Purification of the Recombinant Hepatitis B Core Antigen, and Its Potential Use for the Diagnosis of Hepatitis B Virus Infection

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ABSTRACT

The HBcAg gene of HBV was cloned into the pRIV-2 expression vector for the synthesis of the 22-kD protein. The expressed protein was assembled into particles with a 7% expression level of total *E. coli* proteins present in a 15% SDS-PAGE. HBcAg was purified to 90% by using a combination of two purification steps with ammonium sulphate and a CL-4B sepharose gel filtration chromatography. The total recovery of the proposed methodology was 47%. The use of this protein in the diagnosis of HBV infection was evaluated by ELISA using a panel of positive and negative sera. The values of 99.3% specificity and 97.9% sensitivity demonstrated the potential use of purified HBcAg in the serodiagnosis of HBV infection.

Keywords: HBcAg, HBV, purification, serodiagnosis

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RESUMEN

Purificación del antígeno recombinante del core de la hepatitis B, y su uso potencial en el diagnóstico de la infección con el virus de la hepatitis B. El gen del HBcAg del VHB fue clonado en el vector de expresión pRIV-2 y usado para la síntesis en *E. coli* de la proteína de 22 kD. La proteína expresada fue capaz de ensamblarse en forma de partícula con un nivel de expresión del 7% con respecto al total de proteínas de *E. coli* presentes en un SDS-PAGE del 15%. El HBcAg fue purificado hasta un 90% utilizando una combinación de dos pasos de precipitación con sulfato de amonio y una cromatografía de filtración en gel de sepharose CL-4B. El recobrado total del método propuesto fue del 47%. El uso en el diagnóstico de la infección por el VHB fue evaluado por ELISA mediante un panel de sueros positivos y negativos. Los valores obtenidos de 99,3% de especificidad y 97,9% de sensibilidad demostraron el uso potencial del HBcAg purificado en el serodiagnóstico de la infección con el VHB.

Palabras claves: diagnóstico, HBcAg, purificación, VHB

*I*ntroduction

Hepatitis B virus (HBV), a member of hepadnaviridae family, is a small enveloped DNA virus that replicates by reverse transcription [1]. The 3.5 kb HBV genome is enclosed in a viral nucleocapsid composed of the 22 kD core protein (HBcAg). The viral capsid or core particle has an icosahedral structure and assembles into two spherical shell variants: a smaller capsid composed of 180 core protein copies (90 dimers) with a triangulation number T=3 and a larger one composed of 240 copies (120 dimers) with a T=4 [2]. The HBcAg expressed in heterologous hosts such as *Escherichia coli* [3-5] is able to assemble into a particle, indistinguishable from authentic capsids in terms of antigenicity and morphological structure [6].

Anti-HBcAg test was first used in the middle of the 80's in an effort to identify donors at risk of transmission of post-transfusion non-A, non-B (PT-NANB) hepatitis [7]. Several years later, the HCV was identified, and it was shown to be responsible for most, if not all (>90%) cases of PT-NANB hepatitis [8]. However, the screening of anti-HBcAg donors contributes to the elimination of the potential infectious units from HBV chronically infected individuals with no detectable HBsAg and also the units from donors with acute hepatitis B who are in the window period where HBsAg is not detectable and only the IgM anti-HBcAg response is observed. These characteristics make the anti-HBcAg antibodies a useful serological marker in the diagnosis of HBV infection, considered a serious health problem with as much as 5% of the world's population infected with HBV [8].

The production of HBcAg in a heterologous host like *E. coli* provides a suitable way to obtain enough antigen to be used not only for biophysical and morphological studies but also for the development of anti-HBcAg diagnostic systems. Also, more recently it was successfully used as a carrier particle to enhance the immunogenicity of foreign proteins [9-11]. Most of the commercially available assays for total anti-HBcAg are competitive, based on the presence of an immunodominant epitope on the HBcAg surface, which is virtually recognized by all sera of acute and chronic individuals [12 , 13]. Here, HBcAg was expressed in *E. coli* using a plasmid controlled by a pL promoter. Purification was achieved by the combination of two sequential ammonium sulphate precipitations and gel filtration chromatography on a sepharose CL-4B column, which represents a suitable and efficient way to obtain a highly purified HBcAg. Antigenicity of the purified HBcAg was evaluated by a direct ELISA format. This format was similar to the assay published by Nelles *et al.* [14] using protein A/horseradish peroxi-

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dase (HRP) as the conjugate, instead of monoclonal antibodies specific for human IgG and IgM.

Materials and Methods

Reagents

Sodium azide, tris, ammonium sulfate, HRP, orthophenyldiamine (OPD) and dithiothreitol (DTT) were provided by Sigma Chemical Co (ST Louis, USA). The human anti-HBcAg/HRP from Abbott GmbH (Wiesbaden-Delkenheim, Germany) was used as the conjugate. The 96-well microtiter plates (Polysorp) were purchased from Nunc Inc., Denmark. The bovine serum albumin and Coomassie brilliant blue G-250 were purchased from Spectrum (New Brunswick, USA). The protein A and sepharose CL-4B, used as the gel filtration matrix, were from Amersham Pharmacia Biotech (Sweden).

Cloning the HBcAg gene

The HBcAg gene was amplified by polymerase chain reaction (PCR) from the pR2M6-HBcAg construction [15], using a set of specific primers (sense primer: 5'-CTTGGATCCATGGACATTGACCCTTAT-3', antisense primer: 5'-GAAGGATCCAAGCTTACATTGAGATTCCCGAGA-3'). The reaction mixture (100 µL) contained 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1 %Triton X-100, dNTP mixture 0.2 mM each, 10 pmoles of each primers and 2.5 U Taq Pol. The reaction was incubated at 94 ºC, 55 ºC and 72 ºC for 1 min respectively, 25 cycles. The amplified gene was cloned into the pRIV-2 vector (Pharmacia, Sweden), under the control of a pL promoter, using the restriction sites *Nco*I/*Bam*HI (Biolab, USA). Expression was tested in *E. coli* W3110 cells previously transformed with the pGP1-2 plasmid, which confers kanamycin resistance and codifies for the cI857 protein, the pL promoter repressor.

Expression of HBcAg

Transformed cells were grown in 30 mL of the LB medium [11], supplemented with ampicillin (50 mg/mL) and kanamycin (50 mg/mL) for 12 h at 28 ºC. When cell density reached 1.0 OD units (at 610 nm), the culture was diluted 1:10 in a fresh LB medium, supplemented with ampicillin and kanamicin, and incubated at 28 ºC for 4 h. Induction was made by thermal shock at 42 ºC, for 10 min, followed by incubation at 42 ºC for 5 h.

Cell disruption

One gram of biomass was homogenized in 10 mL of TE 1x buffer (10 mM Tris, 0.1 mM EDTA, pH 7). The suspension was sonicated (U200S sonicator IKA Laborthecnik, Germany) for 3 min intervals at a 70% output in an ice-bath. Disrupted cells were centrifuged for 25 min at 26 000 g to remove the precipitate.

Ammonium sulphate precipitation

The clear supernatant obtained in the previous step was diluted with 80 mL of the TE 1x buffer. At room temperature (RT), 5.04 g of $(NH_4)_2SO_4$ (10 % saturation) was slowly added to the final volume of 90 mL, with continuous shaking using a magnetic stirring bar. After 1 h, the solution was centrifuged for 10 min at 14 000 g and 4 ºC. Then, 16.47 g of $(NH_4)_2SO_4$ (40% saturation) were added to the supernatant, which was incubated under the same conditions shown in the previous step. The solution was then centrifuged for 20 min at 26 000 g and 4 ºC. The precipitate was suspended in 10 mL of the buffer (100 mM Tris, 150 mM NaCl, 2 mM DTT and 0.01% (w/v) sodium azide, pH 7.5), used also as the mobile phase during the chromatography purification step. The suspension was concentrated to 2.5 mL in a stirred ultrafiltration cell (Amicon Inc, Beverly MA, USA), using a YM10 membrane and filtered through a membrane of 0.45 μm pore size (Sartorius, Goettingen, Germany).

Chromatography on the sepharose CL-4B matrix

Finally, 2.5 mL of the filtered antigen was loaded on a sepharose CL-4B column (C 90x1.6 cm), previously equilibrated with the mobile phase described above. The column was eluted at a linear flow of 0.1 cm/min, collecting 2 mL per fraction for 12 h. The equipment used were an uvicord SII 2238, a two-channel recorder rec-480, a fraction collector Frac-100 and a peristaltic pump P-1, all purchased from Amersham Pharmacia Biotech (Sweeden).

The isopycnic CsCl gradient

A a stepwise CsCl density gradient was loaded with 0.5 mL of chromatography purified HBcAg at a concentration of 0.23 mg/mL. The gradient was made by layering from bottom to top 4 mL of CsCl at 1.422 g/mL, 2 mL at 1.288 g/mL, 2 mL at 1.175 g/mL and 2 mL at 1.079 g/mL in 0.1 M Tris-HCL buffer (pH 7.5) plus 0.15 M NaCl. Tubes were centrifuged in a Hitachi SCP70H centrifuge (Japan) using a RPS40T rotor at 160 000 g for 24 h at 4 ºC. Thereafter, the gradient was carefully fractionated, from bottom to top, in 0.5 mL fractions using a peristaltic pump P-1. The refractive index of each fraction was measured using a Sibuya Optical refractometer (Japan).

Fractions containing HBcAg were detected by ELISA. Microtitration plates (Nunc Inc., Denmark) were coated with anti-HBcAg rabbit polyclonal antibody (CIGB, Havana, Cuba) at 10 µg/mL in phosphate buffer saline (PBS). After washing with PBS plus 0.05 % Tween 20 (PBS-T), 10 µL of each fraction were mixed with 90 µL of PBS, and incubated for 1 h at 37 ºC. Then, the plate was washed again and 100 µL of the anti-HBcAg human polyclonal antibody conjugated to HRP (Corzyme, Abbott Laboratories) were added to each well. The plate was incubated at 37 ºC for 1 h and washed again. Finally, 100 µL of the substrate buffer (citrate phosphate buffer pH 5.5, 0.014% H_2O_2, 0.25% OPD) was added to each well. The plate was incubated in the dark for 10 min and colour development was stopped with 50 µL/well of 2.5 M sulphuric acid. The OD was read at 492 nm in a reader plate (SenSident, LabSystem Inc., Finland).

SDS-PAGE and blotting

SDS-PAGE was performed according to the Laemmli method [16]. Proteins were mixed 1:1 with the sample buffer containing 4% SDS, 125 mM Tris-HCl pH 6.9, 20 % glycerol, 0.002% bromophenol blue with (reducing) or without (non-reducing) 10% β-mercaptoethanol. The samples were heated for 5 min at 100 ºC. Poly-

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acrylamide gels of 15% (w/v) were used. Coomassie brilliant blue staining revealed the protein bands.

Immunoblotting analysis was performed by the method of Towbin *et al.* [17]. Briefly, after protein transference the nitrocellulose membrane was blocked with 5% dry skim milk (Unipath LTD, Hampshire, England) in PBS for 1 h at 37 °C. The membrane was exposed to the anti-HBcAg monoclonal antibody (AcM22) conjugated to HRP (CIGB, Havana, Cuba), diluted 1:200 in PBS-T. After washing with PBS-T, the membrane was revealed with 3,3'- diaminobenzidine (Sigma Co., ST Louis, USA).

Immunoassay for anti-HBcAg detection

The antigenic property of the purified antigen was evaluated by ELISA. Microtitration plates (96 wells/plate, Nun Inc., Denmark) were coated with 3.5 µg/mL of purified HBcAg in PBS. The coating was carried out at 45 °C for 90 min. Serum samples (100 µL/well) were diluted 1:20 in PBS, 2% BSA and 2.5 mM DTT. Plates were incubated for 1 h at 37 °C, and washed four times with 200 µL/well of PBS+T. A mixture of protein A/HRP and human anti-IgM monoclonal antibody/HRP (CIGB, Havana; 100 µL/well) was added, and plates were incubated for 1 h at 37 °C. The substrate buffer was added and the reaction was stopped as in the previously described ELISA in the isopycnic gradient topic. A panel of 452 negative sera from volunteer blood donors was used to evaluate the specificity of the assay. Ninety six positive sera were used to assess sensitivity. All sera were previously tested by the radioimmunoassay CIAE SPRIA anti-HBcAg (Beijing, China), according to instructions.

Results

Cloning and protein expression

The HBcAg gene was amplified by PCR using specific primers and cloned in the pRIV-2 *E. coli* expression vector. SDS-PAGE analysis of disrupted W3110 *E. coli* cells shows a protein band at the expected 22 kD size in the induced clones. The expression level was about 7% of the total *E. coli* proteins (Figure 1A). Specificity of the protein was confirmed by Western blot using anti-HBcAg AcM22 (Figure 1B).

Ammonium sulphate precipitation

Initial two-step precipitation with ammonium sulphate is shown in Figure 2. At the first 10% saturation step a considerable amount of *E. coli* contaminant proteins precipitated, while HBcAg was still soluble in the supernatant. After the final 40% saturation step HBcAg was obtained in the precipitate. Using this stepwise precipitation procedure, the HBcAg is enriched from the initial 7% to 35% purity (Figure 2).

Chromatography on sepharose CL-4B

Partially purified HBcAg was chromatographed on a Sepharose CL-4B column (Figure 3A). In the first elution peak (fractions 10-16) three *E. coli* contaminants are mainly eluted and well separated from the HBcAg elution region (fractions 23-38), which was pooled and considered to be the purified HBcAg. Figure 3B shows the immunoblotting, where not only HBcAg was recognized, but also a degradation product can be observed.

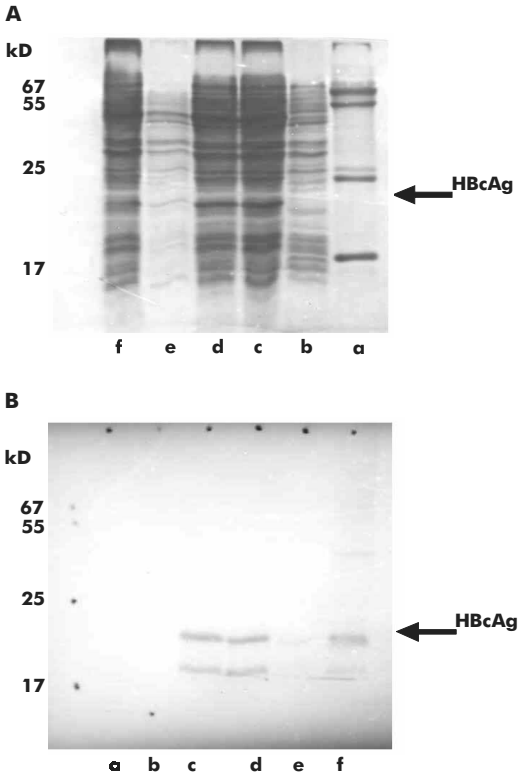

Figure 1. SDS-PAGE (A) and Western blot (B) analysis of HBcAg expression in W3110 *E. coli*. Lanes: a, molecular mass markers (66 kD BSA, 55 kD immunoglobulin heavy chain, 25 kD immunoglobulin light chain, 14.4 kD lisozyme); b, wild type W3110 *E. coli*; c, transformed W3110 expressing HBcAg; d, soluble fraction after sonication; e, insoluble fraction after sonication; f, soluble fraction after sonication assayed under non-reducing conditions.

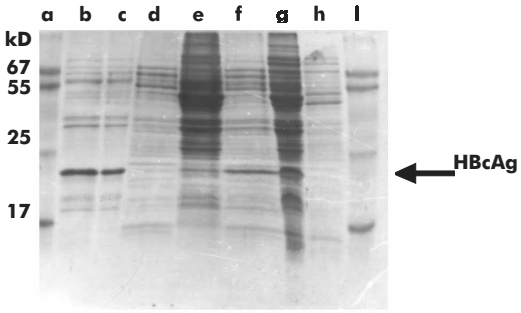

Figure 2. SDS-PAGE analysis of the two-step ammonium sulfate precipitation (equal volume of 10 µL of each fraction obtained was applied on the gel). Lanes: a,i, molecular mass markers; b,c, pellet of 40% saturation; d, supernatant of 40% saturation; e, pellet of 10% saturation; f, supernatant of 10% saturation; g, soluble fraction after sonication; h, wild type W3110 *E. coli*.

Also, above the HBcAg there are some weak bands that were recognized by the anti-HBcAg AcM22. These bands are probably the result of an insufficient monomerization, similar to that observed in Figure 1B, lane f, when the sample was applied under non-reducing condition. The final purified protein had 90% pu-

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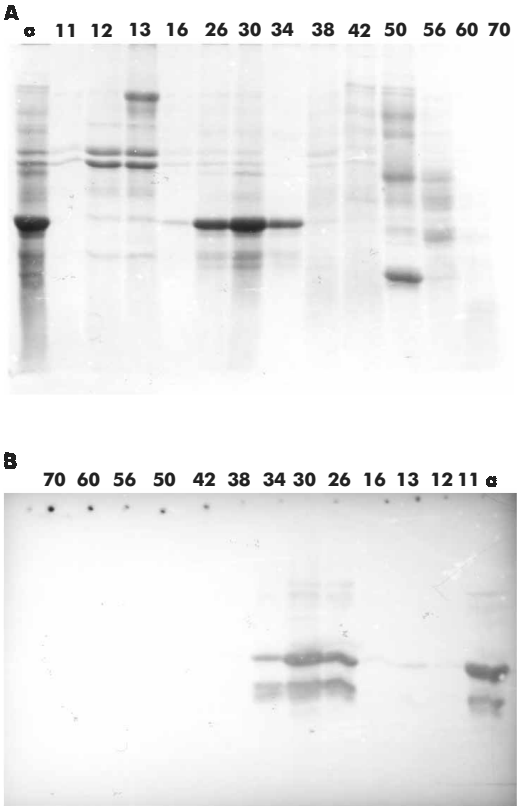

Figure 3. SDS-PAGE (A) and Western blot (B) analysis of the fraction eluted from chromatography on sepharose CL-4B column. Lane: a, protein sample obtained in the 40% saturation pellet. Numbers indicate the chromatography fraction order.

rity according to the data obtained by the Molecular Analyst program (Bio-Rad Laboratories, USA) and the stepwise recoveries that are summarized in the Table. Total protein concentration in the samples was determined by the method described by Bradford [18], using BSA as the standard curve.

When the purified HBcAg was analysed by SDS-PAGE using non-reducing conditions with or without preheating, HBcAg and its degradation products were not able to enter the gel (Figure 4). Preheating in the absence of the reduction agent was not sufficient for the disruption of HBcAg particles.

Isopycnic CsCl gradient

The fact that HBcAg under non-reducing condition does not enter the gel suggests the polymeric nature of the expressed HBcAg. To demonstrate that purified HBcAg assembled into a capsid, the isopycnic CsCl gradient

Table 1. Purification steps and the recovery of HBcAg.

	Total protein (mg)	HBcAg (mg)	Purity (%)	Purification (fold)	Recovery (%)
Extraction	160	12	7.5	1	100
Saline precipitation	18	6.3	35	4.6	52
Purified HBcAg (sepharose CL-4B)	6.2	5.6	90	2.6	47

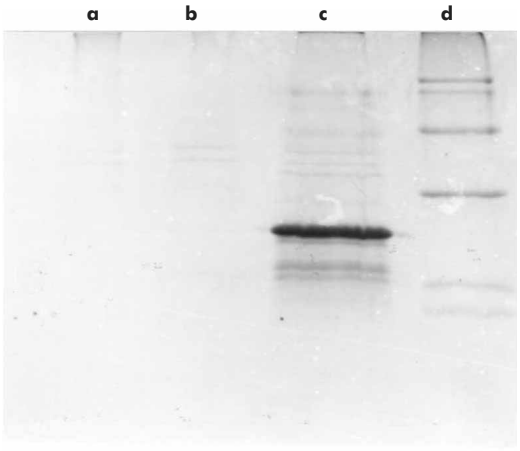

Figure 4. SDS-PAGE of the final purified HBcAg assayed under non-reducing condition. Lanes: a, HBcAg under non-reducing conditions without preheating; b, HBcAg under non-reducing condition with preheating; c, HBcAg under reducing conditions; d, low molecular weight markers (Amersham Pharmacia Biotech, Sweden).

was fractionated and each fraction was assayed by ELISA for HBcAg detection. The maximum optical density was obtained at a 1.36 g/mL density (Figure 5), which agrees well with the range of previously reported densities (1.36 g/mL-1.39 g/mL) [19 , 20].

Immunoassay for anti-HBcAg detection

Purified HBcAg was evaluated in an ELISA format, in order to demonstrate its correct antigenic properties and its potential use in the diagnosis of HBV infection. The analysis of distribution fitting for the whole negative population (452 sera) revealed a log-normal fit (Chi-Square: 21.59, df =16, p = .156). The cut-off line of the ELISA was established as the value representing a 99 percentile of giving a negative distribution. Three out of 452 negative sera were positive according to our ELISA and among the positive sera, two were classified as negative.

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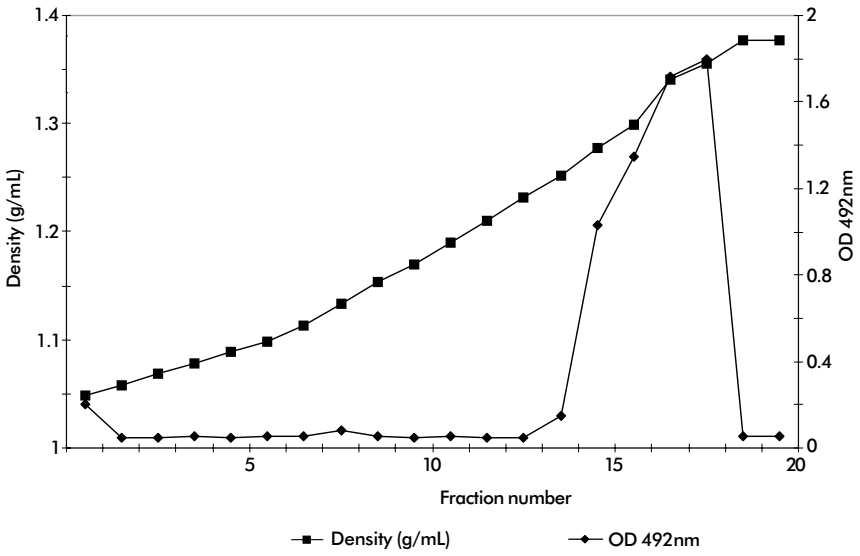

Figure 5. ELISA for the detection of HBcAg in fractions obtained after the CsCl gradient.

Discussion

Initially HBcAg was prepared by isolating Dane particles (42 nm HBV virion) present in the plasma of HBsAg positive donors and then removing the surface antigen by contacting the Dane particle to a nonionic surfactant [21]. This methodology resulted expensive with a high biological hazard. The genetically engineered production of HBcAg in bacteria [3] or in yeast [22], have proposed a suitable way to obtain enough of the recombinant HBcAg for morphological and biophysical studies and also for the development of diagnosis systems for HBV infection.

Different protocols have been used to purify HBcAg expressed in *E. coli*. Most of these protocols are laborious and involve a combination of different conventional purification steps, e.g. multiple ammonium sulfate precipitation, gel filtration, ultracentrifugation gradients on sucrose or CsCl and generally no data of the overall recovery are shown [3-5]. In our work a modified process that only includes two steps: ammonium sulfate precipitation and gel filtration, was used. To clarify the protein extracted after cell disruption, ammonium sulphate precipitation at a high concentration (40-45% saturation), has been successfully used [3, 23]. In our process, we standardized two sequential precipitation steps using ammonium sulfate at 10% and 40% saturation, that enriched 4-5 fold the initial purity of HBcAg with an overall yield of 45% (Figure 2).

After ammonium sulfate precipitation, partially purified HBcAg was applied to gel filtration chromatography on the Sepharose CL-4B column. Several studies have reported that HBcAg, when expressed in *E. coli*, assembles into spherical shells resembling those seen in infected liver. Two size variants of icosahedral shells are produced: a smaller capsid composed of 180 monomers with a diameter of 280-320 Å and a larger capsid composed of 240 monomers of 310-360 Å in diameter [4, 24]. The presence of two HBcAg populations explains the wide region obtained for the eluted HBcAg (fractions 23-38) after gel filtration (Figure 3A). There were mainly three *E. coli* proteins eluted in the first peak (Figure 3A, fractions 10-16). These proteins eluted at a volume lower than the HBcAg suggest that they probably form an aggregation product with a larger molecular size than the HBcAg capsid-like particle.

Finally, purified HBcAg is obtained with 90% purity, as estimated by the anti-HBcAg AcM22 recognized bands in the immunoblotting assay (Figure 3B). The observed degradation product could be a result of the proteolytic digestion of 22 kD HBcAg during its synthesis in the cytoplasm of bacteria and before the particles assemble. The effect of HBcAg proteolytic digestion has been published before by other authors that have found that when HBcAg is treated with trypsin a main band of approximately 16 kD is produced, due to the tryptic cleavage site determined by mass spectroscopy between arginine residues 150 and 151 [4, 23]. Other trypsin recognition sites have been identified by Dalseg [25] in the C-terminal arginine cluster of HBcAg. We speculate that HBcAg degradation probably takes place before the particles assemble, since degraded bands are detected in the fractions corresponding to the HBcAg particle of 5-6 x 10^6 Da after gel filtration chromatography, and disappear when the sample is analyzed by SDS-PAGE under non reducing conditions (Figure 4), suggesting that degraded bands together with intact 22 kD HBcAg participate in particle assembly. The possibility that degraded bands could form a part of the assembled particles is also supported by Zlotnick *et al.* and Beames *et al.* [23, 26], who studied the effect of the HBcAg C-terminus on capsid assembly and demonstrated that truncated HBcAg variants containing at least the first 140 amino acids still conserve the ability to assemble into the capsid.

The immune response to the HBcAg mainly recognizes a highly antigenic immunodominant epitope, located by Salfed *et al.* [27] in the linear locus (amino acid residues 78-83) of HBcAg. More recently, Conway *et al.* [28] using data obtained by cryomicroscopy and Wynne *et al.* [29] found that this region resides on the outer rim of the 30 Å long spikes formed at the surface of capsid shells. The formation of the conformational immunodominant epitope depends on a correct particle assembly, which is not present in the dimer and monomer structures. To demonstrate the correct exposure of the conformational immunodominant domain, essential for anti-HBcAg serodiagnosis, an ELISA format was used. Since its introduction, in the middle of the 80's for blood screening, the current serological tests for anti-HBcAg have presented a significant proportion of results, which are non-specific. The problem is especially apparent in populations at low risk for HBV, such as volunteer blood donors [30]. Robertson *et al.* [31] characterized a reduction-sensitive factor from human plasma, responsible for the apparent false activity in competitive assays for antibodies against HBcAg. This reduction-sensitive factor showed an unspecific IgM anti-HBcAg response in donors with no history of exposure to HBV. Weare *et al.* [32] demonstrated that the addition of reduction agents such as sodium metabisulfite, cystein or DTT during sample incubation could selectively eliminate false-positive reactivity and greatly improve the specificity of competitive anti-HBcAg tests. For this reason, DTT was used during sample incubation in our direct anti-HBcAg ELISA in order to obtain improved specificity. The assayed negative and positive panels showed 99.3% specificity and 97.9% sensitivity, respectively. Hence, the above described methodology offers an efficient mean to purify large amounts of recombinant HBcAg, that could be used in the development of diagnostic systems for the detection of HBV infection.

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