

Identification and Characterization of Heparan Sulphate Binding Proteins of *Entamoeba histolytica*

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Abstract

A large number of microbial pathogens bind to heparan sulphate on eukaryotic cell surfaces. Heparan Sulphate Binding Proteins (HSBPs) from *Entamoeba histolytica* culture lysates were obtained by sequential ammonium sulphate precipitation and Protein purify. SDS-PAGE and immunoblotting experiments indicated the presence of two major extracellular proteins in *E. histolytica* (51.2 kDa and 61.0 kDa). Characterization of HSBPs by 2D Gel electrophoresis of 40% (NH₄)₂SO₄ precipitated lysate of *E. histolytica* revealed that the isoelectric point of 51.2 kDa HSBP was at pH3.0. The protein of 61.0 kDa HSBP showed three spots in 40% (NH₄)₂SO₄ lysate which had isoelectric point between pH 4.0 - 7.0. While in 80% (NH₄)₂SO₄ precipitated lysate, 51.2 kDa HSBP showed only one spot which had isoelectric point at pH 3. However, 61.0 kDa HSBP revealed 2 spots which had isoelectric point between pH 4 and 5. The result showed that this parasite has proteins which interact with heparan sulphate whose molecular formula is C₁₄H₂₃NO₂₁S₃⁻². These proteins may have a role in binding of parasite to heparan sulphate on host cells. Further characterization by MALDI-TOF analysis of HSBPs from *E. histolytica* demonstrated HSBPs to be novel protein in this parasite which has been isolated, purified and characterized first time by our group in the present study.

Keywords

Heparan Sulphate, *Entamoeba histolytica*, Identification, Characterization

1. Introduction

Entamoeba histolytica, the causative agent of human amoebiasis, has a world-wide

distribution with a high morbidity and mortality [1]. Amoebiasis, the cause of dysentery and extra-intestinal abscesses is second fatal parasitic disease in the world [2]. Worldwide 50 million people suffer from amoebiasis, developing disabling colitis or extraintestinal complications leading to 50,000 - 100,000 deaths every year [3] [4]. *E. histolytica* usually resides in the large intestine and its invasion into intestinal epithelium cause ulcerative colitis or disseminate on into other organs, most commonly the liver, leading to abscess formation. Adherence of the parasite to intestinal epithelial cells is a prerequisite for the pathogenesis of disease [5]. While different adhesion molecules have been identified on the surface of *E. histolytica* [6] [7] [8], only 260 kDa galactose and N-acetyl-D-galactosamine-inhibitable adherence lectin (Gal/Gal NAc lectin) has been widely explored and considered to play a crucial role in the interaction between the parasite and an epithelial cell [9] [10]. A large array of glycoproteins, glycolipids and proteoglycans are present on the surface of eukaryotic cells. These proteoglycans include heparan sulphate, dermatan sulphate, chondroitin sulphate, keratan sulphate, heparin and other sulphated molecules, and hyaluronic acid which are found ubiquitously in human body as a part of extra cellular matrix [11]. Protozoan parasites such as *Leishmania amazonensis* [12], *Plasmodium falciparum* [13] and *Trypanosoma cruzi* [14] have also been reported to bind to heparan sulphate on eukaryotic cell surfaces which facilitate their adherence and cellular invasion by these pathogens. The heparan sulphate binding proteins in *E. histolytica* are not yet identified and characterized. It is likely that heparan sulphate binding proteins may have an important role in attachment and lysis of host cells by *E. histolytica*. This study reports the isolation, identification and characterization of this protein from *E. histolytica* with affinity for heparan sulphate proteoglycan.

2. Materials and Methods

2.1. Parasites Strains and *In-Vitro* Culture

The axenic strain of *Entamoeba histolytica* (HM1: IMSS) which was grown in TY-I-S-33 medium was used in the study for preparation of Heparan Sulphate Binding Proteins (HSBPs). Before harvesting, the cells were inoculated in serum free medium for 48 hours. Trophozoites were harvested at 48 - 72 hour (mid-log phase) by chilling and after pelleting at 150× g for 5 min, the cell pellet was lysed in 10 mL of lysis buffer containing 150 mM NaCl, 50 mM Tris, 0.5% (v/v) Nonidet P-40 (Sigma, USA) and 20 µl protease inhibitor cocktail (Sigma, USA). The lysed amoebic trophozoites were microcentrifuged at 10,000 g for 10 min. The supernatant was stored at -20°C and was used for further protein isolation by ammonium sulphate precipitation.

2.2. Protein Precipitation

The proteins were precipitated from the culture supernatants with ammonium sulphate (NH₄)₂SO₄ at 40%, 60%, 80% and 100% saturation. The proteins precipi-

pitated with each concentration of ammonium sulphate were centrifuged (18,000 g, 30 min, 5°C) and finally suspended in distilled water. The proteins were dialyzed against four changes of 0.01 M ammonium bicarbonate using Spectra/POR® Membrane with molecular weight cut off 6 - 8 kDa. The measurement of protein content in each preparation was carried out by using bicinchoninic acid protein assay [15] using protein estimation kit from Bangalore Genei Pvt. Ltd. India, as per the manufacturer's instructions. All the preparations were stored at -20°C until used.

2.3. Protein Purify

The proteins were purified by Protein purify with Heparin Hi-Trap column (Amersham Biosciences Ltd., UK) as described [16]. The ligand in Heparin Sepharose High Performance is a naturally occurring sulfated glucosaminoglycan which is extracted from the native proteoglycan of porcine intestinal mucosa. Heparin consists alternating units of uronic acid and D-glucosamine, most of which are substituted with one or two sulfate groups. The molecular weight of the polymer is distributed over the range 5000 - 30,000. It is covalently coupled to highly cross-linked agarose beads. The coupling method gives high capacity and high performance. The medium is stable over the pH range 5 - 10, and tolerates all commonly used aqueous buffers.

Briefly, protein samples obtained with various concentrations of ammonium sulphate (40% - 100%) were filtered through a 0.45 µm membrane filter and diluted 1:1 with 0.1 M sodium acetate (pH 5.0). One mL of diluted sample was applied to a 5 mL Heparin Hi-Trap column, previously equilibrated with 0.1 M sodium acetate buffer (pH 5.0). Proteins lacking affinity for heparin were washed out with 0.1 M sodium acetate buffer (pH 5.0) at a flow rate of 1 mL/min. Adsorbed proteins were eluted with NaCl (0.25 and 0.5 M) over 30 min at the same flow rate and 1 mL fractions were collected. Finally, the column was washed with 0.01 M NaOH and then was regenerated with distilled water and 0.1 M sodium acetate buffer. The fractions collected were dialyzed extensively against 10 mM ammonium bicarbonate and stored at -20°C until used.

2.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

After affinity chromatography, the protein preparations were electrophoresed [17], using electrophoresis apparatus (Mini-PROTEAN® II Electrophoresis System, Bio-Rad, USA). Protein samples were denatured before electrophoresis for 5 min at 100°C in sample buffer (0.6 M Tris-HCl (pH 6.8), glycerol 10%, SDS 10%, β-metacaproethanol 5% and bromophenol blue 0.05%). Samples containing 5 - 10 µg of total protein and molecular mass standard were loaded on to a discontinuous acrylamide gel (separating gel 12%, stacking gel 4%), electrophoresis was done at 80 V for 2 h and the gel was stained with Coomassie blue to visualize the proteins.

2.5. Immunoblotting

For immunoblotting, Heparan Sulphate (Sigma) was conjugated with Horseradish Peroxidase (HRP) by Periodate Oxidation Method [18]. In brief, 0.1 M solution of sodium periodate (NaIO_4) was prepared in freshly distilled water and 0.2 mL of this solution was added to 1 mL of 5 mg/mL HRP solution in distilled water. The solution was stirred gently for 20 minutes at room temperature. The pH of the solution was raised to 9.5 by addition of 25 μL of 0.2 M sodium carbonate. One mL of heparan sulphate (Sigma) solution (18 mg/mL) in 0.01 M Na_2CO_3 , pH9.5 was added. The mixture was then put on an end to end shaker for 2 hours at RT. After 2 hours, 0.1 mL of sodium borohydride (4 gm/mL) was added and was kept at 4°C for 2 hours. Finally the mixture was stored at -20°C till use.

For immunoblotting, the purified proteins on SDS-PAGE gels were electro transferred onto nitrocellulose membrane by Mini Trans—Blot Electrophoretic Transfer Cell (Bio-Rad, USA). Immunoblotting was performed according to the method adopted by Bustos *et al.*, 2000 using heparan sulphate—HRP conjugate (Sigma, USA). Conjugate bound to heparan sulphate binding proteins on nitrocellulose membranes was visualized with DAB (3,3'-diaminobenzidine) 2.5 mg and H_2O_2 (2.5 μL) in 10 mL of 0.1 M Sodium acetate, pH 5.0. The reaction was stopped with 0.1 M Sodium metabisulphite substrate solution and the results were interpreted on a Gel-Doc System (Uvi pro, UK) using UVI-Photo MW software.

2.6. Characterization of Heparan Sulphate Binding Proteins

1) 2-D Gel Electrophoresis

Separation of proteins in first dimension by isoelectric focusing was performed using the Ettan IPGphor 3 Isoelectric focusing system (GE Healthcare Bio-Sciences, UK). Fifty μL of HSBP antigen preparation, 20 μL of carbamylate marker and 60 μL Rehydration buffer was added to micro centrifuge tubes. Final volume of the mixture was adjusted to 130 μL using rehydration buffer containing 0.02% (w/v) Dithiothreitol as recommended. Rehydration buffer containing sample was added to the strip holder at the anode. The strip holder was placed on Ettan IPGphor 3 Isoelectric focusing system (GE Healthcare Bio-Sciences, UK). The running protocol was rehydration at 20°C for 12 hours at 50 μA per strip followed by isoelectric focusing at 50 μA per strip as follows:

Step 1: 500 V for 5 hours,

Step 2: 4500 V for 1.5 hrs (gradient) and,

Step 3: 4500 V for 7 - 10 hrs till 14000 V hrs.

For the equilibration of the strip, 10 mL of fresh equilibration buffer with 100 mg DTT was prepared for each strip. Strip was incubated in equilibration buffer for 15 min on constant rocker.

For separation of proteins in second dimension based on molecular weight 15% separating acrylamide gel was prepared. The strip was placed across the top

of the gel making sure that the strip is in touch with the gel and air bubbles between the strip and the top of the gel were removed. The warm 1% agarose made with 1X running buffer containing bromophenol blue was added. The agarose was allowed to cool and solidify, before moving to the electrophoresis apparatus. Running buffer (1X) was added to the upper and lower buffer chambers and the gel was placed inside the apparatus. Gel was run for 1.5 - 2 hrs at 15 - 20 mA. Gel was removed and stained with Coomassie Brilliant Blue G-250. The results were analyzed using PD Quest software (Bio-RAD).

2) MALDI TOF Analysis

Further characterization of these HSBPs was done by MALDI-TOF. As the spots in 40% $(\text{NH}_4)_2\text{SO}_4$ precipitated lysate showed the wider range in terms of pH (**Figure 1(a)**), these spots were further processed for MALDI-TOF. In brief, the proteins spots were excised from the 2D gel. The fragments were incubated with 50% acetonitrile (ACN) and dried in a vacuum centrifuge. Trypsinization of proteins was done with porcine trypsin and 5% ACN overnight at 37°C. Gel fragments were sonicated to provoke the release of peptides. The organic phase containing the peptides was re-extracted. For MALDI-TOF mass spectrometry, samples were spotted on a MALDI target plate mixed with matrix (4x-cyano-4-hydroxycinnamic acid solution in 1 mL, 1:1 ACN:ethanol) and dried. Before analysis, the system was calibrated according to standards supplied by the company (Bruker Daltonics Flex). MALDI-TOF spectra was acquired on a Reflex IV in positive reflectron mode in the m/z 500 - 3000 range [19].

3. Results

3.1. Protein Concentration of Purified Heparan Sulphate Binding Proteins Obtained after Affinity Chromatography

Varying amounts of proteins were obtained from culture lysates precipitated with different concentration of ammonium sulphate *i.e.* with 40%, 60%, 80% and 100% followed by dialysis and protein purity with Heparin Hi trap column in *E. histolytica* (**Table 1**).

To get the high protein concentration, the two elutions (0.25 M NaCl and 0.5 M NaCl) of *E. histolytica* lysates precipitated with each concentration of ammonium sulphate were pooled for further analysis by SDS-PAGE and immunoblotting.

3.2. SDS PAGE Analysis

SDS PAGE analysis of purified eluted proteins revealed that in *E. histolytica* all the preparations except the protein precipitated with 100% ammonium sulphate showed protein bands capable of binding to heparin Hi-trap columns.

3.3. Immunoblotting Analysis to Identify Heparan Sulphate Binding Proteins

Immunoblotting analysis with heparan sulphate conjugated with horse radish peroxidase showed the presence of two proteins with heparan sulphate binding

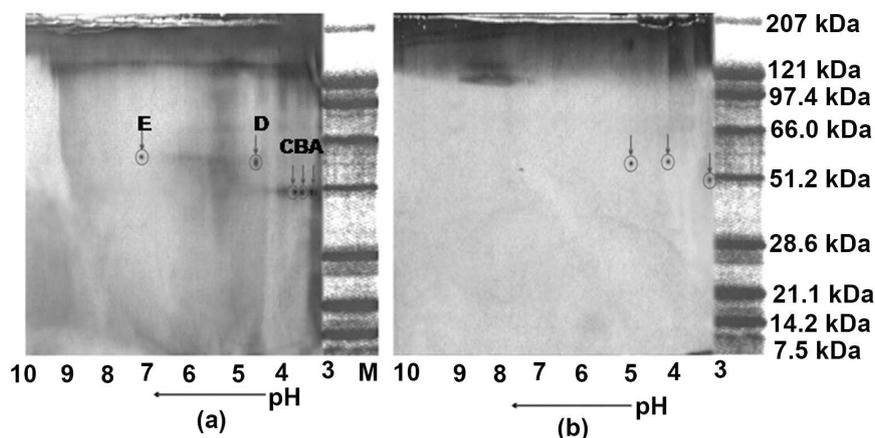


Figure 1. Characterization of HSBPs (51.2 kDa and 61.0 kDa) of *E. histolytica* by 2-D Gel Electrophoresis showing spots corresponding to different pH. The spots are encircled and indicated by arrows. (a) Proteins precipitated with 40% $(\text{NH}_4)_2\text{SO}_4$. (b) Proteins precipitated with 80% $(\text{NH}_4)_2\text{SO}_4$.

Table 1. Protein concentration in culture lysates of *Entamoeba histolytica* precipitated with various concentrations of $(\text{NH}_4)_2\text{SO}_4$ and purified by affinity chromatography with Heparin Hi-Trap column.

$(\text{NH}_4)_2\text{SO}_4^a$ conc ^b	Elution with	Protein conc. of <i>E. histolytica</i>
40%	0.25M NaCl ^c	1.0 mg/mL
40%	0.5M NaCl	0.75 mg/mL
60%	0.25M NaCl	0.8 mg/mL
60%	0.5M NaCl	0.9 mg/mL
80%	0.25M NaCl	0.7 mg/mL
80%	0.5M NaCl	1.5 mg/mL
100%	0.25M NaCl	0.2 mg/mL
100%	0.5M NaCl	0.1 mg/mL

^a: Ammonium Sulphate; ^b: Concentration; ^c: Sodium Chloride.

activity at 51.2 kDa and 61.0 kDa both in 40% and 80% preparations in *E. histolytica*.

3.4. D Gel Electrophoresis

PD Quest software (Bio-RAD) was used to analyze the isoelectric points of the protein spots obtained in the 2D-PAGE (Amersham Biosciences, Ltd., UK). On 2-D Gel electrophoresis analysis of HSBP obtained at 51.2 kDa in 40% $(\text{NH}_4)_2\text{SO}_4$ precipitated lysate, 3 spots were identified as indicated by arrows in **Figure 1(a)**. These spots appeared in the region of pH between 3 and 4 which indicated three isoforms of this protein (**Figure 1(a)**). Another 2 spots were also seen at 61.0 kDa. These had isoelectric point between pH 4 and 7 (**Figure 1(a)**).

In 80% $(\text{NH}_4)_2\text{SO}_4$ precipitated lysate of *E. histolytica*, 51.2 kDa HSBP showed only one spot which had isoelectric point at pH 3 (**Figure 1(b)**). However, 61.0

kDa HSBP revealed 2 spots which appeared in the pH range of 4 and 5 (**Figure 1(b)**).

3.5. MALDI-TOF Analysis of the Proteins

The mass spectrums obtained after MALDI-TOF analysis of 40% $(\text{NH}_4)_2\text{SO}_4$ precipitated HSBPs of 51.2 kDa (around pH 3 *i.e.* 3 spots) and 61.0 kDa (between pH 4 and 7 *i.e.* 2 spots) are represented in **Figures 2-4** and **Figure 5** and **Figure 6** respectively.

The data of trypsinized protein mass fragments obtained from MALDI-TOF was analyzed with Matrix Science *i.e.* with MASCOT and was further searched on various protein search engines like Uniport, Swissprot and NCBI. The analysis revealed that peptide mass fragments of this protein were not available in the Data Basics. Thus, the current data of the proteins from all the five spots did not match with any previous data of available proteins from eukaryotes. Peptide mass fingerprinting of the Heparan Sulphate Binding Proteins of *Entamoeba histolytica* is not submitted to the above mentioned sites so far. The results indicated that it is a novel protein. This led to the conclusion that this protein is identified first time in our experiments.

However, immunogenicity and protective efficacy of this antigen is already reported by us [20].

4. Discussion

Heparan Sulphate, a proteoglycan present on all eukaryotic cell surfaces [11], (Wadstrom and Ljungh, 1999), has been shown to be involved in adherence/invasion of several parasites like *Leishmania amazonensis*, *Plasmodium*

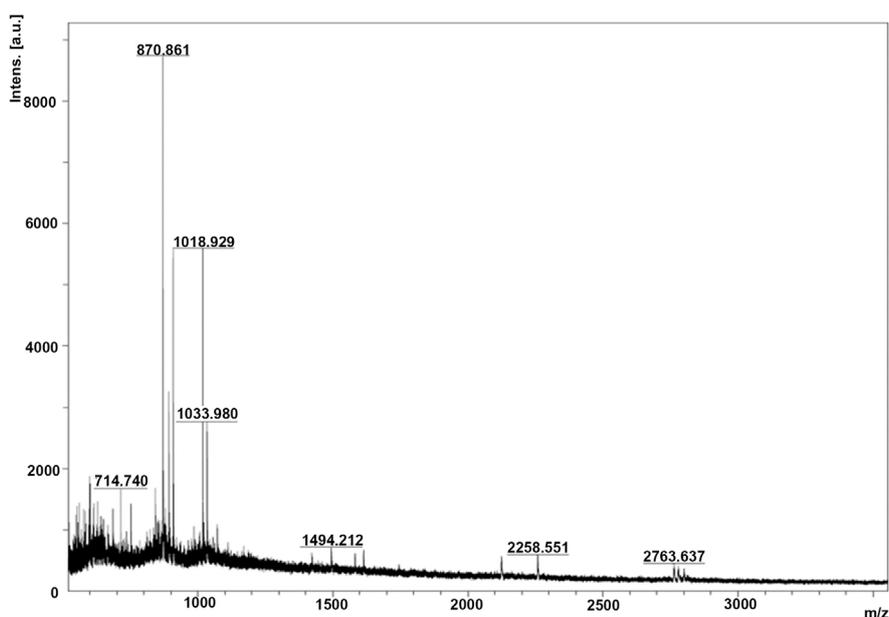


Figure 2. Mass Spectrum of protein spot of 51.2 kDa HSBP at pH 3 in 2 D Gel Electrophoresis (Spot A in **Figure 1(a)**).

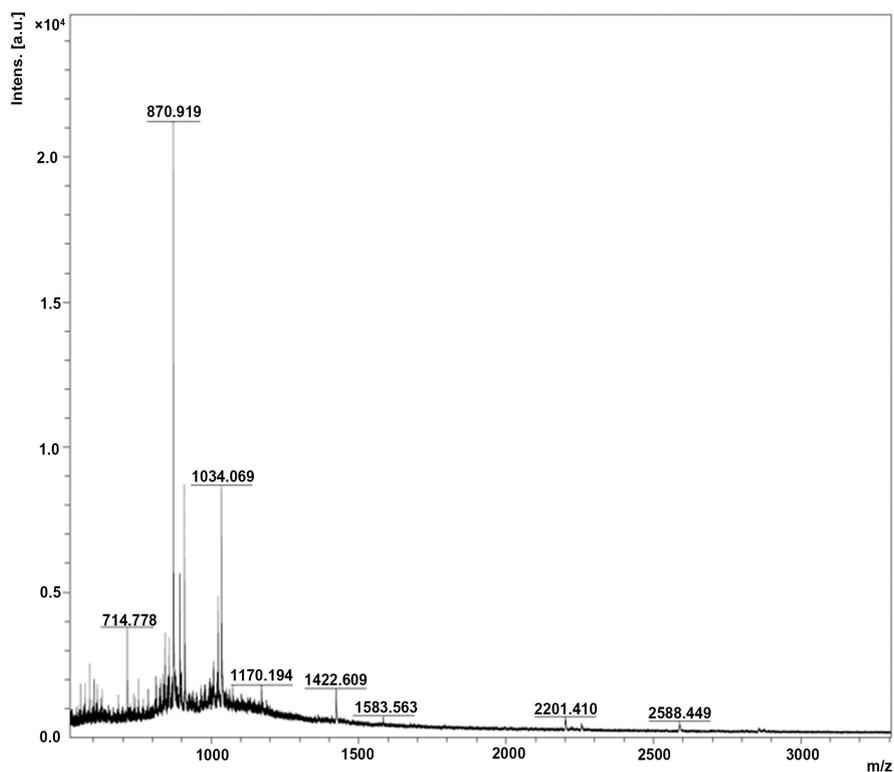


Figure 3. Mass Spectrum of protein spot of 51.2 kDa HSBP at pH 3 in 2 D Gel Electrophoresis (Spot B in **Figure 1(a)**).

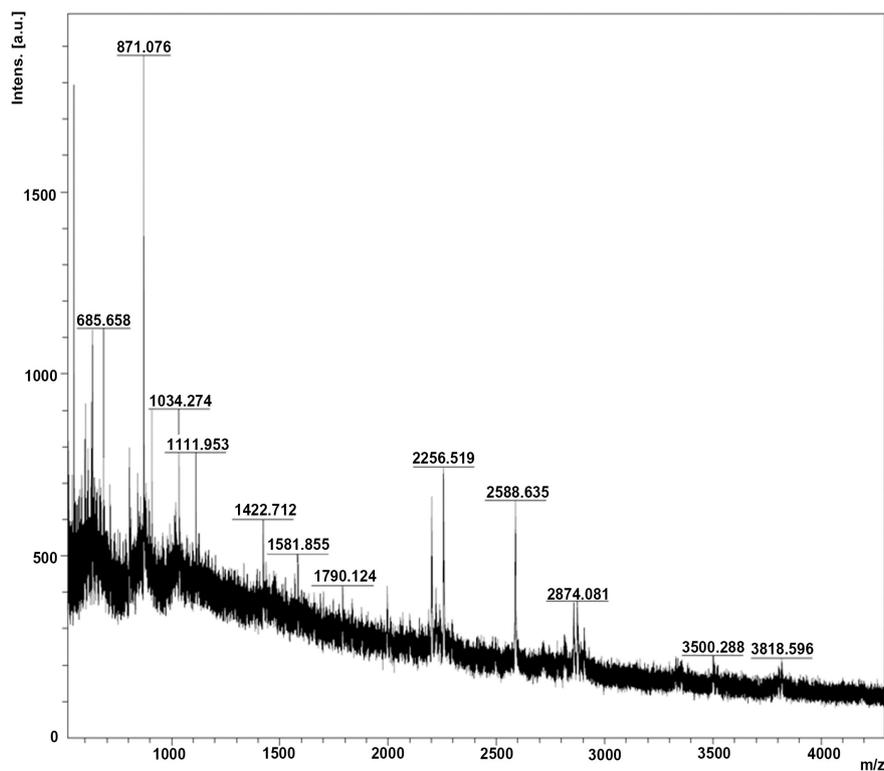


Figure 4. Mass Spectrum of protein spot of 51.2 kDa HSBP at pH 3 in 2 D Gel Electrophoresis (Spot C in **Figure 1(a)**).

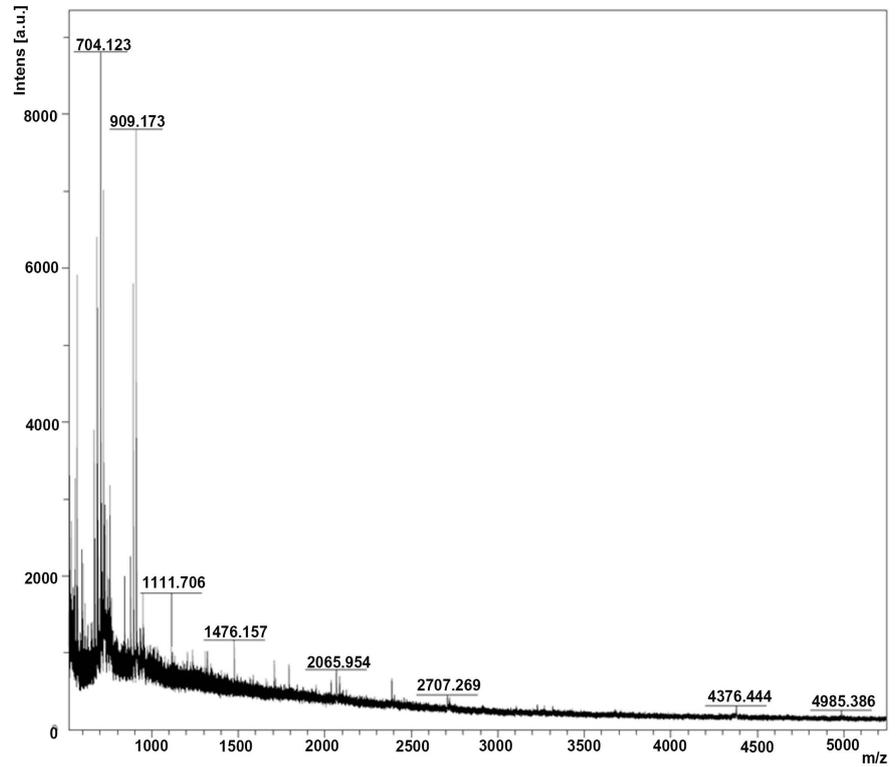


Figure 5. Mass Spectrum of protein spot of 61.0 kDa HSBP at pH 3 in 2 D Gel Electrophoresis (Spot D in **Figure 1(a)**).

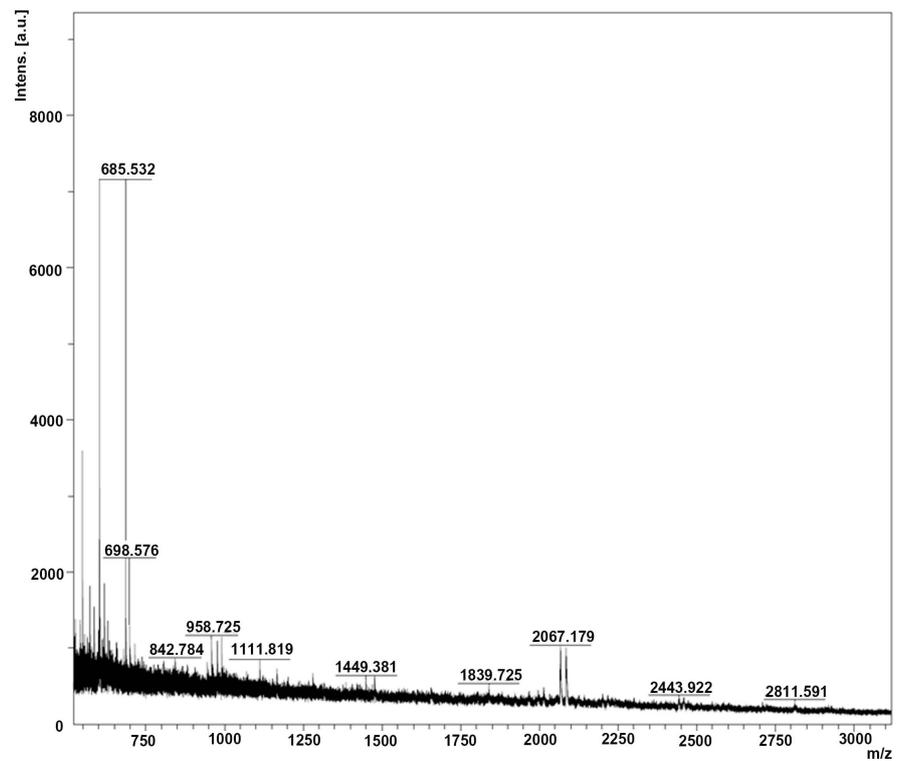


Figure 6. Mass Spectrum of protein spot of 61.0 kDa HSBP at pH 3 in 2 D Gel Electrophoresis (Spot E in **Figure 1(a)**).

falciparum and *Trypanosoma cruzi* [12]. Love *et al.* (1993) identified a high affinity heparin-binding activity on the surface of the amastigote form of *Leishmania*. Amastigotes of *Leishmania amazonensis* bound approximately 120,000 molecules of heparin per cell, with a K_d of 8.8×10^{-8} M. This heparin-binding site mediates the adhesion of amastigotes to mammalian cells via heparan sulphate proteoglycan, which are expressed on the surface of mammalian cells [12] (Love *et al.*, 1993). Pancake *et al.* (1992) [13] reported that recombinant *Plasmodium yoelli* circumsporozoite protein bound avidly to heparin, fucoidan and dextran sulfate-Sepharose, but bound comparatively poorly to chondroitin sulfate A or C Sepharose [14]. Ortega and Pereira (1991) studied the role of host heparin and heparan sulfate glycosaminoglycans (GAG) in *T. cruzi* invasion using three proteoglycan-deficient mutants of Chinese hamster ovary (CHO) cells. All three mutants supported adhesion and infection to a much lower extent than the parental CHO cells. One of the mutants, pgsD-677, did not express heparan sulfate but contained three-to four-fold excess chondroitin sulfate, yet the cell line was a poor substrate for *T. cruzi* adhesion.

No information is yet available on heparan sulphate binding amoebic protein(s) which can be envisaged as key players in tissue adhesion and invasion into deeper tissues by *Entamoeba histolytica*. It was reported [21] that the interactions between *E. histolytica* and intestinal epithelial cells involve a CD44 cross reactive 80 kDa membrane protein expressed on the parasite surface. The principal receptor known for CD44 is hyaluronic acid (HA) which is glycosaminoglycan and is found ubiquitously on extracellular matrix of human cells [21]. Besides HA binding proteins, *E. histolytica* may have on its surface other glycosaminoglycan binding proteins *i.e.* those that bind to heparan sulphate, chondroitin sulphate etc. With this hypothesis, the present study was undertaken to isolate, purify, identify and characterize heparan sulphate binding amoebic proteins.

In the present study, the affinity chromatography in other words protein purity of amoebic lysates with heparin allowed the purification of a set of proteins with different molecular masses which showed high reactivity towards heparan sulphate. The findings in the present study indicate that *E. histolytica* has mainly two proteins at 51.2 kDa and 61.0 kDa molecular mass which react with heparan sulphate. The characterization of HSBPs obtained from both the parasites, *Entamoeba histolytica* with 2-D gel electrophoresis revealed that HSBPs are acidic in nature as all the spots on gel were at $\text{pH} \leq 7$. In a study on proteomic analysis of *E. histolytica*, it was observed that most of the amoebic proteins separated within a pH range of 4 to 7 have isoelectric point of 5 to 6 [22]. Moreover, heparan sulphate proteoglycan itself is acidic in nature [23]. Further, characterization of HSBPs from *Entamoeba histolytica* proved these as novel proteins.

Thus, to the best of our knowledge, we have demonstrated for the first time, presence of heparan sulphate binding proteins in *E. histolytica*. It is possible that amoebic HSBPs may have a role in adherence of parasite to host cell and patho-

genesis. It was important to study the immunogenicity and protective efficacy of HSBPs of *E. histolytica* in an animal model by our group [20] which may show potential of these proteins as vaccine candidates for amoebiasis.

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Ethical Approval

The study has been approved by the Institutional Ethics committee.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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