

Purification of recombinant hepatitis delta antigen expressed in *E. coli* cells

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Recombinant DNA technology enables the massive production of recombinant hepatitis delta antigen (recHDAg) retaining immunological properties and transport functions. However, purification procedures of the recombinant delta antigen have, to date, not been described in the literature. We present a purification procedure allowing one to obtain highly purified recHDAg from bacterial cells expressing the hepatitis delta antigen.

Hepatitis delta antigen; Fusion protein; Purification

1. INTRODUCTION

HDV is a human pathogen that was discovered by Rizzetto and coworkers in HBV infected patients experiencing severe liver diseases [1]. The HDV genome consists of a circular, single stranded RNA approximately 1.7 kb in length [2–4] with the ability to fold on itself by intramolecular base pairing to form a double stranded rod-like structure [5]. The HDV genome and an highly basic phosphoprotein, HDAg [6,7], encoded by anti-genomic strand of HDV, are packed within an envelope composed of HBsAg [8]. HDAg is the only protein encoded by HDV and exists in infected hepatocytes in two forms 24 kDa and 27 kDa [8]. Furthermore the larger form (214 amino acids) represents a 19 amino acids carboxyl-terminal extension of the smaller form (195 amino acids) [9, 10].

Recombinant DNA technology enables a massive production of recombinant delta antigen retaining immunological properties and transport functions [11–15]. However, purification procedures of the recHDAg have, to date, not been described in the literature.

In this paper we present a recHDAg purification procedure which allow yields of more than 1 mg of recom-

binant protein from 1 liter of *E. coli* cells transformed with an expression plasmid harboring recHDAg gene.

2. MATERIALS AND METHODS

2.1. Materials

Cultures media, salts, and HPLC solvents were purchased from Sigma (USA); HPLC reverse phase columns were purchased from Waters (USA) and gel-filtration columns were purchased from Biorad (USA). HDAg detection kits and the natural antigen were supplied by SORIN Biomedica (I).

2.2. Methods

2.2.1. Cloning and expression of recHDAg in *E. coli*

The cloning strategy and the expression procedure have been previously described [14].

2.2.2. Bacterial paste lysis

E. coli cells (2 g), strain W3110 (ATCC 27325), transformed with pSORIN-Delta [14], was suspended in solution A (7 M CH₃CN, 0.1% TFA) (5 ml). The suspension was sonicated (3 × 1 min, 100 W burst, 5°C), incubated for 30 min at 60°C and then clarified by centrifugation (30 min 10,000 × g, 10°C). Cellular debris were reextracted with buffer A (5 ml) and the extraction solution clarified by centrifugation as described above.

2.2.3. Organic precipitation

The lysis supernatant and the cellular debris extraction solution were pooled and diluted 1:1 with solution B (50% CH₃CN, 0.2% TFA). The solution was incubated at 5°C for 30 min, with stirring, and subsequently clarified by centrifugation as described above. An aliquot of the clarified solution (0.1 ml) was dried under vacuum, dissolved in water (0.1 ml) dialyzed against buffer C (8 M urea, 50 mM sodium acetate, pH 5.5). 1 μl of the dialyzed solution (1.5 ml) was loaded on a 12.5% acrylamide SDS-PAGE gel (Fig. 1, lane A)

2.2.4. Reverse phase chromatography

The clear supernatant, after organic precipitation, was loaded on a reverse phase column (19 × 300 mm, C18-Deltapak) equilibrated in 0.5 × solution B (flow rate = 10 ml/min, detector 215 nm). The un-

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Abbreviations: HD, hepatitis delta, HDV, hepatitis delta virus; HBV, hepatitis B virus; HDAg, delta antigen; recHDAg, recombinant delta antigen; HBsAg, hepatitis B surface antigen; TFA, trifluoroacetic acid; CH₃CN, guanidinium chloride; CH₃CN, acetonitrile, kDa, kilodalton; kb, kilobase; anti-HD, anti-HDV antibodies; BSA, bovine serum albumin; OVA, ovin serum albumin.

bound material was washed out with $0.5 \times$ solution B until the baseline returned to zero. The adsorbed proteins were eluted with a 30 min linear gradient of CH_3CN (25% to 50%, flow rate=10 ml/min). The peak fraction containing the higher level of recHDAG immunological reactivity (hatched peak, Fig. 2) was dried under vacuum and dissolved in 2% acetic acid solution (1 ml); $1 \mu\text{l}$ was loaded on a 12.5% acrylamide SDS-PAGE gel (Fig. 1, lane B).

2.2.5. Gel-filtration chromatography

Partially purified recHDAG was loaded onto a silica based gel-filtration column (7.8 \times 600 mm, Biosil-250) equilibrated in solution B (flow rate=1 ml/min, detector 215 nm) the eluted material was collected in 0.25 ml fractions. The fractions, corresponding to molecular weights 35 to 30 kDa, containing the HDAG immunoreactivity, were pooled; $1 \mu\text{l}$ of the immunoreactive pool was loaded on a 12.5% acrylamide SDS-PAGE gel (Fig. 1, lane C).

2.3. Western blot and ELISA immunoassays

Western blot analysis was done on purified recHDAG (data not shown) using a modified Towbin's protocol [16]. RecHDAG immunoreactivity detection in column eluates (data not shown) was performed using a commercial ELISA sandwich immunoassay for detection of HDAG (ETI-DELTA-K). ELISA dilution curve immunoassays (Fig. 3) were performed using a commercial ELISA single step immunoinhibition kit (ETI-ABDELTA-K). All the assays were performed on recHDAG diluted in anti-HD negative human serum and following the procedures recommended by the manufacturer.

2.4. RNA Binding assays

The recHDAG RNA binding activity was evaluated with the procedure described by F. Poisson et al. [17]. RecHDAG was coated on NUNC microtiter plates at 12.5 ng/well, 25 ng/well, 50 ng/well, 100 ng/well and 200 ng/well as described elsewhere [17].

3. RESULTS AND DISCUSSION

We have developed a purification procedure for a recHDAG expressed in *E. coli*. The purified recHDAG is a fusion protein consisting of 8 amino acids of the NH_2 -terminus of β -galactosidase, HDAG sequence truncated at aminoacid 204 and a trailer of 49 amino acids encoded by *tet* gene [14]. The antigen is very hydrophilic, is expressed in the cytosol of *E. coli* [14] and represents about 1% of the total *E. coli* proteins (R. Calogero et al., personal communication). We have used harsh conditions for the purification to reduce proteolytic degradation of the recombinant antigen. We purified 1 to 2 mg of recHDAG from 1 liter bacterial culture. The recHDAG had a purity higher than 95% (found out by scanning on a Coomassie stained gels). RecHDAG has a molecular weight of 30 kDa (Fig. 1, lane C) and is soluble in acidic buffers, forming insoluble aggregates at pHs higher than 7 (data not shown). The sequencing of the first 20 amino acids at the NH_2 -terminus confirmed the sequence predicted from the DNA sequencing. The recHDAG, from an immunological point of view, is more stable than the natural one (Table I). A substantial decrease (7%) in the immunoreactivity of the natural HDAG as compared to the recombinant antigen is observed after 7 days at 37° C. We observed a more drastic effect (> 20%) after incubation for 3 days at 45° C (Table I).

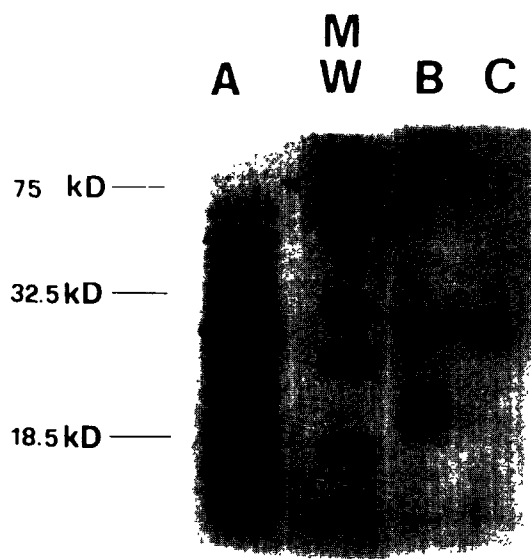


Fig. 1. In the SDS page gel recHDAG is indicated by an arrow. Lane A: *E. coli* clarified lysate after CH_3CN addition. Lane MW: Biorad prestained low molecular weight markers. Lane B: peak fraction of reverse phase chromatography associated with HDAG immunoreactivity. Lane C: gel-filtration pool of the 30 kDa purified recHDAG.

The recombinant antigen has shown an immunological behavior similar to the natural one, as demonstrated in endpoint immunoinhibition assays, where a sensibility identical to the natural HDAG was achieved (Fig. 3).

The recombinant antigen retained specific HD-genome RNA binding activity (Fig. 4). The RNA binding to recHDAG was evaluated using digoxigenin labeled RNAs [17]. The recHDAG bound more efficiently HDV RNAs (Fig. 4, open and closed triangles) respect to the control RNAs (Fig. 4, open and closed squares). Furthermore, the recHDAG bound more efficiently the antigenomic strand (Fig. 4, closed triangles) than the genomic strand (Fig. 4, open triangles). Those genomic and antigenomic strands contain a rod-like structure that has been shown to allow HDAG binding [18].

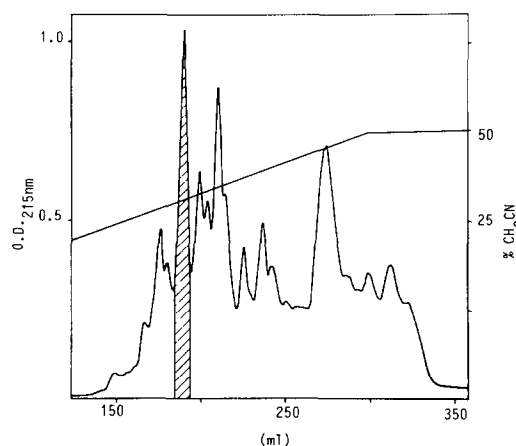


Fig. 2. Elution profile of the reverse phase column (see Methods).

Table I

The retention of immunoreactivity of the natural and recombinant antigen was analyzed in accelerated stability studies

Sera	Temperatures					
	4°C (7 days)		37°C (7 days)		45°C (3 days)	
	HDAg	rechDAg	HDAg	rechDAg	HDAg	rechDAg
NC-0	100	100	81.5	98.8	71.2	95.4
NC-1	100	100	80.4	94.8	69.8	92.0
NC-2	100	100	84.0	95.7	67.0	93.5
PS-01	100	100	94.3	85.0	85.7	100.1
PS-65	100	100	95.0	90.3	75.8	90.5
PS-66	100	100	82.2	94.7	76.2	100.3
mean	100	100	86.2	93.2	74.3	96.9

Each condition was tested in triplicates on 3 negative sera (NC-0, NC-1, NC-2) and 3 positive sera (PS-01, PS-65, PS-66), comparing various batches of recombinant and natural antigen. The rechDAg shows greater stability to thermal shock than the natural one. The loss of binding capacity for the rechDAg is within - 5% ($\pm 1\%$) as compared to - 20% ($\pm 1\%$) for the natural HDAg.

The level of purity and the yield of protein, which we have achieved, will allow the use of rechDAg in the development of new assays for the *in vitro* detection of HDV infection in human patients. Moreover, substitution of rechDAg in test formats would prevent the present hazards to workers involved in the purification of the natural one from large quantities of HBV/HDV infected material.

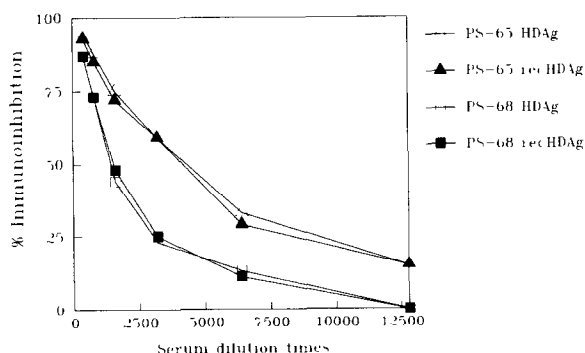


Fig. 3. Immunoreactivity of rechDAg as compared to that of the natural antigen was evaluated finding out the endpoint titers of two anti-HD positive human sera (PS-65, PS-66) representing moderately positive specimens. Each specimen was serially diluted in negative human sera and tested in duplicate. The sensitivities found for each of the antigen types were essentially identical.

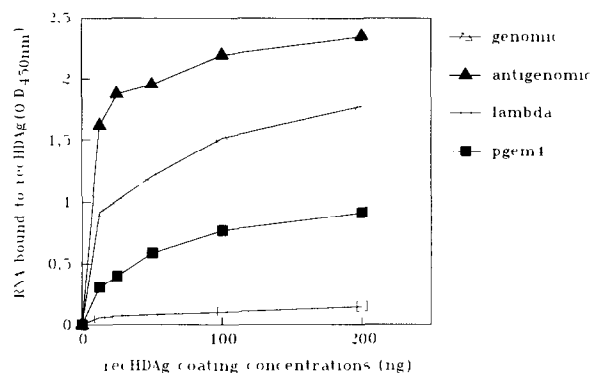


Fig. 4. The results of the rechDAg RNA binding activity are given in absorbance values at 450 nm referring to various rechDAg coating concentrations (see Methods). Genomic strand (open triangles) is a 1220 nucleotides long RNA containing nucleotides 1420 to 1679 and nucleotides 1 to 962 of HDV genome. Antigenomic strand (closed triangles) contains the sequences complementary to the genomic strand. Lambda (open square) is a 1.37 kb RNA generated by transcription of a lambda fragment inserted in a pGEM plasmid [17]. pgem4 (closed square) is a 2.6 kb RNA obtained by transcription of the *NheI* cut pGEM-4 plasmid [17]. BSA and OVA do not bind any of the as mentioned above RNAs (data not shown).

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