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Simple immunoaffinity method to purify recombinant hepatitis B surface antigen secreted by transfected mammalian cells

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Abstract

Purification of recombinant hepatitis B surface antigen (*rec*HBsAg) produced in a stable Chinese hamster ovary (CHO) cell line was evaluated using Linx Affinity Purification System (Invitrogen, USA). To purify HBsAg secreted by this cell line, a murine monoclonal antibody (MAbAH1) raised against native HBsAg was used. The purified AH1MAb was conjugated with phenyldiboronic acid (PDBA) and immobilized on the immunoaffinity chromatographic support. Using an optimized protocol the affinity column was able to purify *rec*HBsAg from supernatant of mammalian cells cultures with more than 80% purity. This method showed to be simple and quicker than the current ultracentrifugation methods. The method is also efficient and economical in obtaining purified *rec*HBsAg.

Keywords: Purification; Recombinant hepatitis B surface antigen

1. Introduction

The envelope of hepatitis B virus (HBV) contains three proteins encoded by the S gene known as major, middle, and large proteins [1]. Each of these proteins is present in two forms: the major protein exists in a nonglycosylated (P24) and glycosylated (GP27) form [2]; the middle protein is a glycoprotein carrying one (GP33) or two glycans (GP36) [3–5]; and the large protein is present in a nonglycosylated (P39) and glycosylated (GP42) form [5]. The S gene has been expressed in a number of different systems such as procaryotic cells [6], yeast [7], mammalian cells [8], insect cells following infection with recombinant baculoviruses [9] and plants [10]. Methods describing purification of

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HBsAg obtained from plasma have been described in the literature since 1975 [11–13]. Particles are present in human plasma in higher levels than in the supernatant of mammalian cells expressing them; all the methods cited above describe purification of HBsAg from plasma or yeast that represent a source of huge quantities of particles in contrast with mammalian cells that express HBsAg particles to the supernatant in very small quantities. Purification of recombinant HBsAg (*rec*HBsAg) particles produced by stably transfected mammalian cell cultures until now has been achieved by ultracentrifugation [14], column chromatography and ultracentrifugation [15] but not by immunoaffinity chromatography. Immunoaffinity chromatography with monoclonal antibodies (MAbs) has been widely used for the purification of proteins. It has proved to be a powerful tool in several purification procedures, mainly because of the high selectivity of this technique.

The present paper describes a purification method for the isolation of *rec*HBsAg from supernatant of CHO cells containing 5% of fetal serum bovine, using a commercial immunoaffinity system-Linx Affinity Purification System produced by Invitrogen (USA). The purity, immunogenicity and protein recovery were analysed. The method presented here is based on the description of an affinity purification technique, which is suitable for small quantities of protein, is rapid and involves one single step of purification in contrast to the other methods that describe two steps.

2. Experimental

2.1. Materials

All chemicals used in this work were of analytical-reagent grade and purchased from Merck (Darmstadt, Germany). Tween 20 was obtained from Sigma (USA).

2.2. Construction of pDM14 vector

A preS2/S sequence was amplified from HBV viral DNA, genotype D, subtype *ayw3*, extracted from an HBV chronic carrier using primers PS2-S1 [16,17]. The PCR product was cloned into pCR2.1 TOPO vector (Invitrogen, USA) and subcloned into a mammalian cell expression vector pcDNA 3 (Invitrogen, USA) in the polylinker region after digestion of both vectors with endonuclease *Eco*RI. The pcDNA3 vector containing preS2/S HBV sequence was named pDM14 and is under the control of a CMV promoter.

2.3. Transfection of CHO cells

CHO cells were transfected with pDM14 vector previously purified by ultracentrifugation in a CsC1 gradient according to Sambrook and Russell [18]. A cellular clone which secretes HBsAg particles in the supernatant of CHO cultures was selected by enzyme-linked-immunosorbent assay, using Hepanostika HBsAg uniform II (Organon Teknika, The Netherlands).

2.4. Collection and concentration of recHBsAg particles

CHO cell cultures producing *rec*HBsAg were grown in roller bottles (890 cm^2 in surface area, Corning, USA) using 50 ml of Earle's 199 medium (Gibco, USA) supplemented with 5% (v/v) fetal bovine serum per bottle. Five days after cells reached confluence the supernatant was collected and concentrated 50-fold by precipitation. An effective precipitation was accomplished by slowly adding 10% (w/v) PEG powder (6000–9000) while stirring at room temperature for 30 min and leaving the resulting admixture overnight (O.N.) at 4 °C. The admixture was then centrifuged at 10 000 *g* for 30 min and the pellet was dissolved in a minimum volume of phosphate-buffered saline (pH 7.4, PBS). Concentrated *rec*HBsAg was dialyzed against water before using.

2.5. Optimization of elution step of the recHBsAg purification process

The optimization of elution step of the *rec*HBsAg purification was performed as described previously [13] with slight modifications. A polystyrene microtiter plate (Nunc, USA) was coated with 100 μl

per well of 10 μg/ml AH1 MAb in 0.1 *M* sodium hydrogen carbonate buffer (pH 9.6) and incubated overnight at 4 °C. MAb solution was removed and the plate was washed three times using 0.05% Tween 20 in PBS. Subsequently 100 μl per well of 200 ng/ml purified *rec*HBsAg diluted in 0.05% Tween 20 in PBS was added. After incubation for 1 h at 37 °C the plate was washed in the same manner as described before and elution from the plate was carried out by adding 100 μl per well of different elution buffers: (a) 3.5 *M* $MgC1_2$, (b) 20 m*M* MOPS, (c) 0.1 *M* $NaHC_3$ pH 11.2, (d) 3 *M* KSCN and (e) 3 *M* NaSCN. After incubation for 5 min the plate was washed five times in the same manner as described before and incubated at 37 °C for 1 h with 100 μl of anti-HBs peroxidase-conjugate produced in-house. After washing, the color was developed using 100 μl of 0.05% *o*-phenylenediamine and 0.015% hydrogen peroxide in citrate buffer (pH 5.0). The reaction was stopped after 20 min with 50 μl of 1.25 *M* H_2SO_4. The optical densities were measured in a Multiskan system (Titertek, Helsinki, Finland) at 492 nm.

2.6. Purifications of recHBsAg by affinity

MAb AH1 was conjugated with PDBA as recommended by the manufacturer. To determine the conjugation number, the conjugation reaction was desalted by gel filtration chromatography using a PD-10 desalting column (Pharmacia Biotech, Sweden). Calculation of conjugation number was performed as recommended by the manufacturer by removing unreacted PDBA–NHS. The coupling was performed by adding all the PDBA–AH1MAb conjugation reaction to the column and by equilibrating with washing-buffer containing 0.1 *M* sodium bicarbonate, pH 8.0. After incubation for 30 min at room temperature the column was exhaustively washed and the flow-through analysed for the presence of ligand using an appropriate assay according to the manufacturer instructions. The *rec*HBsAg obtained by concentration on step 2.4 diluted 5-fold in washing-buffer containing 0.5 *M* NaCl was filtered on 0.45 μm Millex-HA filters and loaded into the column. A portion of the diluted *rec*HBsAg was saved before purification for further analysis. The column was incubated for 30 min at room temperature and the flow-through collected for further analysis. The wash was performed by using washing-buffer containing 0.5 *M* NaCl followed wash buffer without NaCl. The *rec*HBsAg was eluted by adding small volumes of elution buffer containing 0.1 *M* sodium bicarbonate (pH 11.2). Six fractions of 1 ml (time after time) were collected and analysed. Analyses were performed by enzyme immunoassay (Organon, Teknika, The Netherlands) in order to determine antigen recovery and by SDS–PAGE to evaluate purity.

2.7. Evaluation of recHBsAg recovery and purity

The concentration of *rec*HBsAg recovery was determined by enzyme immunoassay (Organon Teknika, The Netherlands). The amount of *rec*HBsAg before and after the purification process was calibrated against an international panel containing different dilutions of standard HBsAg (Boston Biomedica, USA). Gels stained with Coomassie Blue containing purified bands of *rec*HBsAg were quantified using Image Master 1D (Pharmacia Biotech).

2.8. Protein sequence analysis

The sequencing experiments were performed according to De Simone et al. [19]. The *rec*HBsAg was subjected to SDS–PAGE (12%) and the protein blotted onto a polyvinylidene fluoride membrane (0.2 μm, Bio-Rad). After electroblotting the membrane was stained for 5–10 min with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and destained with 60% (v/v) methanol. N-terminal amino acid sequence analysis was carried out on a Model PSQ-1 gas-phase protein sequence system, consisting of an Edman reaction unit, an on-line phenylthiohydantoin (PTH)-amino acid analyzer and C-R4A chromatogram integrator (Shimadzu, Kyoto, Japan). Edman degradation was performed according to the standard program supplied by Shimadzu. The released PTH-amino acid derivatives were identified using an on-line HPLC system (Model 6A) with a Wako-Pak WS-PTH column (250×4.6 mm I.D.) (Wako, Osaka, Japan).

2.9. Preparation and characterisation of MAb to HBsAg

Inbred adult, female, BALB/c mice were immunized twice subcutaneously with HBsAg particles purified from a human serum infected with an *ayw*[2] serotype as described previously [20]. The first immunization was with 2.5 μg protein using incomplete Freund's adjuvant. Four weeks later the animal was boosted intravenously with 7 μg of protein. After 4 days the mouse spleen cells were fused with murine myeloma cells using 40% (w/v) polyethylene glycol 1500 [21]. The resulting clones were screened for the production of anti-HBs by enzyme immunoassay (Organon Teknika, The Netherlands). Anti-HBs antibody-producing cells were cloned twice by limiting dilution and ascitic fluids were produced by i.p. inoculation of nude mice with $5–10\times10^5$ hybridoma cells. The subclass of the MAb was determined by enzyme immunoassay, using anti-subclass antibodies coated on polystyrene microtiter plate and detection of bound MAb was achieved with HRP-conjugated goat anti-mouse immunoglobulin. The AH1 MAb IgG1 subclass was selected to *rec*HBsAg purification process.

2.10. Purification of anti-HBs antibodies using the Affi-prep protein A MAPS ®II buffers

Polyclonal antibodies developed against a recombinant yeast vaccine (TGP-943), expressing middle S of an *adr* serotype has been characterized previously [20]. Anti-HBs polyclonal and MAb AH1 monoclonal antibodies were purified by Affi-Prep protein A MAPS® II buffers (Bio-Rad, USA) as recommended by the manufacturer. They were diluted 1:2 in binding buffer MAPS II (BioRad, USA) and filtrated in 0.45-μm Millex-HA filters. Protein-A column was washed and equilibrated with the same binding buffer. The diluted antibodies were applied and the flow-rate was adjusted to 1.5 ml/min. The column was washed once more with binding buffer and 15 fractions of 1.0 ml were eluted with elution buffer MAPS II (BioRad, USA). The antibody-containing fractions were detected by reading the optical density at 280 nm. The purity of antibody fractions was checked by SDS–polyacrylamide gel electrophoresis.

2.11. Determination of protein concentration

Protein concentration was determined by a modified Lowry method [22] using bovine serum albumin (BSA) as a standard.

2.12. SDS–PAGE and Western blotting analysis

Electrophoresis was run in 12.5% gels according to Laemmli [23]. The gels were stained with Coomassie Brilliant Blue R-250.

For Western blot analysis after electrophoresis, non-stained gels were transferred for 20 min at 16 V to a 0.22-μm nitrocellulose membrane (BioRad, USA) using a semi-dry system (BioRad, USA). The membranes were incubated with blocking reagent (5% milk in PBS) at room temperature for 2 h or O.N. at 4 °C and subsequently processed for 1 h with anti-HBs polyclonal antibody or normal rabbit serum (control membrane) diluted in 10 m*M* Tris–HCl (pH 8.0), 0.15 *M* NaCl and 0.05% Tween 20. After washing out primary antibody, membranes were processed with an anti-rabbit HRP-conjugated immunoglobulin (Sigma, USA). Reacting bands were detected by 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, USA).

3. Results and discussion

3.1. Obtention of recHBsAg particles from supernatant of CHO cultures

The generation of stably transfected clonal CHO cells, expressing and secreting reasonable quantities of *rec*HBsAg particles has been described [24–26]. Recombinant particles are being widely used in the development of vaccines that illicit better responses. For this purpose the expression model using mammalian cells is ideal due to fidelity in originating particles similar to those originated during a natural infection. Our main objective was to obtain *rec*-HBsAg with a reasonable degree of purity for use in enzyme immunoassay for detection of antibodies anti-HBs in patient sera through a fast immunoaffinity method using an antibody that was not previously characterized for epitope specificity.

Due to the low expression level of recombinant

particles when compared with other expression systems as yeasts and bacteria, the use of a protocol of concentration of recombinant particles contained in the supernatant of CHO cells was necessary. The conditions for protein precipitation by PEG were optimized for the culture medium originally containing 5% fetal bovine serum. The precipitation protocol [15] used was chosen in order to achieve maximum recovery of *rec*HBsAg and minimum contamination, particularly with BSA, the major contaminant in this case. We have examined the difference in recovery using whether PEG or ammonium sulphate. The results were similar. However we elected PEG precipitation in this process because the stability of *rec*HBsAg was affected by ammonium sulphate. Also some features regarding the stability of *rec*HBsAg were analyzed (results not shown). Storage at 4 or −20 °C of *rec*HBsAg purified solution in PBS preserved the antigenicity for several months. A relative loss was observed after 1 year of storage at 4 °C (25%) and −20 °C (15%). We have lyophilized non-purified *rec*HBsAg supernatant to use as a positive control of HBsAg detection kit produced by BioManguinhosFiocruz. This procedure has also showed sufficient stability for several months.

The analysis of supernatant samples containing *rec*HBsAg particles, before and after the precipitation process with PEG 6000–9000, using the enzyme immunoassay commercial kit showed a recovery of *rec*HBsAg of about 80%. Although the *rec*HBsAg loss in the pellet has been probably due to interactions formed among some *rec*HBsAg molecules with BSA present in the fetal serum as already described elsewhere [27], almost all BSA was retained on the discarded supernatant (Fig. 1). The reproducibility of the precipitation method selected was evaluated in three pilot assays using the conditions of precipitation as described previously [15]. We considered that minor variations found between the assays were not significant (Table 1).

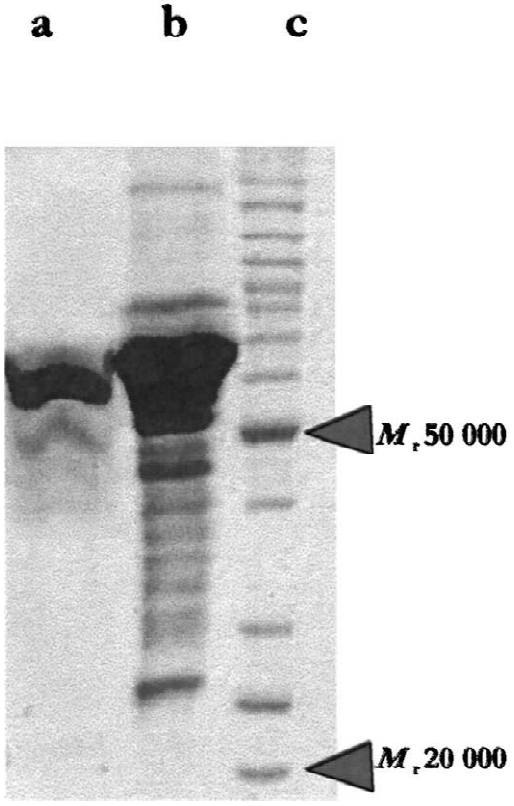

Fig. 1. SDS–PAGE gel analysis of *rec*HBsAg supernatant after precipitation with PEG. (a) Supernatant fraction after PEG precipitation and centrifugation; (b) starting material; (c) molecular mass marker BenchMark protein ladder (Invitrogen, USA). Approximately 20 μg of protein were applied per sample.

3.2. Purification of recHBsAg using Linx Affinity Purification System

We have standardized a purification system to be applied in the supernatant of CHO cells containing *rec*HBsAg. Initially it was necessary the production and purification of the specific antibody, which was conjugated and coupled to the affinity resin. The Linx Affinity Purification System is based on the specific interaction between two families of molecules named phenyldiboronic acid (PDBA, a bivalent derivate of phenylboronic acid (PBA)) and salicylhydroxamic acid (SHA). PDBA presents greater avidity for SHA and the system uses the PDBA–NHS ester to derive a protein with primary amine groups. The PDBA-conjugated protein is then bound to SHA-resin. The key to successful generation of an affinity column using this system is the reaction condition during conjugation of PDBA to the ligand as well as the binding of PDBA-conjugated ligand to SHA-resin. The determination of the conjugation number was a way to check for success-

Table 1
Evaluation by enzyme immunoassay of *rec*HBsAg recovery after precipitation process in three pilot assays

Assay number	Starting material	Supernatant fraction after PEG precipitation and centrifugation	Pellet fraction after PEG precipitation and centrifugation	*rec*HBsAg recovery (%)
01	0.339	0.070	0.301	89
02	0.342	0.069	0.277	81
03	0.347	0.066	0.247	71

Specific absorbance values shown in columns 2, 3 and 4 were measured at 450 nm. One hundred microlitres of each sample from a final volume of 80 ml were used for analysis.

ful conjugation. Depending on the number of exposed lysines and on the molar input ratio, a good conjugation number is between 4 and 12. We found a conjugation number of 10 as satisfactory. In order to check for successful coupling of PDBA–AH1MAb to SHA-resin the absorbance at 280 nm of flow-through was determined before and after adding all the PDBA–AH1MAb conjugation reaction. Less than 1% of total protein was recovered showing an effective coupling. With regard to PDBA–SHA binding stability we decided to use 0.1 *M* sodium bicarbonate, pH 8.0, to process conjugation and coupling because binding rate using this buffer is about 96% according to manufacturer, although other buffers such as tris or glycylglycine could be used. Despite effective conjugation process and coupling of PDBA–AH1Mab to SHA-resin the yield of *rec*HBsAg purified using Linx Affinity Purification System calculated as described on step 2.10 was 42% and the purity almost 90% (Table 2). A significant loss of *rec*HBsAg was found mainly in the non-bound fraction. Because AH1MAb is a monoclonal antibody with specificity to *ayw*^{2} subtype [17] and *rec*HBsAg is from *ayw*^{3} subtype we believe that the use of new monoclonal antibodies that demonstrate greater specificity to *rec*HBsAg purified by Linx Affinity Purification System could probably solve this problem. We are characterizing other monoclonal antibodies produced in our laboratory, but due to the great diversity of determinants of HBsAg, this work is laborious and time-consuming and it was not possible to select an *ayw*^{3} antibody to confirm that the difference in subtypes could be responsible for low yields.

The second step was to choose the best elution buffer to be used in the Linx Affinity Purification System. In the screening for appropriate elution buffers using 96-well plates 100 m*M* $NaHCO_3$ pH 8.0 showed the best elution efficiency, recovering 100% of bound antigen and was chosen as the elution buffer to be used in this assay. The elution buffers 3 *M* KSCN and 3 *M* NaSCN were found to interfere with the commercial enzyme immunoassay, in spite of well-known HBsAg particle stabilizer [28].

The results obtained after this purification procedure are shown in Fig. 2. The SDS–PAGE analysis after antigen purification with 100 m*M* $NaHCO_3$, pH 8.0, showed a band of M_r 27 000 with N-terminal sequence corresponding to the small HBsAg (M-E-N-I-T-S-G-F-L). We could also observe an additional band of higher molecular mass (M_r 50 000) in all preparations (Fig. 2). The possibility of contamination of the purified *rec*HBsAg with AH1MAb had been eluted during the purification process and consecutively was investigated by using an enzyme

Table 2
Purification results of *rec*HBsAg for three assays using Linx Affinity Purification System

Purification (number)	Amount (mg)				
	Applied	Non-bound	Washing	Elution	*rec*HBsAg yield (%)
1	1.9	1.00	0.03	0.8	42
2	2.9	1.50	0.03	1.3	44
3	3.6	1.77	0.09	1.5	42

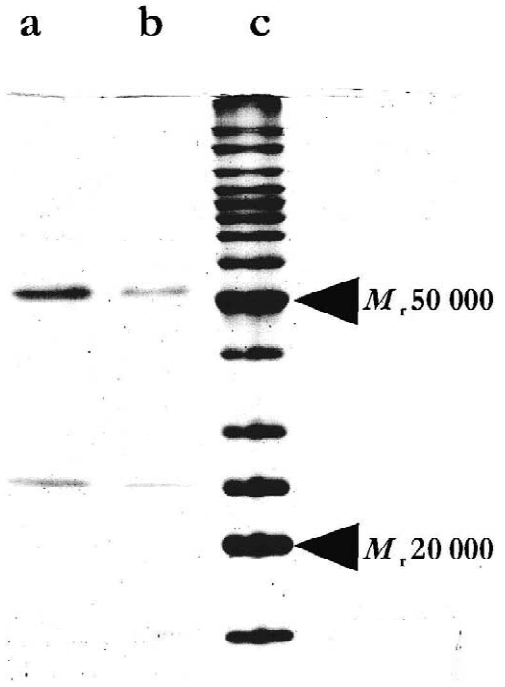

Fig. 2. 12.5% SDS–PAGE under reducing conditions of *rec*-HBsAg purified using Linx Affinity Purification System. (a,b) Eluted fractions with approximately 20 and 10 μg of protein, respectively; (c) molecular mass marker BenchMark protein ladder (Invitrogen, USA).

immunoassay (Organon). We have not found anti-HBs in the purified *rec*HBsAg. The reducing conditions used in the affinity-purified system may favour the formation of band of HBsAg multimers, as described previously [13,29]. The specificity of purified *rec*HBsAg was evaluated by Western-blotting analysis using anti-TGP polyclonal serum, which reacted only with *rec*HBsAg. There was not reaction with *rec*HBsAg when a normal rabbit serum was used confirming the specificity of purified *rec*HBsAg (Fig. 3).

For the production of a kit for detection of antibodies anti-HBs in patient sera it was necessary to develop a purification process in average scale, fast and simple and that could replace classic methods such as ultracentrifugation in sucrose or cesium chloride (CsCl) gradients that are time consuming delicate procedures. On the other hand this affinity purification method can be completed in 1 h and requires little sophisticated manipulation. In substitution to the kit of the Invitrogen Linx Affinity we have used the Versalinx affinity purification system (Prolinx, USA) that uses the same purification strategy. The efficiency of the chromatography affinity method to purify *rec*HBsAg from supernatant of mammalian cells containing BSA was confirmed.

3.3. Purification of antibodies anti-HBs

The AH1 MAb IgG1 subclass was tested using the enzyme immunoassay commercial kit (Organon) and showed specific activity up to 1:64 000 dilution before the purification process. The AH1 MAb was purified from ascitic fluid by affinity chromatography using Affi-Prep protein A MAPS II buffers (Bio-Rad, USA). This system consists of highly purified protein A covalently coupled to an unique macroporous polymer matrix. The utility of protein purification for murine monoclonal antibodies has been limited because, according to published methods, IgG1 retention represents a significant purification problem. Standard protocols result in highly purified products but in a low yield for IgG1 subclass. In agreement with the manufacturer the MAPS buffer system for protein A affinity chromatography was developed to optimize the binding and recovery of many immunoglobulins, especially mouse monoclonal antibodies subclass IgG1.

The purity of the final AH1 MAb preparation was over 90% as assessed by SDS–PAGE stained by Coomassie Blue densitometry. Only two bands of a molecular mass (M_r) 50 000 and 25 000 were detected corresponding, respectively, to the heavy and light chains of IgG antibody molecule (Fig. 4). Similar results were obtained for anti-TGP polyclonal antibody.

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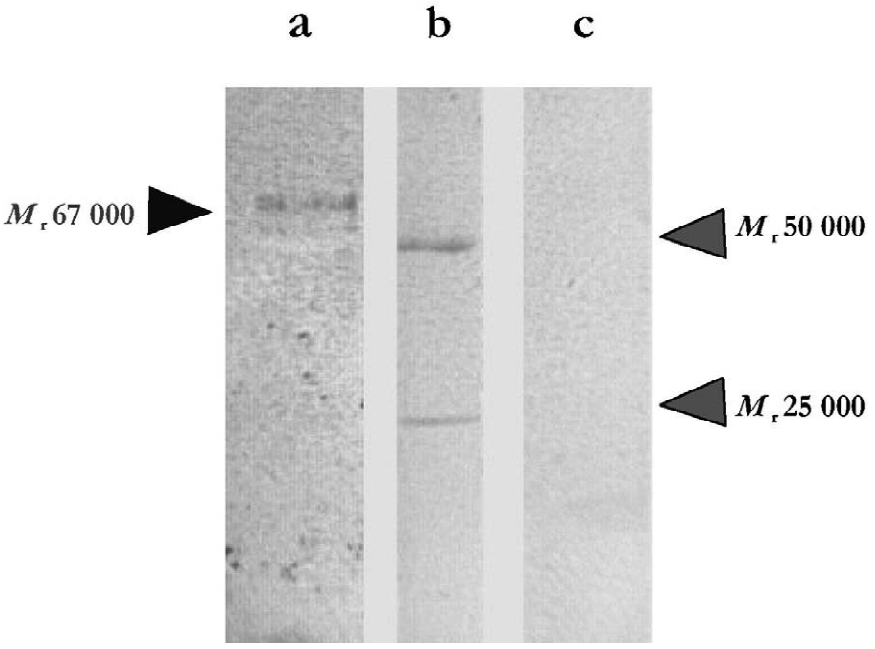

Fig. 3. Western-blotting analysis of purified *rec*HBsAg. (a) Commercial bovine albumin (Sigma, USA) reacted with non-purified normal rabbit serum (control); (b,c) *rec*HBsAg purified using Linx Affinity Purification System, respectively, reacted with anti-TGP polyclonal antibody and reacted with non-purified normal rabbit serum.

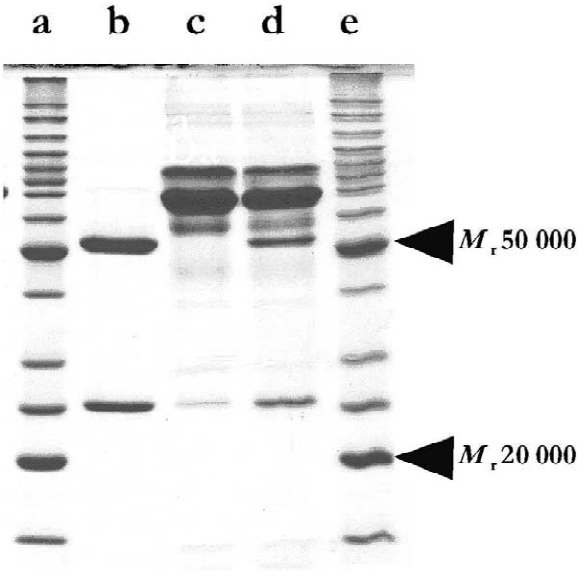

Fig. 4. SDS–PAGE (12.5%) under reducing conditions of AH1 MAb purified using protein A resin. (a,e) Molecular mass marker BenchMark protein ladder (Invitrogen, USA); (b) eluted fraction; (c) non-bound fraction; (d) starting material. Approximately 20 μg of protein were applied per sample.

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