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CHARACTERIZATION OF STORAGE PROTEIN, ARYLOPHORIN FROM THE HEMOLYMPHOF THE PEST, *HELICOVERPA ARMIGERA*

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ABSTRACT
Storage proteins, such as arylophorin, are synthesized from the fat body of a wide range of lepidopteran and dipteran larvae and also in other insect orders. Storage protein from hemolymph of *H. armigera* was obtained by ammonium sulphate fractionation and anion-exchange chromatography. From 1,500 mg of total protein, 7.35 mg of purified protein was acquired with 24.4% recovery. Subunit molecular weight of purified storage protein was estimated to be 66 kDa. The native molecular weight of the protein was determined to be 400 kDa. Amino acid composition showed that the protein was characterized by the presence of aromatic amino acids. Multiple amino acid sequence alignment of obtained LCMS sequence of the purified protein of *H. armigera* indicated that the protein is arylophorin. The deduced amino acid sequence was found to more homology with the arylphorins of *Spodoptera litura, Manduca sexta, Hyalophora cecropia* and *Antherae apernyi*.

INTRODUCTION

Storage proteins, such as arylophorin, are synthesized from the fat body of a wide range of lepidopteran and dipteran larvae and also in other insect orders. They are the major proteins in the hemolymph. Storage proteins are taken up by the fat body shortly before pupation and stored in protein granules. The central function of the proteins seems to be a storage pool of the amino acid resources for complete development of the adult. There are at least two kinds of storage proteins in lepidoptera. They form loose clusters on a bivariate plot of the proportion of aromatic amino acid (tyrosine plus phenylalanine) versus methionine (Hou et al. 2013; Telfer and Kunkel, 1991). Arylphorin is rich in aromatic amino acids, and the second protein is referred to as methionine-rich storage protein.

Insects prepare for the synthetic demands of molting, metamorphosis and reproduction by accumulating hexamerins in their hemolymph and fat body. First to be described were two soluble storage hexamerins isolated from larvae of *Calliphora erythrocephala* (Munn and Greville, 1969). Two or more hexamerins were later reported in the hemolymph and fat body of many other insects, with some lepidopterans having as many as four. Hexamerins occurring within a single species can differ in amino acid composition, stage of synthesis, distribution between hemolymph and fat body, timing of clearance from the hemolymph, and antigenic reactivity (Telfer and Kunkle, 1991). Storage proteins have mostly been studied in Diptera and Lepidoptera, but more recently also in other insect orders. Storage proteins have also been found in other insect orders; arylphorins as well as other hexamerins were described in bees (Shipman *et al.,* 1987), ants (Wheeler and Martinez, 1995), beetles (DeKort and Koopmanschap, 1994), but also in the hemimetabolous locusts (DeKort and Koopmanschap, 1987) and cockroaches (Jamroz *et al.,* 1996). Hou *et al.* (2013) have reported the crystal structure of *Bombyx mori* arylophorins at 2.8 A°, which displays a heterohexameric structural arrangement formed by trimerization of dimers comprising two structural similar arylphorins. Hansen *et al.* (2002) have demonstrated that anterior fat body protein interacts with the hexamerin receptor and plays a key role in regulation of hexamerin uptake by fat body cells along the anterior-posterior axis.

The cotton bollworm or tomato fruit borer *Helicoverpa armigera* (Hubner) is a polyphagous lepidopteron pest that infests important crops like cotton, tomato, sunflower and corn all over the world (Madhusudan *et al.,* 2011; Gadhiya *et al.,* 2014).This pest has developed resistance to all the major insecticide classes and it has become increasingly difficult to control their population in India (Kranthi *et al.* 1997; Srinivas *et al.,* 2004). Hexamerins in five plecopteran species was studied and obtained partial cDNA sequences from *Perla marginata* (Perlidae), *Nemoura sp.* (Nemouridae), *Taeniopteryx burksi* (*Taenioptery gidae*), *Allocapnia vivipara* (Capniidae), and *Diamphipnopsiss amali* (Diamphipnoidae) (Hagner-Holler *et al.,* 2007). In the present study we report the purification and characterization of storage proteins from the pest, *Helicoverpa armigera*.

MATERIALS AND METHODS

Chemicals

DEAE-cellulose, Coomassie brilliant blue R and sudan black were purchased from Sigma Aldrich (Mumbai, India). Tris-HCl and EDTA were purchased from Himedia (Mumbai, India), while the other chemicals used were of analytical grade.

Insect culture

Larvae of *H. armigera* were obtained from the insect rearing laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. The larvae were reared on a chickpea based semi-synthetic diet under laboratory conditions as described earlier (Akbar *et al.,* 2012)

Collection of hemolymph

Hemolymph was collected from the *H. armigera* larvae according to Charalambidis *et al*. (1995). Hemolymph samples (approx. 10 ml from 3 days old pupae) were dialyzed in 250 mL of buffer (50 mMTris-HCl and 1 mM EDTA pH 7) for approximately 5h. and then centrifuged again at 7,800 g.

Purification of storage protein

Ammonium sulfate was added to 50 mL of hemolymph extracted from the third- and fourth-instar larvae (1,500 mg of protein) to give 40% saturation. The solution was kept for overnight stirring at 4 ºC, and the precipitate was removed by centrifugation for 30 min at 10,000 g, and the supernatant was brought to 60% ammonium sulfate saturation. After overnight stirring at 4ºC, salt precipitated proteins were collected by centrifugation for 30 min at 10,000 g, and dissolved in buffer 50 mMTris-HCl buffer, pH 7.4, containing 1 mM EDTA, and dialyzed for 18 h against the preceding buffer at 4ºC. This pool was applied to DEAE cellulose (2 x 10 cm) previously equilibrated with 50 mMTris-HCl buffer, pH 7.4, containing 1 mM EDTA. After the column was washed with 100 ml of the same buffer, proteins were eluted with a step gradient of 0 to 0.5 M NaCl and the fractions were collected each of 3 mL (Solvak and Repka, 1993). The eluent was monitored at 280nm.

Molecular weight determination

SDS-PAGE was carried out in 10% polyacrylamide in non-reducing conditions according to the method of Laemmli (1970). Native-PAGE was performed in 5 - 8% gradient polyacrylamide gels. The gels were stained after electrophoresis with Coomassie brilliant blue R and sudan black separately (Slovak and Repka, 1993).

LCMS sequence of storage protein

For determination of LCMS sequence, purified protein was subjected to SDS-PAGE. The storage protein was detected in gel by sudan black staining (Slovak and Repka, 1993). Areas of brown stain were excised and sent for sequence analysis at HSC Advanced Protein Technology Center, Department of Structural Biology and Biochemistry, Toronto, Canada.

Phylogenetic tree of the storage protein

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.10543165 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 79 positions in the final dataset. Cladistic analysis was performed using the programs provided with MEGA 4 (Tamura *et al.,* 2007).

RESULTS

Identification of storage protein

Total hemolymph proteins were screened by SDS-PAGE in order to identify storage proteins. On the basis of Coomassie blue staining, these proteins increased in relative abundance in hemolymph throughout the final instars. Sudan black staining was used to stain the lipopolypeptides.

Purification of storage protein

Hemolymph from the late instars served as the starting material for the purification of storage proteins. An indispensable first step was fractionation by ammonium sulphate. At first, the dialyzed hemolymph was saturated with 40%, later the precipitation was raised to 60%. The precipitated proteins were subjected to anion exchange chromatography. The storage protein was eluted as a single peak from DEAE-cellulose, and gave a single band when analyzed by SDS-PAGE. From 1,500 mg of total protein, 7.35 mg of purified protein was obtained with 24.4% recovery (Table. 1).

Table 1: Purification of storage proteins

S. No.	Fraction	Total protein content (mg/mL)	Recovery (%)
1.	Crude hemolymph	30.12 ± 3.54	100
2.	60% $(NH_4)_2SO_4$ ppt	27.84 ± 1.23	92.4
3.	DEAE cellulose	7.35 ± 0.74	24.4

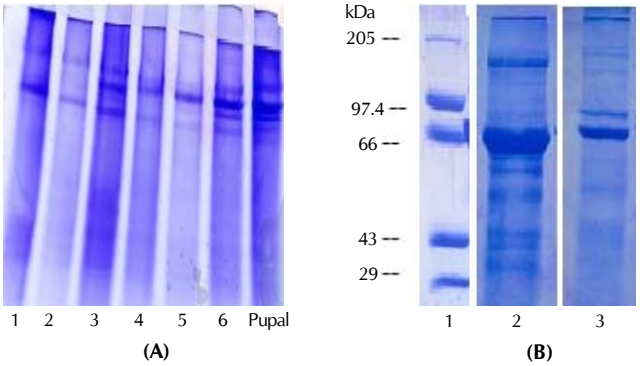

Figure 1: (A) Native gel electrophoresis of hemolymph proteins from 1 to 6 (lane 1 to 6) instars and pupae of *H. armigera*. (B) SDS PAGE of hemolymph proteins of *H. armigera* (lane 1, molecular weight markers; lane 2, 60% ammonium sulphate precipitated hemolymph extract and lane 3, purified protein). Other experimental conditions were given in materials and methods

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Hyalophora cecropia(Arylophorin)    FQDVDQVNVNDEYYKIGKDYDVEANIDNYTNKKAVEEFLKLYRTGYLPKY
Antheraea pernyi (Arylophorin)      FQDVDQLNTNDEYYKIGKDYDIEANIDNYTNKKAVEEFLKMYRCGFLPKY
H.armigera                          ------------------DYDVEANXDNYTNK------------------
Spodoptera litura(Arylophorin)      FENSEQLDYHSDYYKVGKDYDIEANIQNYSNKQAVEEFLLLYRTGTLPKY
Manduca sexta(Arylophorin)          FQDVDQVNPNDEYYKIGKEYNIEANIDNYSNKKAVEEFLQLYRTGFLPKY
                                                  .*::*** :.*::*

Hyalophora cecropia(Arylophorin)    FEFSIFHHKLREEAIALFHLFYYAKDFETFYKSAAFARVHLNEGQFLYAY
Antheraea pernyi (Arylophorin)      NEFSVFHDKLRDEAIALFHLFYYAKDFDTFYKSAAFARVHLNQGQFLYAY
H.armigera                          --FSXFYER-------------------------------------------
Spodoptera litura(Arylophorin)      YKFSIFYERMRDEAIALFHVLYYAKDFETFYKTAAWAKVYMNEEQFFYAY
Manduca sexta(Arylophorin)          YEFSPFYDRLRDEAIGVFHLFYYAKDFDTFYKSAAWARVYLNEGQFLYAY
                                      ** *:.:

Hyalophora cecropia(Arylophorin)    YIAVIQRNDTHGFVLPAPYEVYPQFFVNMDTTVRIYRTKMQDGILHPTKA
Antheraea pernyi (Arylophorin)      YIAIIQRKDTYGIVLPAPYEIYPELFVNIDTTYKMFRTKMQNGLINPEAA
H.armigera                          ------------------------------------------------
Spodoptera litura(Arylophorin)      YIAIVQRADTDGIVLPAPYEVYPQFFFNSEIMHKLYRTKMQNNVFDEKLA
Manduca sexta(Arylophorin)          YIAVIQRKDTQGFVVPAPYEVYPQFFANLNTMLKVYRTKMQDGVVSADLA

Hyalophora cecropia(Arylophorin)    INYGIVKEEEHYVYYANYSNTFLYNNEEQRLTYLTEDIGFNSYYYYFHSH
Antheraea pernyi (Arylophorin)      VEYGIVKEDNHYVYYSNYSNAITYYNEEQRLAYFTEDIGLNAYYFFFHIH
H.armigera                          ------------------------------------------------
Spodoptera litura(Arylophorin)      AQYGMVKDNNNYVFYANYSNSLSYPNKEQKLSYFTEDIGLNSYYFYFHSQ
Manduca sexta(Arylophorin)          AQHGIVKEKNYYVYYANYSNSLVYNNEEQRLSYFTEDIGLNSYYYYFHSH

Hyalophora cecropia(Arylophorin)    LPFWWT-SERYGNLKHRRGEIYYYFYQQLLTRYYFERLTNGLGSIPEFSW
Antheraea pernyi (Arylophorin)      LPFWWT-AEKYGNLKERRGEMYHYFYDQLLTRYYFERLTNGLGTIPEFSW
H.armigera                          ------------------------------------------------
Spodoptera litura(Arylophorin)      MPFWWK-SEKLNILKDRLGEVFFYYYQQLLARYYLERLPHGLGEIPEFSW
Manduca sexta(Arylophorin)          LPFWWN-SERYGALKSRRGEIYYYFYQQLIARYYFERLSNGLGDIPEFSW

Hyalophora cecropia(Arylophorin)    YSPIKTG-YYPLMTSYYYPFAQRPENYNLHSVKNYEAIRFLDIFEKTFVQ
Antheraea pernyi (Arylophorin)      YSPVKTG-HYPLLTSYYTPFSQRPNFYNVHSEENYEKIRFLDAYENYFVQ
H.armigera                          ----------------------SNNYNXHSEKNYEYXRFXDTYEKTFFQ
Spodoptera litura(Arylophorin)      YSKFKSG-YYPQLPAHYINYVQRSNDYNLHNEKNYEYIRFLDTYEKTFFQ
Manduca sexta(Arylophorin)          YSPVKSG-YYPLMSSYYYPFAQRPNYWNVHSEENYEKVRFLDTYEMSFLQ
                                                .: :   . .* *  :* * :*  *.*

Hyalophora cecropia(Arylophorin)    SLQKGQFESNGKKIDFHDEKAINFVGNYWQENADLYGEEVTKDYQR-SYE
Antheraea pernyi (Arylophorin)      ALQKGVFEGFGQTIYLNDSKANSFVGNYWQDNADLYGEEVTKDYQR-SYE
H.armigera                          FXQK------------------------------------DXHQYSYE
Spodoptera litura(Arylophorin)      FLQKAEFKSPEKEMNYVG--------NYWHMNSDLYAEQSNKDLHQYSYE
Manduca sexta(Arylophorin)          FLQNGHFKAFDQKIDFHDFKAINFVGNYWQDNADLYGEEVTKDYQR-SYE
                                      *.                                  :  : ***

Hyalophora cecropia(Arylophorin)    IVARHVLGAAPKPFDKHIFMPSALDFYQTALRDPAFYQLYNRIVGYINAF
Antheraea pernyi (Arylophorin)      IVARQVLGAAPKPFDKYTFMPSALDFYQTSLRDPTFYQLYNRIIGYFNQF
H.armigera                          XXARHVXGGSPKPFDK----------------DPAFYQXYQRXVDYXXAY
Spodoptera litura(Arylophorin)      IIARHVLGASPKPFDKYTFMPSALDFYQTSLRDPAFYQLYQRIIDYLIDY
Manduca sexta(Arylophorin)          IIARQVLGAAPKPFDKYTFMPSALDFYQTSLRDPMFYQLYNRILKYIYEY
                                      **:* *.:*:. .:                 ** *** *.: : *   :

Hyalophora cecropia(Arylophorin)    KHYLVPYPQEKLHFVG-VKINDVVVEKLVTFFEYYEFDATNGVFMTEKEI
Antheraea pernyi (Arylophorin)      KQYLEPHSQEKLHFVG-VKVNNVVVDKLVTFFEYYDFDATNTVFLTEEEL
H.armigera                          K-------------------------------------------------
Spodoptera litura(Arylophorin)      KQYVKPYNHNDLHFVG-VKINDVEVSELVTYFEYFDFNVTNSAYYNKEEL
Manduca sexta(Arylophorin)          KQYLQPYSSEKLAFKG-VKVVDVVVDKLVTFFEYYDFDASNSVFWSKEEV
                                    *

Hyalophora cecropia(Arylophorin)    KNSYPHNFKVRQPRLNHKSFNVNIEVKSDVATDAVLKIFMGPKYDDNGFP
Antheraea pernyi (Arylophorin)      KTKYPHNLKVRQPRLNHQPFNINIDIKADVATDAVVKIFMGPKYNENGFP
H.armigera                          ----------XNHKPFTVSVDXKSDVASDAVVKXFXGPK-------
Spodoptera litura(Arylophorin)      K-AYPVNYIVRQPRINHKPFNVKINVKSDVASDAVFKIFIGPKYHANGYP
Manduca sexta(Arylophorin)          KSSYPHDFKIRQPRLNHKPFSVSIDIKSEAAVDAVVKIFMAPKYDDNGFP
                                              **:.*.:.:: *::   :*..* * .**

Hyalophora cecropia(Arylophorin)    ITLEENWNKFFELDWFTHKITPGQNKIVRNSNEFLIFKEDSLPLTDLLKL
Antheraea pernyi (Arylophorin)      ITLENDRMKFFEMEWFTHKITPGQNTIVRNSNEFVIFKEDSLPSTELYKL
H.armigera                          ---------FYEXDWFVQK--------------------------------
Spodoptera litura(Arylophorin)      VNIEDDWMKFYELDWFVQKLVPGENKIERKSSEFVLFKDDSVSINEIYKW
Manduca sexta(Arylophorin)          LKLENNWNKFFELDWFTYKFVAGDNKIVRNSNDFLIFKDDSVPMTELYKL
                                          * * :**. *
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Figure 2: Multiple deduced amino acid sequence alignment of obtainedsequence of *H. armigera* with *Hyalophora cercopia, Antherae apernyi, Spodoptera lutira and Manduca sexta*

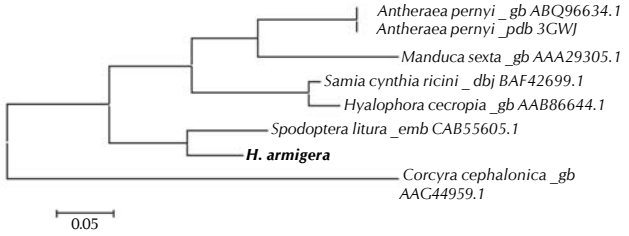

Figure 3: Phylogenetic tree for the arylphorin protein of *H. armigera*. Other experimental details were given in materials and methods

Molecular weight determination

Subunit molecular weight of purified storage protein was estimated to be 66 kDa (Fig. 1). The native molecular weight of the protein was determined to be 400 kDa.

Alignment of LCMS sequence

An initial alignment of LCMS sequence was generated by the program Clustal X software package. For multiple sequence alignment, the complete sequences for different organisms were obtained from NCBI website, and the sequence was manually corrected and aligned by NCBI BLAST (Fig. 2). Upon the sequence homology, the protein was identified as arylphorin.

Phylogenetic tree of the arylphorin of *H. armigera*

Phylogenetic analysis showed that the isolate had maximum similarity (more than 99%) with *Spodoptera litura* and *Manduca sexta* among the eight taxas (Fig. 3).

DISCUSSION

Arylphorins are a class of insect storage proteins that have an unusually high content of the aromatic amino acids phenylalanine and tyrosine and belong to the larger class of storage proteins known as the hexamerins. The arylphorins are high molecular weight complexes composed of six similar (or identical) subunits. All have molecular masses of nearly 500 kDa and are hexamers composed of approximately 80 kDa subunits. They are related to the arthropod hemocyanins, and more distantly to the phenyloxidases (Burmester, 2002). Apart from their role in serving amino acids during the non-feeding stage, hexamerins have also been identified as constituents of the sclerotizing system of the insect's cuticle (Peter and Scheller, 1991). They may also serve as carriers for ecdysteroids (Enderle *et al.,* 1983), juvenile hormone (Braun and Wyatt, 1996) and otherorganic compounds such as riboflavin (Magee *et al.,* 1994). More recent studies suggest that hexamerins function incaste differentiation of termites via regulation of the juvenile hormone levels (Zhou *et al.,* 2006; 2007). There is also evidence that some hexamerins play a role in the insect's humoral immune response (Phipps *et al.,* 1994; Beresford *et al.,* 1997).

Subunit molecular weight of the arylphorin *in H. armigera* was found to be 66 kDa (Fig. 1). Hexamerin structure has generally been inferred from molecular weight estimates for native and dissociated forms of the proteins in polyacrylamide gel electrophoresis (PAGE) (Telfer and Kunkel, 1991). The molecular properties of *H. armigera* are in agreement with those of the *B. mori*, where the native molecular weight was reported to be 500 kDa, with a subunit mass of approximately, 85 kDa. The storage proteins of four Diptera (*C. erythrocephala, C. stygia, L. cuprina and D. melanogaster*) also consist of subunits of 80-85 kDa, associated with either hexamers (mol. wt. 500 kDa) or trimers (250 kDa) (Wolfe *et al.,* 1977). The concentration of storage protein increased in the hemolymph from the first instar to the final instar of larval development. The concentrations of the storage proteins in the hemolymph changed during development of insect larvae from the final instar to the larva-pupal ecdysis. Such changes are essentially similar to those of two storage proteins in *H. cecropia* (Tojo *et al.*, 1978) and *B. mori* (Tojo *et al.,* 1980). In *B. mori* it has been reported that the two storage proteins (SP-1 and SP-2) increase in the hemolymph from the final instar and reach maximal levels at time of spinning and then during the larval-pupal transformation decrease in the hemolymph (Tojo *et al.,* 1980).

Amino acid composition showed that the protein was characterized by the presence of aromatic amino acids (Table 2). Munn *et al.* (1971) proposed that a high aromatic amino acid content is related to demands for these constituents during sclerotization of the cuticle. SP-2 (arylphorin) of *B. mori* has been reported to contain high quantity of tyrosine and phenylalanine (Tojo *et al.,* 1980). Dipteran protein isolated from *Calliphora erythrocephala* (Munn and Greville, 1969) had both, aromatic and methionine high, and given the name calliphorin. Calliphorin was shown to be synthesized by the fat body (Munn *et al.,* 1969) and secreted into the hemolymph, beginning about half way through the larval growth period. Arylphorin purified was identified by data obtained by LCMS sequence. The deduced amino acid sequence was found to bore homology with the arylphorins of *Spodopteralitura, Manducasexta, Hyalophoracecropia* and *Antheraeapernyi* (Fig. 2). Molecular phylogenetic analyses have shown a complex pattern of hexamerin evolution insome insect orders, with several hexamerin subtypes, such as the highly aromatic arylphorins or methionine-rich hexamerins occurring in parallel in a single species (Burmester, 1999, 2001). Hexamerin sequences have been successfully used as markers for the inference of insect phylogeny (Burmester, 1999).

In conclusion, arylphorin, a storage protein, one of the key in metamorphosis, reproduction and insect development, are purified, partially sequenced and its amino acid composition deduced. Insect storage proteins with their broader role in insects' sclerotizing system in cuticle, as a carrier of ecdysteroids, juvenile hormone, riboflavin, and caste differentiation, demands biochemical documentation from all the orders of insectato understand their various role in insect physiology and deduce insect phylogeny for pest management and sustainable crop production. Now a days disruption of the regulation of storage proteins is a potential method to control this pest.

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APPLICATION FORM

NATIONAL ENVIRONMENTALISTS ASSOCIATION (N.E.A.)

To,
The Secretary,
National Environmentalists Association,
D-13, H.H.Colony,
Ranchi - 834 002, Jharkhand, India

Sir,
I wish to become an Annual / Life member and Fellow* of the association and will abide by the rules and regulations of the association

Name _____

Mailing Address _____

_____

Official Address _____

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E-mail _____Ph. No._____(R)_____(O)

Date of Birth _____ Mobile No. _____

Qualification _____

Field of specialization & research _____

Extension work (if done) _____

_____

Please find enclosed a D/D of Rs..... No. ..... Dated ..... as an *Annual / Life membership fee*.

*Attach **Bio-data and some recent publications along with the application form when applying for the Fellowship of the association.**

Correspondance for membership and/ or Fellowship should be done on the following address :

SECRETARY,
National Environmentalists Association,
D-13, H.H.Colony,
Ranchi - 834002
Jharkhand, India
E-mails : m_psinha@yahoo.com Cell : 9431360645
dr.mp.sinha@gmail.com Ph. : 0651-2244071