Partial purification and characterization of acetylcholinesterase isozymes from adult bovine filarial parasite *Setaria cervi*

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Filariasis is a major health problem, affecting millions of people in tropical and sub-tropical regions of the world. The isolation and characterization of parasite-specific enzyme targets is essential for developing effective control measures against filariasis. Acetylcholinesterase (AchE, E.C. 3.1.1.7), an important enzyme of neuromuscular transmission is found in a number of helminths including filarial parasites and may be playing a role in host-parasite interactions. Earlier, we demonstrated the presence of two isozymes of AchE, different from the host enzyme in the human (*Brugia malayi*) and bovine (*Setaria cervi*) filarial parasites. In the present study, two isozymes of AchE (pAchE1 and pAchE2) were isolated from *S. cervi* adults and characterized biochemically and immunochemically. The AchE was partially purified on Con-A Sepharose column and then subjected to preparative polyacrylamide gel electrophoresis (PAGE) for separation of the isozymes. The AchE activity was localized by the staining of gel and the isozymes were isolated from the PAGE strips by electroleution. Both isozymes preferentially utilized acetylcholine iodide as substrate and were strongly inhibited by the true AchE inhibitor (BW284c51), suggesting that they were true AchE. The polyclonal antibodies produced against the isozymes showed significant cross-reactivity with *B. malayi* AchE, but not against the host enzyme. These findings suggested that both the isozymes were biochemically (in terms of their substrate specificity and inhibitor sensitivity) and immunochemically similar, but different from the host enzyme.

**Keywords**: *Setaria cervi*, *Brugia malayi*, Acetylcholinesterase, Bovine filarial parasite, Human filarial parasite, Filarial acetylcholinesterase

Filariasis is a major public health problem in tropical and subtropical countries affecting millions of people worldwide. Effective chemical and immunological remedies against filariasis are not available and definitive diagnosis of the disease is still a major problem. Development of new chemotherapeutic, immunodiagnostic and immunoprophylactic measures is required for effective control of the disease. The basic stumbling block in the design of the suitable anti-filarial drug is beset with our poor knowledge about the metabolic activities of adult and various development stages of human filarial parasites, as well as of the disorder generated in the host harboring the infection. Thus, isolation and characterization of functional components/enzymes of the parasites is important for rational design of suitable anti-filarial drugs.

Acetylcholinesterase (AchE), an important enzyme of neuromuscular transmission, is found in many species of helminth parasites including filarial parasites¹⁻⁵. In nematodes, its secretion has been one of the parameters used in the evaluation of the effectiveness of anthelmintics⁶⁻⁹. In schistosomes, AchE is demonstrated to be a functional protein involved in multi-faceted activities such as for vaccine development, drug design and diagnostic purposes. AchE inhibitors significantly decrease the amplitude of muscle contraction in various parasites. The cholinesterase inhibitors are of potential interest in anthelmintic chemotherapy¹⁰. Multiple molecular forms of AchE have been reported from vertebrates¹¹⁻¹³, free-living nematode *Caenorhabditis elegans*¹⁴ and parasites like *Nippostrongylus brasiliensis*⁹ and *Schistosoma mansoni*¹⁵. These forms

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Abbreviations: AchE, acetylcholinesterase; ATi, acetylthiocholine iodide; BTi, butyrylthiocholine iodide; BchE, butyrylcholinesterase; BW248c51, 1,5-bis(4-allyldimethyl ammonium-phenyl)pentan-3-one dibromide DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); ELISA, enzyme-linked immunosorbent assay; Iso-OMPA, tetra(monoisopropyl) pyrophosphotetramide; PAGE, polyacrylamide gel electro-phoresis; PBS, phosphate buffered saline.

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of AchE have been intensively studied in *C. elegans* and schistosome parasites. In vertebrates, AchE is encoded by a single gene, while separate genes encode a number of AchEs in nematode parasites. In *C. elegans* four AChE genes have been identified and characterized.

Earlier, we have demonstrated the presence of AchE activity in adult, microfilariae and excretory-secretory products of *Setaria cervi* (bovine filarial parasites). Recently, we have identified two isozymic forms of AchE in human (*Brugia malayi*) and bovine (*S. cervi*) filarial parasites. In the present study, two AchE isozymes from *S. cervi* adults have been partially purified and isolated employing Con-A Sepharose affinity column chromatography and native preparative-polyacrylamide gel electrophoresis (PAGE). The isozymes have been biochemically and immunochemically characterized using specific substrates, inhibitors and polyclonal antibodies.

**Materials and Methods**

**Parasites**

Adult motile *Setaria cervi* (female and male) were collected from the peritoneal folds of freshly slaughtered buffaloes at a local slaughterhouse and brought to the laboratory in normal saline. The parasites were washed extensively with normal saline before use. The human filarial parasite (*Brugia malayi*) was maintained in *Mastomys natalensis* at our Institute and adult worms of *B. malayi* were obtained from the peritoneal cavity of infected mastomys.

**Preparation of enzyme extract**

A 20% extract of *S. cervi* adult worms was prepared as described earlier. Briefly, the adult worms were homogenized with 50 mM phosphate buffer, pH 8.0 containing 0.5% Triton X-100. The extraction was done on ice for 1.5 h with occasional vortexing. The extract was centrifuged at 1,05,000 × g for 60 min and the supernatant obtained was used for isolation of parasite AchE isoforms. The normal bovine serum obtained from the fresh normal buffalo blood collected from the local abattoir was used as source of host enzyme.

**Measurement of enzyme activity**

The AchE activity was measured as described previously. Briefly, to each tube was added 800 µl of 0.05 M phosphate buffer pH 8.0, 50 µl of 40 mM acetylthiocholine iodide (ATI, Sigma) and 10-100 µl of enzyme preparation. The reaction was started by adding 50 µl of 2 mM DTNB [5,5'-dithiobis(2-nitrobenzoic acid), Sigma, USA] and the change in absorbance was recorded at 30 s interval at 412 nm in Spectrophotometer (Labomed, USA). The enzyme unit was expressed as µmoles of substrate hydrolyzed per min.

**Con-A Sepharose column chromatography**

The supernatant (1,05,000 × g) was concentrated by Amicon filtration and 10 ml of the concentrated fraction was applied to Con-A Sepharose affinity column, pre-equilibrated with 50 mM phosphate buffer (pH 8.0) containing 0.01% Triton X-100. The column was washed with about 10 column volumes of the same buffer until the absorption (at 280 nm) of the last fraction became zero. The enzyme was then eluted with 0.4 M α-methyl mannospyranoside prepared in 50 mM phosphate buffer (pH 8.0) containing 0.01% Triton X-100. Twenty fractions of 3 ml each were collected and OD280 nm was taken of the eluted fractions. The AchE activity was assayed in the eluted fractions and the fractions containing high AchE activity were pooled, concentrated and applied to preparative PAGE.

**Preparative PAGE**

Preparative-PAGE of *S. cervi* Con-A fraction was performed under non-denaturing conditions (using Bio-rad Vertical Minigel electrophoresis apparatus) following the method previously described and modified by Watts et al. The Con-A fraction was run on 6% separating and 4% stacking gel at pH 8.3. Sample was prepared by mixing (1:1) with sample buffer containing 20% glycerol and 0.002% bromophenol blue and electrophoresis was carried out at 50 V/gel for 2-3 h. The running buffer used was Tris-glycine (pH 8.3) containing 0.025 M Tris and 0.192 M glycine. Temperature was maintained between 4-8°C by running the electrophoresis in refrigerator. After electrophoresis, one strip of gel was stained for the AchE activity and the remaining portion of gel was kept at 4°C until used for enzyme elution.

The *S. cervi* AchE isoforms separated by native preparative-PAGE were eluted from the gel strips by electrolution (using Bio-Rad Electroleuter). Briefly, the gel strips containing AchE activity (after matching with the activity stained strip) were cut horizontally and chopped into small pieces and were transferred to
the glass tubes-membrane assembly of the gel eluter. The elution was carried out at 20 V/tube using Tris-glycine buffer, pH 8.3.

Staining of AchE activity was done according to previously described method with slight modifications. After electrophoresis, gels were transferred to 25 ml of 0.1 M phosphate buffer (pH 6.0) to which following were added sequentially: 1 ml of substrate solution (20 mg/ml ATI), 5 ml of 0.1 M sodium citrate, 2.5 ml of 0.1 M CuSO₄, 2.5 ml of 0.01 M potassium ferricyanide in a total volume of 50 ml with intermittent shaking. Thereafter, gels were incubated at room temperature with gentle agitation until sufficient intensity of the characteristic brown colour bands was developed and finally washed with distilled water. The activity staining of gels was also done using butyrylthiocholine iodide (BTI, 0.4 mg/ml of staining solution) as substrate. For studying the effect of inhibitors, AchE isoforms were run on native-PAGE and enzyme activity staining was done on the gel in presence of inhibitors.

Effect of inhibitors on enzyme activity
The effect of AchE inhibitors 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW248c51; a true AchE inhibitor) and tetra-(monoisopropyl) pyrophosphortetramide (Iso-OMPA, a BchE-specific inhibitor) on the enzyme activity was studied by incubating the enzyme with different concentrations (0.05-50 μM) of inhibitors for 10 min at room temperature (around 25°C) and then measuring the enzyme activity. The enzyme incubated without inhibitor was used as control. The percent inhibition was calculated from the enzyme activity in presence of inhibitors as compared to control.

Immune rabbit sera
In order to raise the polyvalent hyper immune rabbit serum against S. cervi AchE isoforms, two albino rabbits were immunized intramuscularly one with each isoform (1-2 μg protein/rabbit) emulsified in Freund's adjuvant. Immunization was done over a period of 4 months. Rabbits were bled from the marginal ear vein, 1 week after each injection starting from the second injection. The serum was collected and stored at -20°C until used.

ELISA
Enzyme-linked immunosorbent assay (ELISA) was performed in 96-well plates according to previously described method with slight modifications.

Briefly, the wells of microtitre plates were coated with purified pAchE1/pAchE2 (50 ng/well) diluted in PBS by incubating the plates at 37°C for 1 h and then overnight at 4°C. The plates were blocked by incubation with 300 μl of 3% non-fat dry milk powder (w/v, prepared in PBS) at 37°C for 2 h. After three washes with PBS-Tween (PBS-T), the plates were incubated with 100 μl of appropriately diluted polyclonal antibodies (diluted in 1% milk-PBS) for 2 h at 37°C. Thereafter, plates were washed thrice with PBS-T, followed by 1.5 h incubation with peroxidase conjugated secondary antibody (Sigma, 1:2000 diluted in 1% milk-PBS). After washing the plates with PBS-T, the colour was developed by adding the substrate solution (1 mg/ml, o-phenylenediamine in citrate-phosphate buffer (pH 5.0) containing 1 μl/ml H₂O₂). The reaction was stopped after 10 min by adding 5 N H₂SO₄ and the absorbance was read at 490 nm using Molecular Devices Spectramax 190 Plus microplate ELISA reader.

Protein estimation
Protein contents of enzyme extract was measured by the modified method of Deans et al. and that of purified enzyme preparations by the Bradford method.

Results
Purification of AchE isoforms
The S. cervi AchE isoforms were isolated using a combination of Con-A Sepharose affinity chromatography and preparative-PAGE. The S. cervi soluble fraction was applied to Con-A Sepharose column and the bound enzyme was eluted with 0.4 M α-methyl mannopyranoside. The elution profile is shown in Fig. 1. The fractions (3rd-6th) showing AchE activities were pooled, concentrated and dialyzed using centricon-30 (Millipore, USA). The enzyme fraction from affinity column was run on native-PAGE and staining of gel revealed two AchE activity bands — slow and fast-moving AchE bands designated as parasite AchE1 (pAchE1) and AchE2 (pAchE2) respectively and were electroeluted separately from the gel. The recovery of AchE activity in eluted fractions was around 80%, of which 55% activity was of pAchE1 and 45% of pAchE2. The eluted fractions were run again on native-PAGE, followed by enzyme staining. Both fractions showed a
Fig. 1—Isolation of *S. cervi* Acetylcholinesterase using Con-A Sepharose column chromatography [Concentrated soluble fraction was applied to Con-A Sepharose column. The enzyme was eluted with 0.4 M α-methyl mannosylpyranoside prepared in 50 mM phosphate buffer, pH 8.0 containing 0.01% Triton x-100. The AchE activity and OD at 280 nm were measured for each fraction as described in ‘Materials and Methods’]

Fig. 2—Native-PAGE of purified AchE isozymes of *S. cervi* [Con-A purified AchE fraction (lane A), pAchE1 (lane B) and pAchE2 (lane C) gel eluted fractions analyzed on native PAGE. AchE activity staining was done as described in ‘Materials and Methods’]

Table 1—Substrate specificity of *S. cervi* acetylcholinesterase isozymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ATI Activity</th>
<th>% Activity</th>
<th>BTI Activity</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAchE1</td>
<td>0.314</td>
<td>81.8</td>
<td>0.070</td>
<td>18.2</td>
</tr>
<tr>
<td>pAchE1</td>
<td>0.264</td>
<td>80.2</td>
<td>0.065</td>
<td>19.8</td>
</tr>
<tr>
<td>hAchE</td>
<td>0.386</td>
<td>39.6</td>
<td>0.589</td>
<td>60.4</td>
</tr>
</tbody>
</table>

ATI, Acetylthiocholine iodide; BTI, butyrylthiocholine iodide; pAchE1, parasite AchE1; pAchE2, parasite AchE2; hAchE, host AchE

with BTI and ATI respectively (Table 1). These results suggested that both isozymes were true AchE, as both preferentially utilized ATI as substrate.

**Effect of inhibitors on *S. cervi* AchE isozymes**

The effect of two inhibitors BW284c51 (a true AchE inhibitor) and Iso-OMPA (a pseudo AchE inhibitor) on parasite AchE isozymes activity in gels was studied using the staining solution containing 100 µM of the inhibitors. The BW284c51 inhibited the enzyme activity and no AchE bands were observed on staining for both pAchE1 and pAchE2, while IsoOMPA had no effect on the enzyme activity staining of AchE isozyme bands (Fig. 4a). Effect of different concentrations (0.05, 0.5, 5.0 and 50 µM) of these inhibitors on enzyme activity of two isozymes was studied spectrophotometrically and the results are shown in Fig. 4b. The BW284c51 inhibited the
activities of both isozymes; around 20% inhibition at 0.05 µM, 50% at 0.5 µM and 80-90% at 5 µM concentration of BW284c51 was observed for both the isozymes. The Iso-OMPA did not have any significant inhibitory effect even at 50 µM concentration (Fig. 4b). The strong inhibition by BW284c51 and not by Iso-OMPA suggested that both isozymes were true AchE.

Immunoreactivities of *S. cervi* AchE isozymes

Immunoreactivity of *S. cervi* AchE isozymes was studied in ELISA using immune sera against pAchE1 and pAchE2. The rabbit anti-pAchE1 (IRS1) and rabbit anti-pAchE2 (IRS2) sera were tested at 1:1000 and 1:5000 dilutions. The immune sera showed high reactivity with both the isozymes of *S. cervi* AchE even at 1:5000 dilution (OD₄₉₀ >1.5) in ELISA, while the host AchE did not show any significant reactivity with these immune rabbit sera. Both the immune rabbit sera (IRS1 an IRS2) at 1:5000 dilution also showed high reactivity with *B. malayi* AchE in ELISA (Fig. 5).

Discussion

The occurrence of multiple molecular forms of AchE has been reported in vertebrates, invertebrates and helminth parasites. The molecular forms of nematode AchE have been extensively studied using free-living nematode *C. elegans* and differ in their quaternary structure and mode of anchoring to the cell surface. The *S. cervi* adult worm possesses two electrophoretically distinct isozymes of AchE as shown by our earlier study. In order to characterize these AchE isozymes, we have partially purified and separated the two AchE isozymes employing Con-A Sepharose affinity column and preparative PAGE. On using Con-A Sepharose column, only one sharp peak of AchE (containing both isozymes) was obtained, indicating that both isozymes had equal affinity for α-methyl manno-pyranoside and were glycoprotein in nature. We also attempted to separate these two AchE isozymes of *S. cervi* adult worm by gel filtration and ion-exchange chromatography (data not shown).

On using Sephacryl S-500 column, both isozymic forms were localized in the first protein peak coming just after the void volume. On using DEAE-cellulose column, pAchE1 could be separated from pAchE2 by eluting the column with 0.2 M salt concentration, but pAchE2 which was eluted at 0.4 M salt concentration also contained pAchE1 activity (data not shown).
However, in an earlier study, two isoenzymes of AchE from *S. cervi* microfilariae excretory-secretory (E-S) products were separated using DEAE-sepharose column and employing 0.1 and 0.2 M salt concentrations. This might be due to the difference in binding affinities of the isozymes present in the adult worm extract and E-S products, as we used the somatic extracts of adult worms in the present study, while Sharma and Rathaur utilized the microfilarial E-S products.

In vertebrates, mainly two types of cholinesterases (AchE and butyrylcholinesterase) have been demonstrated based on their substrate specificity and inhibitor sensitivity. The true AchE (EC 3.1.1.7) specifically catalyzes the hydrolysis of acetylcholine and is selectively inhibited by the AchE-specific inhibitor BW284c51. The butyrylcholinesterase or pseudocholinesterase (BchE; EC 3.1.1.8) degrades a wider range of choline esters and is specifically inhibited by Iso-OMPA. In the present study, two isozymes of *S. cervi* AchE (pAchE1 and pAchE2) have been characterized in terms of their substrate specificity and inhibitor sensitivity and found to be true AchE, as revealed by preferential utilization of acetylthiocholine iodide and strong inhibition by BW284c51. The presence of true AchE is also reported in other helminth parasites, such as *N. brasiliensis* and *Hymenolepis diminuta*.

The *S. cervi* AchE isozymes have also been characterized immunochemically using polyclonal antibodies raised against these isozymic forms. The antibodies against both isozymes show reactivity with filarial AchE (*S. cervi* and *B. malayi*), but no significant reactivity with host AchE, suggesting their specificity to parasite AchE. However, these polyclonal antibodies (anti-pAchE1 and anti-pAchE2) could not differentiate the two isozymes. Recently, we also generated monoclonal antibodies against the filarial parasite AchE and one monoclonal antibody, though strongly inhibited the activity specifically of parasite AchE, but could not differentiate the two isozymes of *S. cervi* AchE (Kaushal et al., unpublished observation). The schistosoma AchE has also been shown to be immunologically different from the host enzyme and considered as target for vaccine development.

In conclusion, two isozymes of AchE from *S. cervi* adults have been partially purified, separated and characterized. These isozymes could not be differentiated on the basis of substrate specificity, inhibitor sensitivity and immunoreactivity with polyclonal and monoclonal antibodies produced against *S. cervi* AchE. But, both isozymes are found to be biochemically and immunologically different from the host enzyme and can be used as target in designing effective anti-filarial agents on a rational basis. Further, molecular characterization at gene level may help in identifying the structural differences between the isozymes of parasite AchE.

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