rabbit was immunized with excised strips of NCP carrying the protein dissolved in dimethyl sulphoxide and emulsified in Freund's complete adjuvant.

Serum 1093X was raised by immunizing a rabbit subcutaneously with glutathione-S-transferase (GST) which had been purified from *S. japonicum* worm homogenate by affinity chromatography on glutathione-conjugated agarose (Simons & Van der Jagt, 1981) and emulsified in Freund's complete adjuvant. When tested by Western blotting, the antiserum reacted with equal intensity against both the 26 kDa and 28 kDa *S. japonicum* GST isoenzymes.

As a negative control, sera were obtained from rabbits immunized as above but with horse albumin in Freund's adjuvant. All antisera were blocked by treating with total *E. coli* lysate before use.

Isolation of genomic DNA

S. mansoni cercariae (0·2 g) were collected as described previously (Baba *et al.* 1977) and incubated for 3 h at 50 °C in 20 ml of lysis buffer (10 mM EDTA (pH 8·0), 10 mM Tris–HCl (pH 8·0), 0·5 % (w/v) SDS, 50 μg/ml Proteinase K (Sigma, Poole, UK)). One volume of TE-saturated phenol (pH 8·0) was added and mixed by gentle inversion of the tube. The solution was centrifuged for 10 min at 5000 ***g***. The upper aqueous layer was taken, and the step repeated twice. One volume of water-saturated chloroform was added, and the solution centrifuged for 10 min at 5000 ***g***. The upper aqueous layer was taken, and the step repeated twice. One-tenth of a vol. of 3 M sodium acetate (pH 6·0) and 2 vols of absolute ethanol were added to the resulting aqueous phase and incubated at −80 °C for 1 h before centrifuging at 12000 ***g*** for 20 min at 4 °C. The nucleic acid pellet was resuspended in 1 ml of TE buffer (10 mM Tris–HCl (pH 8·0), 1 mM EDTA (pH 8·0)) and RNase (Sigma) was added to a concentration of 50 μg/ml. After incubation at 37 °C for 30 min, the solution was phenol/chloroform extracted and ethanol precipitated as above. The DNA pellet was resuspended in 1 ml of TE buffer and stored at 4 °C.

Cloning the cercarial protease gene

Oligonucleotide primers were designed, based on the sequence of the cercarial protease cDNA clone isolated previously (Newport *et al.* 1988). Primers 1 and 2 (see Materials and Methods section) had alignment with the 3′ and 5′ ends of the mature protein coding sequence between amino acid residues 21 and 265 of the open reading frame.

PCR (Mullis & Faloona, 1987) was used to amplify the protein coding sequence from *S. mansoni* genomic DNA. The total reaction volume was 50 μl, containing 10 mM Tris–HCl, pH 9·0, 2·5 mM $MgCl_2$, 50 mM KCl, 0·1 % Triton X–100 (v/v), 40 pmol each of primers 1 and 2, 0·05 μg of genomic DNA, 0·25 mM each of dATP, dCTP, dGTP and dTTP (Promega) and 0·5 units of *Taq* DNA Polymerase (Perkin-Elmer Cetus, Berks, UK). Negative control tubes were set up containing either no DNA or no primer. Amplification was performed on a thermocycler, with an initial denaturing step at 95 °C for 5 min, and then for 20 cycles (92°C: 30 sec, 55 °C: 1 min, 74 °C: 1 min).

The amplified product was subjected to phenol/chloroform extraction and ethanol precipitation, as above. The amplified DNA was then treated with Klenow fragment of *E. coli* DNA polymerase I to give blunt-ended fragments. The total reaction volume was 30 μl, containing 30 mM Tris–HCl, pH 7·2, 10 mM $MgSO_4$, 1 mM DTT, 0·2 mM each of dATP, dCTP, dGTP and dTTP, 1 μg DNA and 2 units of Klenow. The solution was incubated at 37 °C for 5 min, then at 70 °C for 10 min. The DNA was ligated into *Sma*I-digested pT7T3α18 vector, in a total volume of 20 μl, containing 0·5 μg of digested plasmid DNA, 0·2 μg of digested insert DNA, 30 mM

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Tris–HCl, pH 7·8, 10 mM $MgCl_2$, 10 mM DTT, 1·5 mM ATP, 4% (w/v) PEG 8000 and 2 units of T4 ligase (Promega). The solution was incubated at 16 °C overnight, then at 70 °C for 10 min. Transformation was performed by electroporation at 2500 V into *E. coli* strain XL1Blue, using standard procedures (Potter, 1988). Selected transformants were screened for the protease gene insert by the use of cells as a source of DNA for PCR. A small number of cells were resuspended in 100 μl of sterile filtered water, placed in a water bath at 100 °C for 3 min, and 10 μl used in PCR as above, in place of genomic DNA (Lorens, 1991).

Three independent clones, designated as pHP1, pHP2 and pHP3, were subjected to dideoxynucleotide sequencing (Sanger, Nicklen & Coulson, 1977), using a Sequenase Version 2·0 kit (Amersham USB, Bucks, UK) and M13 universal sequencing primers (Genosys). Additional data were produced by subcloning *Eco*RV-digested fragments of the inserts into *Sma*I-digested pT7T3α18, and performing DNA sequencing as above. Resulting data were aligned with the previously described cercarial protease cDNA clone sequence (Newport *et al*. 1988) using Gene Jockey Sequence Processor (Biosoft, Cambridge, UK) on an Apple Macintosh computer.

Cloning the 3 exons of the gene

Oligonucleotide primers (see Materials and Methods section) were designed to align with the 3′ and 5′ ends of the 3 exons of the gene, amino acid residues 28–78, 79–184 and 185–265 (numbers based on the open reading frame). Restriction sites were incorporated into the primers to facilitate cloning (1st exon-primers 3 and 4, with *Bam*HI and *Xba*I; 2nd exon-primers 5 and 6, with *Sac*I and *Eco*RI; 3rd exon-primers 7 and 2, with *Xho*I and a Klenow-filled blunt end) PCR was used to amplify the exons from *S. mansoni* genomic DNA. The total reaction volume was 50 μl, containing 10 mM Tris–HCl, pH 9·0, 3 mM $MgCl_2$, 50 mM KCl, 0·1% (w/v) Triton X–100, 60 pmol of each primer, 0·05 μg of genomic DNA, 0·25 mM each of dATP, dCTP, dGTP and dTTP (Promega) and 0·5 units of *Taq* DNA Polymerase (Perkin-Elmer Cetus). Negative control tubes were set up as above. Amplification was performed on a thermocycler, with an initial denaturing step at 95 °C for 5 min, then for 25 cycles (92 °C: 30 sec, 50 °C: 1 min, 74 °C: 1 min).

The amplified fragments were subjected to phenol/chloroform extraction and ethanol precipitation as above. The fragments were digested with appropriate restriction enzymes and ligated into double-digested pGEX-KG plasmid vector, in a total volume of 20 μl, containing 0·5 μg of digested plasmid DNA, 0·2 μg of digested insert DNA, 30 mM Tris–HCl, pH 7·8, 10 mM $MgCl_2$, 10 mM DTT, 1·5 mM ATP, 4% (w/v) PEG 8000 and 2 units of T4 ligase (Promega). Transformation and screening of colonies by the use of PCR was performed as above.

Clones of the 3 exons were digested with *Bam*HI and *Hin*dIII, and the inserts were subcloned into *Bam*HI-*Hin*dIII digested pT7T3α18 vector to facilitate analysis by DNA sequencing as described above. Clones carrying the expected GST-exon fusions were thus identified and were designated as pGEXON1, pGEXON2 and pGEXON3 respectively.

Production of fusion proteins

E. coli strain JM109 was transformed by electroporation, as above, with each of the 3 constructs pGEXON1, pGEXON2 and pGEXON3. Single colonies of each were grown overnight at 37 °C in 5 ml of 2-YT (1·6 g Tryptone, 1·0 g Yeast Extract and 0·5 g of NaCl/100 ml) containing 50 μg/ml ampicillin and 50 μg/ml methicillin (Sigma). One ml of each culture was added to 50 ml of 5-YT (4·8 g Tryptone, 2·5 g Yeast Extract and 1·25 g NaCl/ 100 ml) containing antibiotics as above.

After incubation at 37 °C until an OD_{600} of 1·0 was reached, the bacteria were induced by adding filter sterilized isopropyl-β-D-thiogalactopyranoside, IPTG (Sigma) to a final concentration of 0·5 mM. Cells were grown for a further 2 h at 37 °C (Smith & Johnson, 1988). Control cultures of cells transformed with pGEX-KG and untransformed cells, were also grown and induced as above. Samples (200 μl) from the induced bacterial cell cultures were centrifuged at 5000 ***g*** for 5 min. The cell pellets were resuspended in 50 μl of cell lysis buffer (2% (w/v) SDS, 10 mM EDTA, 50 μg/ml PMSF, freshly prepared) and boiled for 3 min. A 15 μl sample of each cell lysate was added to 15 μl of loading buffer (100 mM Tris–HCl, pH 6·8, 20 mM DTT, 20% (v/v) glycerol, 10 mM EDTA, 4% (w/v) SDS, 0·2% (w/v) bromophenol blue) and boiled for a further 3 min. The samples were then subjected to electrophoresis on a 10% polyacrylamide–sodium dodecyl sulphate (SDS–PAGE) gel and stained with 0·5% (w/v) Coomassie brilliant blue (Laemmli & Favre, 1973).

Western blotting

SDS-PAGE was performed using samples of induced cell lysates, as above. Proteins were electrophoretically transferred onto Hybond-C Super nitrocellulose paper (Amersham-USB) (Burnette, 1981) using a BioRad (Hemel Hempstead, Herts, UK) transblot electroblotter, run at 40 mA for 2 h. Blots were incubated in 100 ml of TTBS (20 mM Tris–HCl, pH 8·0, 300 mM NaCl, 0·1% (v/v) Tween 20) with 1 g dried milk powder for 1 h, then washed with TTBS. This was followed by incubation

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  67 GTG TCA ACC TGG TTG ATA CGT AGT GGT GAA CCT GTG CAA CAC CGC ACT GAA TTC CCG TTC
 127 ATC GCA TTC TTA ACG ACA GAG AGA ACA ATG TGT ACA GGT TCA CTA GTC TCA ACG AGA GCA
 187 GTA CTC ACA GCT GGT CAT TGT GTT TGC TCA CCA TTG CCA GTG ATT CGG gtaagagatcgactg
 250 aaacacattgttgctcaatgtaatcgattgatttcacagacagtgtttgcgtgtgcgtgcgtttgtgtttgtgtatgca
 329 gtgggtttgcatagattttaatgtctatatacttggtcttatttcag GTT TCA TTT CTC ACA CTG AGG AAT
 400 GGC GAC CAA CAA GGC ATC CAT CAC CAA CCG TCT GGA GTT AAG GTG GCA CCA GGA TAC ATG
 460 CCC TCT TGT ATG TCG GCA CGA CAG AGG AGA CCA ATC GCA CAG ACA CTC AGT GGA TTC GAT
 520 ATT GCA ATT GTA ATG CTG GCT CAA ATG GTC AAC TTA CAG AGT GGA ATC AGA GTG ATC AGT
 580 CTG CCA CAG CCA TCG GAT ATC CCG CCA CCT GGA ACT GGT GTT TTC ATT GTT GGT TAT GGA
 640 AGG GAT GAT AAC GAC CGT GAT CCG TCA CGT AAG AAT GGT GGA ATA TTG AAG AAA gtgagtt
 701 gttggtgaataaacgacatgactcagtcgtcagtcagatgtcagtcagttattctgtgtgtctgtgtatctgtttgtgt
 780 gtctgtctgtctacctgatccggttgttgtattggtcagagccttgataataacaactgtgtttggatgactttgtgac
 859 agttcagtagcagagtgatttccatctcggtcattgtgttggtgaggtgaggtgacgtgatgtgaggtgagttgaggta
 938 gattggatgggatggaatgtgatgtgatgggatgattgagaccacttggaggagagaagactcatgaaatatctatgca
1017 aacgatggaagtgtgttgtgtacatgaagtagggggtcaatgtgtttgagtatgtgtttggagagtggtgagatggaga
1096 gtgacttgatcgtcgaatatagtgcacatgtgattgtatgtggactattgttgtgtgggtagtgtgaagggtggatatt
1175 gtgccagttgatattttcgaaattcacttgtgtgttttgttttgttccgttgtgttttgttctttctcatctgtactgt
1254 acgttgttgtactgtactgttgttgtggttgtttgtttgcaccacag GGT CGA GCG ACT ATA ATG GAA TGC
1325 CGA CAT GCG ACC AAT GGC AAT CCT ATA TGT GTG AAA GCA GGT CAG AAT TTC GGA CAG TTA
1385 CCC GCT CCA GGT GAC AGT GGT GGA CCT CTC CTC CCA TCC CTT CAA GGT CCA GTA CTC GGT
1445 GTC GTA TCA CAT GGT GTC ACA CTG CCA AAC CTT CCC GAT ATC ATT GTC GAG TAT GCC AGT
1505 GTG GCT AGA ATG TTG GAT TTT GTA CGC TCC AAT ATT TGA
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Fig. 1. Consensus sequence of 3 PCR clones of *Schistosoma mansoni* cercarial elastase gene. Nucleotide numbers correspond to the open reading frame of the cDNA sequence reported by Newport *et al.* (1988). The 2 introns are shown in lower case text. Splice signal and branch formation sequences are shown in underlined text.

in 1:1000 dilution of rabbit serum BR67 (raised against *S. mansoni* partially purified cercarial protease from cercarial transformation fluid) or rabbit serum 1093X (raised against native *S. japonicum* GST) in TTBS for 3 h. After washing with TTBS, the blots were incubated for 2 h in 1:1000 dilution of goat anti-rabbit conjugate serum (GAR.IgG(H+L)PO, Nordic Immunoconjugates, Maidenhead, Berks, UK) in TTBS. After washing with TTBS, the blots were placed in developing solution (20 mg 4-chloro-1-naphthol in 4 ml of methanol, 20 ml of TBS (20 mM Tris–HCl, (pH 8·0), 300 mM NaCl) and 10 μl of 30% (v/v) hydrogen peroxide for 5 min, then washed in distilled water.

RESULTS

Sequence and analysis of genomic clones

Analysis of the consensus sequence derived from the 3 plasmids pHP1, pHP2 and pHP3 revealed that a DNA fragment of 1477 base pairs had been amplified from *S. mansoni* genomic DNA using primers that aligned with the 3′ and 5′ ends of the mature protein coding sequence, using PCR. This consensus sequence is shown in Fig. 1. Alignment of this genomic composite sequence with the cDNA sequence revealed the presence of 2 introns in the amplified gene. The first intron is 141 bp in length, separating amino acids 78 and 79. The second intron is 604 bp in length, separating residues 184 and 185. The determined sequence was deposited in the EMBL DNA database and has been allocated the accession number Z70296.

In order to demonstrate that the use of pooled DNA did not effect the amplification of an illegitimate product, PCR of the cercarial elastase gene was performed on single *S. mansoni* adult male worms, as described previously (Charrier-Ferrara, Djabali & Goudot-Crozel, 1991). PCR of worm samples from the 4 geographical strains present in the pooled DNA all produced DNA fragments of the same length and restriction map when analysed using *Eco*RI and *Eco*RV restriction enzymes (data not shown).

The consensus sequences of the 3 exons of the gene were found to be identical to that of the cDNA sequence (Newport *et al.* 1988). The cloned sequence contains the expected Ser 218, His 68 and Asp 126 of the catalytic triad, characteristic of the serine proteases. Two changes were found in our nucleotide sequence, replacing amino acid residues Pro 37 (CCC) and Ala 38 (GCT) with Arg (CGC) and Thr (ACT), respectively, in agreement with a sequence published recently (Pierrot, Capron & Khalife,

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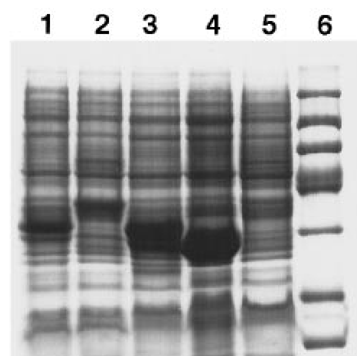

Fig. 2. Overexpression of elastase exon-GST fusion proteins. Coomassie blue-stained SDS–PAGE analysis of total proteins from recombinant *E. coli* carrying plasmids pEXON1, pEXON2 and pEXON3 (Lanes 1–3 respectively), pGEX-KG (Lane 4) and no plasmid (Lane 5). Molecular weight markers are shown in Lane 6, (approximate sizes; 97·4, 66·2, 55·0, 42·7, 40·0, 31·0, 21·5 and 14·4 kDa).

1995). These two positions were also reported to be ambiguous in the original cloned cDNA sequences reported by Newport *et al*. (1988). The deduced *S. mansoni* protease sequence reported by Pierrot *et al*. (1995) shared 84% identity at the amino acid and nucleotide levels with the sequence reported here.

Cloning and expression of individual exons

Using the sequence data obtained, the 3 exons of the cercarial protease gene were amplified separately using PCR. The exons were over-expressed as GST-fusion proteins in *E. coli*. The results are shown in Fig. 2. As can be seen from the Coomassie-stained SDS-PAGE analysis of the induced cells, prominent protein bands of 32, 37 and 34 kDa appeared in the IPTG-induced *E. coli* containing the constructs pGEXON1, pGEXON2 and pGEXON3, respectively. A control lane is also presented which shows the size of the carrier GST protein produced on induction of cells carrying the plain, unmodified pGEX-GK vector plasmid alone. The yield of purified recombinant fusion proteins was approximately 2–3 mg/g of cell paste.

Antigenicity of recombinant fusion proteins

Western blots of induced bacterial cell cultures were probed with sera raised against *S. japonicum* native GST and *S. mansoni* native cercarial protease. The exon-GST fusion proteins were recognized by both types of sera, whereas GST alone was recognized only by the anti-GST serum. A negative control, rabbit antiserum raised against an unrelated antigen (horse albumin), was also used to probe the blots. As expected, this latter antiserum reacted only with it's cognate antigen and not with the *E. coli* or *S. mansoni* derived proteins. These results are shown in Fig. 3.

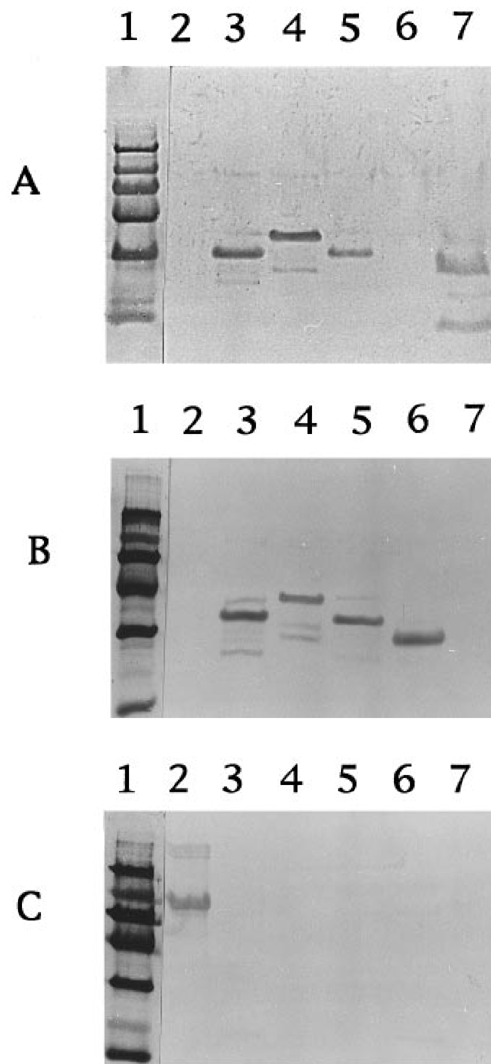

Fig. 3. Western blots of elastase exon-GST fusion proteins. Cercarial transformation fluid (CTF, Lane 7), horse albumin (Lane 2) and total proteins from recombinant *E. coli* carrying plasmids pEXON1 (Lane 3), pEXON2 (Lane 4), pEXON3 (Lane 5), or pGEX-KG (Lane 6) were separated by SDS-PAGE and Western blotted as described in the Materials and Methods section. The blots were then probed with either anti-cercarial protease (A), anti-GST (B), or rabbit anti-horse albumin (C) and processed as described in the Materials and Methods section. Stained molecular weight markers of 97·4, 66·2, 55·0, 42·7, 40·0, 31·0, 21·5 and 14·4 kDa are also shown in each panel (Lane 1).

DISCUSSION

The results show that a 1477 bp elastase gene fragment was amplified from *S. mansoni* genomic DNA. The previously determined cDNA sequence (Newport *et al*. 1988) from which the PCR primers were designed is only 720 bp in length. Alignment of the genomic composite sequence with the cDNA sequence revealed the presence of 2 introns of 141 and 604 bps in length. These findings are in contrast to original suggestions that the protease gene contains

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only 1 intron of 150 bp, separating residues 77 and 78 (McKerrow, Newport & Fishelson, 1991). However, recent independent findings by another research group confirm the existence of another related elastase gene possessing 2 introns (Pierrot, Capron & Khalife, 1995).

Both introns contain the 5′-GT and 3′-AG consensus splice signal sequences. Both introns contain sequences with a high degree of similarity to the 5′ donor site sequence MAGGTRAGT (M = A or C, R = A or G) described previously (Ohshima & Gotoh, 1987) and the first intron has the sequence TTTCAG, which has been identified as a 3′ splice acceptor sequence for *trans*-splicing in nematode actin genes (Krause & Hirsh, 1987). Both introns have a pyrimidine-rich tract upstream of the 3′ end, and both possess the sequence YNYTRAY (R = A or G, Y = C or T, N = A, C, G or T) used for branch formation in vertebrates, i.e. TTTTAAT in intron 1 and CCTTGAT in intron 2, as described previously (Ohshima & Gotoh, 1987). Also, the second intron is located at the interface between hydrophilic and hydrophobic domains of the protein, as described previously for introns of the schistosomal hypoxanthine–guanine phosphoribosyl transferase gene (Craig *et al*. 1989). The presence of these characteristics within the introns strengthens the validity of the 2 intron structure of the cercarial elastase gene.

Other related serine protease genes sequenced to date, including trypsin, chymotrypsin and elastase, contain introns in conserved positions (Craik, Rutter & Fletterick, 1983). In the cercarial elastase gene, the position of the first intron is consistent with those of related genes, whereas the second intron is positioned 18 amino acid residues downstream of a predicted 'missing' intron site (McKerrow *et al*. 1991). A preliminary communication regarding the 2 intron structure of the gene has already been made (Price, Doenhoff & Sayers, 1996). It is quite possible that the differences between our results and those originally obtained by other workers is due to the existence of a second elastase gene carrying 2 introns. Alternatively, these discrepancies may be due to the genetic diversity of the various isolates used by different laboratories, i.e. the strain originally used carried a slightly different elastase gene implying some degree of polymorphism at this site.

All 3 recombinant GST-exon fusions were expressed to readily detectable levels as monitored by SDS-PAGE. These recombinant exon fusion proteins are all recognized by the anti-elastase sera indicating that neither expression in *E. coli* nor truncation and fusion have abolished the antigenicity of the recombinant products. The results also suggest that epitopes on all 3 exons can be recognized by rabbits immunized with native elastase. Western blotting did reveal minor products of lower molecular weight, particularly in the case of the second exon fusion. Such bands presumably arise due to degradation by native proteases or may be the result of incomplete synthesis.

Thus, by using an increasingly popular technique (reviewed by Muller & Felleisen, 1995) the production of a recombinant source of antigenically active protein fragments corresponding to the residues 28–78, 79–184 and 185–265 of cercarial elastase has been possible. These fragments can be produced on as large a scale as required and as they have evident antigenicity, the proteins, once purified, can be evaluated as candidate vaccines for schistosomiasis. Naturally, the individual exons are extremely unlikely to have the same disadvantage as the native antigen, i.e. the individual recombinant polypeptides will be unable to cleave immunoglobulins. It is also unlikely that the 3 fusion proteins in combination would reconstitute active protease. Thus, we intend to evaluate the efficacy of the fusion proteins both singly and in combination. The GST portion of the fusion protein may be removed by cleavage with thrombin (Smith & Johnson, 1988), or possibly retained, with the advantage that the GST is relatively immunogenic and may improve responses to the cercarial elastase portion which is a relatively poor immunogen (Oettinger, Pasqueline & Bernfield, 1992). We hope that the fusion proteins may be superior in provoking an immune response which may prove protective against parasite infection.

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REFERENCES

BABA, E. H., HOMEWOOD, C. A., GAZINELLI, G. & ATKINSON, E. M. (1977). Comparison of proteolytic enzymes from cercarial extract and secretion. *Comparative Biochemistry and Physiology B-Biochemistry and Molecular Biology* **57B**, 55–57.

BICKLE, Q. D. & DOENHOFF, M. J. (1987). Comparison of the live vaccine potential of different geographic isolates of *Schistosoma mansoni*. *Journal of Helminthology* **61**, 191–195.

BURNETTE, W. N. (1981). Western blotting – electrophoretic transfer of proteins from sodium dodecyl sulfate – polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein-A. *Analytical Biochemistry* **112**, 195–205.

CAMPBELL, D. L., FRAPPAOLO, P. J. F., STIREWALT, M. A. & DRESDEN, M. H. (1976). *Schistosoma mansoni*: partial characterization of enzyme(s) secreted from the preacetabular glands of cercariae. *Experimental Parasitology* **40**, 33–40.

CHARRIER-FERRARA, S., DJABALI, M. & GOUDOT-CROZEL, V. (1991). *Schistosoma mansoni* – a simple method for the extraction of DNA from single worms for PCR amplification. *Experimental Parasitology* **73**, 384.

https://doi.org/10.1017/S0031182096008657 Published online by Cambridge University Press

CRAIG, S. P., MURALIDHAR, M. G., McKERROW, J. H. & WANG, C. C. (1989). Evidence for a class of very small introns in the gene for hypoxanthine-guanine phosphoribosyl transferase in *Schistosoma mansoni*. *Nucleic Acids Research* **17**, 1635–1647.

CRAIK, C. S., RUTTER, W. J. & FLETTERICK, R. (1983). Splice junctions: association with variation in protein structure. *Science* **220**, 1125–1129.

CURTIS, R. H. C. (1988). Characterisation of some *Schistosoma mansoni* hydrolases and other surface antigens. *Ph.D. thesis*, *University of London*, *UK*.

DOENHOFF, M. J., CURTIS, R. H., NGAIZA, J. & MODHA, J. (1990). Proteases in the schistosome life cycle: a paradigm for tumour metastasis. *Cancer and Metastasis Reviews* **9**, 381–392.

GUAN, K. L. & DIXON, J. E. (1991). Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Analytical Biochemistry* **192**, 262–267.

KRAUSE, M. & HIRSH, D. (1987). A trans-spliced leader sequence on actin messenger-RNA in *C. elegans*. *Cell* **49**, 753–761.

KUMAR, S. & PRITCHARD, D. I. (1992). The partial characterization of proteases present in the excretory/secretory products and exsheathing fluid of the infective (L3) larva of *Necator americanus*. *International Journal for Parasitology* **22**, 563–572.

LAEMMLI, U. K. & FAVRE, M. (1973). Maturation of the head of bacteriophage T4. I. DNA packaging events. *Journal of Molecular Biology* **80**, 575–599.

LANDSPERGER, W. J., STIREWALT, M. A. & DRESDEN, M. H. (1982). Purification and properties of a proteolytic enzyme from the cercariae of the human trematode parasite *Schistosoma mansoni*. *The Biochemical Journal* **201**, 137–144.

LEWERT, R. M. & LEE, C. L. (1956). Quantitative studies of the collagenase-like enzymes of cercariae of *Schistosoma mansoni* and the larvae of *Strongyloides ratti*. *Journal of Infectious Diseases* **99**, 1–14.

LORENS, J. B. (1991). Rapid and reliable cloning of PCR products. *PCR Methods and Applications* **1**, 140–141.

McKERROW, J. H. & DOENHOFF, M. J. (1988). Schistosome proteases. *Parasitology Today* **4**, 334–340.

McKERROW, J. H., JONES, P., SAGE, H. & PINO-HEISS, S. (1985*a*). Proteinases from invasive larvae of the trematode parasite *Schistosoma mansoni* degrade connective-tissue and basement-membrane macromolecules. *The Biochemical Journal* **231**, 47–51.

McKERROW, J. H., KEENE, W. E., JEONG, K. H. & WERB, Z. (1983). Degradation of extracellular matrix by larvae of *Schistosoma mansoni*. I. Degradation by cercariae as a model for initial parasite invasion of host. *Laboratory Investigation* **49**, 195–200.

McKERROW, J. H., NEWPORT, G. & FISHELSON, Z. (1991). Minireview. Recent insights into the structure and function of a larval proteinase involved in host infection by a multicellular parasite. *Proceedings of the Society for Experimental Biology and Medicine* **197**, 119–124.

McKERROW, J. H., PINO-HEISS, S., LINDQUIST, R. & WERB, Z. (1985*b*). Purification and characterization of an elastolytic proteinase secreted by cercariae of *Schistosoma mansoni*. *Journal of Biological Chemistry* **260**, 3703–3707.

MULLER, N. & FELLEISEN, R. (1995). Bacterial expression systems as tools for the production of immuno-diagnostic parasite antigens. *Parasitology Today* **11**, 476–480.

MULLIS, K. B. & FALOONA, F. A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods in Enzymology* **155**, 335–350.

NEWPORT, G. R., McKERROW, J. H., HEDSTROM, R., PETITT, M., McGARRIGLE, L., BARR, P. J. & AGABIAN, N. (1988). Cloning of the proteinase that facilitates infection by schistosome parasites. *Journal of Biological Chemistry* **263**, 13179–13184.

OETTINGER, H. F., PASQUELINE, R. & BERNFIELD, M. (1992). Recombinant peptides as immunogens – a comparison of protocols for antisera production using the pGEX system. *Biotechniques* **12**, 544–549.

OHSHIMA, Y. & GOTOH, Y. (1987). Signals for the selection of a splice site in pre-mRNA. Computer analysis of splice junction sequences and like sequences. *Journal of Molecular Biology* **195**, 247–259.

PIERROT, C., CAPRON, A. & KHALIFE, J. (1995). Cloning and characterization of two genes encoding *Schistosoma mansoni* elastase. *Molecular and Biochemical Parasitology* **75**, 113–117.

POTTER, H. (1988). Electroporation in biology – methods, applications and instrumentation. *Analytical Biochemistry* **174**, 361–373.

PRICE, H. P., DOENHOFF, M. J. & SAYERS, J. R. (1996). Expression and antigenicity of a schistosome cercarial protease. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90**, 7.

SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**, 5463–5467.

SIMONS, P. C. & VAN DER JAGT, D. L. (1981). Purification of glutathione S-transferases by glutathione-affinity chromatography. *Methods in Enzymology* **77**, 235–237.

SMITH, D. B. & JOHNSON, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferases. *Gene* **67**, 31–40.

STIREWALT, M. A. & FREGEAU, W. A. (1966). An invasive enzyme system present in cercariae but absent in schistosomules of *Schistosoma mansoni*. *Experimental Parasitology* **19**, 206–215.

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