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Short communication

Comparison of different elution conditions for the immunopurification of recombinant hepatitis B surface antigen

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Abstract

An immunoaffinity chromatographic method was developed using a mAb immunosorbent to purify recombinant hepatitis B surface antigen (r-HBsAg) from yeast. Elution conditions using a mAb-coated ELISA were improved to select the best conditions to purify r-HBsAg. The optimum results in terms of total quantitative recovery were obtained using 20 m*M* Tris pH 11.6. An increase in the CB.Hep-1 mAb (anti-HBsAg) useful immunosorbents half-life and in its yield per cycle was obtained when alkaline elution conditions were used. Moreover, the basic conditions do not affect either the antigenic characteristics or the purity or the molecular integrity of r-HBsAg.

Keywords: Immunoaffinity; Recombinant hepatitis B surface antigen

1. Introduction

Hepatitis B surface antigen (HBsAg), the envelope of the hepatitis B virus (HBV), is a macromolecular structure composed of proteins, carbohydrates and host-derived lipids [1]. A sequence of steps to purify r-HBsAg has been previously described [2]. Because of the excellent immunogenicity of these particles, they have become the basis of the first human recombinant vaccine licensed for widespread use [3,4]. Isolation and purification of HBsAg with specific monoclonal antibodies (mAbs) has also been described [5–8].

Immunoaffinity chromatography (IAC) is a separation method based on specific and reversible interactions between an antigen and a matrix bound antibody [9]. This method has proved to be an extremely powerful technique and has been increasingly successful since the advent of mAbs [10].

The limitations for this otherwise ideal process are generally centered around the dissociation stage, after the ligand has bound onto the affinity matrix. All variables in the association stage and affinity matrix are controllable and well understood. However, the same can not be claimed for the dissociation stage. In general, the efficiency of dissociation and recovery of immunoaffinity-bound ligands depends on the balance between specific activity (SpAct) and total recovery of the product. A gentle dissociating agent may give a high yield but, because of partial denaturation, a low SpAct.

Various reagents and conditions for elution immunoaffinity-bound ligands have been reported [11–15]. With a few exceptions, selection of elution conditions has been empirical, balancing the stability

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of the antigen and the antibody against those conditions, which provides effective elution.

The aim of the present work was to select the optimum dissociating reagent to immunopurify r-HBsAg.

2. Experimental

2.1. Source of r-HBsAg

A recombinant strain of *Pichia pastoris* (C-226) was fermented in a saline medium supplemented with glycerol and its expression was induced with methanol [16]. The r-HBsAg was recovered and submitted to initial purification steps as previously described [17]. A material with 10–25% purity was used as a starting material for immunoaffinity experiments.

2.2. Monoclonal antibody

The CB.Hep-1 mAb (IgG2b) was generated by standard fusion procedures after immunization of Balb/c mice with purified HBsAg, obtained from plasma of chronic HBV carriers [18]. The antibodies were purified from ascitic fluid by Protein A affinity chromatography [19].

2.3. Immunoassay with analyte recovery

A polystyrene (PE) microplate (Costar, Cambridge, USA) was coated with 1 μg per well of CB.Hep-1 mAb in 0.1 *M* sodium hydrogencarbonate buffer overnight at 4°C. The wells were washed using 0.05% Tween 20 in 10 m*M* phosphate-buffered saline solution (PBS-T), and after blocking for 1 h at 37°C with 1% non-fat milk, 0.5 μg/well of purified r-HBsAg was added and the plate was incubated for 1 h at 37°C.

After three washings with PBS-T the plate was incubated for 15 min at 37°C with the following elution reagents: (a) 20 m*M* Tris pH 11.6; (b) 1.0 *M* Na_2CO_3/0.5 *M* NaCl pH 11.6; (c) 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA pH 7.0; (d) 0.2 *M* glycine pH 3.5; (e) 4.5 *M* $MgCl_{2;}$ (f) 8 *M* Urea pH 7.0 (positive control) and (g) PBS pH 7.0 (negative control). The plate was washed thrice and was incubated for 1 h at 37°C with the biotin-labeled CB.Hep-1 mAb.

After three washings the wells were incubated with 100 μl of horseradish peroxidase (HRP)-streptavidin conjugate (Sigma Chemical Co., St. Louis, USA). The reaction was revealed using 100 μl/well of 0.05% orto-phenylenediamine (OPD) and 0.015% hydrogen peroxide (H_2O_2) in citrate buffer (pH 5.0). After 20 min the reaction was stopped with 50 μl/well of 1.25 *M* H_2SO_4. The absorbance was measured in a Multiskan ELISA reader (Labsystems, Helsinki, Finland) using a 492 nm filter. The elution percentages (P) were evaluated according to the following formula:

$$P = [100 - (O - Q/R - Q)] \times 100$$

where O is the absorbance at 492 nm, Q is the absorbance without r-HBsAg and R is the absorbance of the negative control (wells eluted with PBS).

2.4. Column immunoaffinity chromatography

Sepharose CL-4B (Pharmacia-LKB, Uppsala, Sweden) was activated by the CNBr method [20] and supplied by Dr. Rodés (Department of Solid Surface Chemistry, CIGB). The CB.Hep-1 mAb was coupled as recommended by the manufacturer [21]. The amount of coupled antibody was determined measuring the total protein before and after the coupling reaction (about 5 mg/ml of gel for each immunogel). The final products were washed with PBS and stored at 4°C. Packed gel volumes were determined by low-speed centrifugation (250 g for 1 min). Gels were packed into analytical columns (5×0.7 cm I.D., Pierce, Rockford, USA) and equilibrated with 20 m*M* Tris–HCl/3 m*M* EDTA/1 *M* NaCl pH 7.8. The flow-rates were 20 cm/h and 35 cm/h for adsorption and elution, respectively.

The columns were loaded with an excess of a partially purified r-HBsAg preparation according to the previously standardized conditions (2 mg r-HBsAg/5 mg mAb) [22] in the equilibrium buffer containing 1 *M* NaCl. After washing, the bound r-HBsAg was eluted with the following reagents: (a) 20 m*M* Tris pH 11.6 and (b) 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA pH 7.0 (positive control).

Ligand leakage was determined by a sandwich ELISA. Briefly, a plate was coated with a sheep anti-mouse polyclonal immunoglobulin overnight at 4°C, the plate was blocked 30 min at 37°C, the wells were washed and the eluted samples from the immunosorbents were added, the plate was incubated during 3 h at 37°C with 1% non-fat milk in PBS. After washings the plate was incubated with 100 μl/well of a sheep anti-mouse polyclonal immunoglobulin-HRP conjugate (Sigma Chemical Co., St. Louis, USA). The reaction was revealed and stopped as described in Section 2.3.

2.5. High performance size exclusion liquid chromatography (HPLC–SEC).

An analytical column TSK gel-5000 PW (600 mm×7.5 mm I.D., Toso Haas, Japan) with 17 μm pore size was used for characterizing r-HBsAg eluted from the affinity columns. The isocratic chromatographic mobile phase was phosphate buffer adjusted to pH 7.0. The r-HBsAg samples dissolved in PBS, were directly applied into the system. The flow-rate was 0.2 ml/min and the chromatograms were recorded and analyzed by a Biocrom interface board (CIGB, Havana, Cuba).

2.6. SDS–PAGE and Western blot

The r-HBsAg eluted from the immunoaffinity matrixes was electrophoresed in a 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions [23]. The proteins were transferred by a semi-dry electrophoretic transfer with the transfer buffer (25 m*M* Tris, 192 m*M* glycine, 20% methanol) at 25 V for 20 min onto a nitrocellulose membrane (Schleicher & Schuell, Daseel, Germany) [24,25]. After incubating with 1% non-fat milk in PBS for 1 h at 37°C, the membrane was incubated with a CB.Hep-1 mAb solution (10 μg/ml) for another hour at 37°C.

The membrane was washed three times in PBS-T, and incubated for 1 h at room temperature with 100 μl of sheep anti-mouse IgG conjugated to HRP (Sigma Chemical Co., St. Louis, USA).

Bands were visualized by reacting with the substrate solution (5 mg 3,3′ diaminobenzidine, 10 μl 30% H_2O_2 and 10 ml PBS). The reaction was stopped with deionized water.

2.7. Determination of r-HBsAg concentrations

The active r-HBsAg eluted from the CB.Hep-1 mAb immunosorbents was measured by a sandwich ELISA using a sheep anti-mouse polyclonal immunoglobulin against r-HBsAg, both as capture antibody and as an HRP conjugate.

2.8. Determination of protein concentrations

Protein concentrations were determined according to the procedure of Lowry et al. [26].

2.9. Stability of CB-Hep-1 mAb in elution buffers

The stability of the CB.Hep-1 mAb in both reagents, 20 m*M* Tris pH 11.6 and 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA pH 7.0 was analyzed every hour during 24 h at room temperature. We measured the specific activity of mAb by a direct ELISA. Briefly, a plate was coated with r-HBsAg (10 μg/ml) overnight at 4°C, the wells were washed and the samples of CB.Hep-1 mAb were added, the plate was incubated during 30 min at 37°C. The wells were incubated with 100 μl of a sheep anti-mouse polyclonal immunoglobulin-HRP conjugate. After washings, the reaction was revealed and stopped as described Section 2.3.

3. Results and discussion

3.1. Microtiter plate elution

The total quantitative yield of six dissociating reagents was initially evaluated in a PE support, the versatility of this method as an alternative to CNBr-activated Sepharose for affinity chromatography in order to evaluate several elution conditions has been demonstrated [27,28]. Table 1 shows the total protein recovered using various dissociation reagents. From the data we conclude that 20 m*M* Tris pH 11.6, 1.0 *M* Na_2CO_3, 0.5 *M* NaCl pH 11.6, 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA and 8 *M* urea pH 7.0 are the most stripping reagents for bound r-HBsAg with

Table 1
Immunoassay analyte recovery. A sandwich ELISA was performed using PE Microplates which were coated with CB.Hep-1 mAb, the r-HBsAg was added and the plates were incubated with the elution reagents, the plates were incubated with the biotynilated CB.Hep-1 mAb and finally the horseradish peroxidase-streptavidin conjugate was added. The reaction was revealed using OPD and H_2O_2 in citrate buffer

Elution agent	Recovery (%)
20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA	94
4.5 *M* $MgCl_2$	17
Glycine pH 3.5	41
8 *M* Urea	94
1 *M* Na_2CO_3/0.5 *M* NaCl pH 11.6	94
Tris pH 11.6	94
PBS pH 7.2	0

more than 90% of recovered antigen. Moreover, 4.5 *M* $MgCl_2$ pH 7.0 and 0.2 *M* glycine pH 3.5 showed the lowest elution strength, recovering 19% and 40% respectively. In contrast to the results obtained by other authors [8], the interaction CB.Hep-1 mAb/r-HBsAg seems to be more sensitive to basic pH than to chaotropic conditions and ionic strength. We selected 20 m*M* Tris pH 11.6 and 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA pH 7.0 as elution conditions for the immunosorbent assays.

3.2. Stability of CB.Hep-1 mAb in immunosorbent elution conditions

The performance of CB.Hep-1 mAb for the immunogel elution conditions was monitored during 24 h. As the Fig. 1 shows, for the antibody treated with 20 m*M* Tris pH 11.6, the recognition capacity was not reduced and the purity was higher than 95%. Conversely for mAb treated with 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA pH 7.0 the purity was also higher than 95%, but the recognition capacity for r-HBsAg diminished to minimal values from the third hour on. It could be due to the ability of the SCN^- ion for partly unfolding protein structures, at high concentration (3 *M*) [29].

3.3. Column elution of bound antigen using two different reagents

The ability/advantages of the CB.Hep-1 mAb immunoaffinity chromatography system to purify r-HBsAg from semi-purified starting material (10–25% purity) has been demonstrated [8,22].

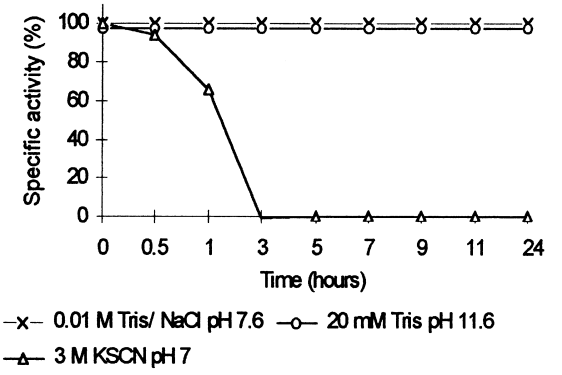

Fig. 1. Specific activity profile of CB.Hep-1 mAb under different conditions. The mAb was incubated at room temperature during 24 h with 3 *M* KSCN/20 m*M* Tris/3 m*M* EDTA pH 7.0, 20 m*M* Tris pH 11.6 and 0.01 *M* Tris/NaCl pH 7.6. A classical ELISA was performed to determine the specific activity of CB.Hep-1 mAb.

Once the optimum elution conditions to PE support were selected, we assayed the CNBr-activated Sepharose CL-4B immunogel. The Fig. 2 shows a comparison between basic and chaotropic conditions during 15 cycles. The amount of eluted antigens per milliliter of gel, for both reagents, decreased with the increment of the cycles. In contrast to earlier work [8], the amount of r-HBsAg eluted from the matrix using 20 m*M* Tris pH 11.6 was 1.7-fold higher than using 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA pH 7.0.

It has been known that the mAb leakage from IAC

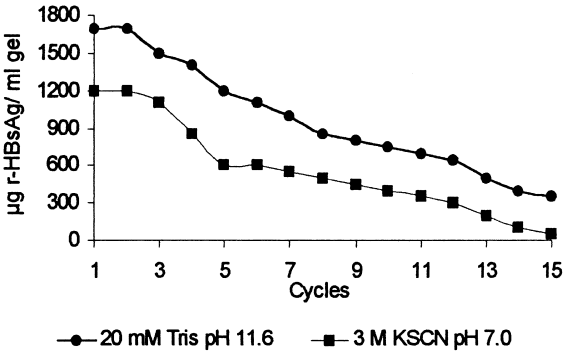

Fig. 2. Elution profile during 15 cycles using two different agents. We used a 2-ml column (5 mg of CB.Hep-1 mAb was covalently coupled to each ml of Sepharose CL-4B). A sample size of 40 ml of semi-purified r-HBsAg was applied to each immunosorbent.

matrices is potentially a serious drawback to the use of such matrices. Leakage of mAbs may also limit the useful immunosorbents half-life thereby rendering the process uneconomic. Taking into consideration that the antigen eluted from the columns is intended for therapeutic use, this parameter raise a great role because leakage of mAb can lead to contamination of the product which could compromise its safety for therapeutic use. We measured the ligand leakage in each cycle and it was below 3 ng IgG/μg r-HBsAg in all cases.

The best performance obtained with the matrix treated with the alkaline solution might be a consequence of two possible factors: firstly, for a rapid diminishing of the binding constant at high pH conditions, it has been previously reported [30], such behavior has not been seen using the SCN^- ion; secondly, the loss of recognition capacity for r-HBsAg showed by CB.Hep-1 mAb under chaotropic conditions. The loss of antibody function has been found to be the major contributor to the degradation of immunosorbents performance [29].

The estimated immunosorbent longevity in the literature varies widely. Reports on immunosorbent reuse range from five to hundreds of cycles, with capacity loss ranging from ''negligible'' to 75% of its initial capacity [31]. One of the possible explanations for the column degradation following two cycles could be by the poor mass transfer of r-HBsAg into the Sepharose CL-4B gel. The fractionation range reported for Sepharose-4B extends up to M_r 2.0×10^7 and the average molecular mass of r-HBsAg is about 2.4×10^6. The ratio between these values is less than 10, which suggests that some restriction to the free diffusion of r-HBsAg exists in this system [8]. The use of Sepharose CL-2B should solve this limitation.

3.4. Analysis of eluted antigens

It has been known that after treatment with 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA, r-HBsAg particles are stabilized [5], therefore we compared the antigen treated using the selected elution agent with the antigen treated with 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA. The eluted antigens were analyzed by SDS–PAGE (Fig. 3a). High purity levels were obtained in all cases (about 95%). This result suggests that the new elution procedure is not only more efficient in terms of total quantitative recovery but is also similar to chaotropic conditions in terms of antigen purity levels.

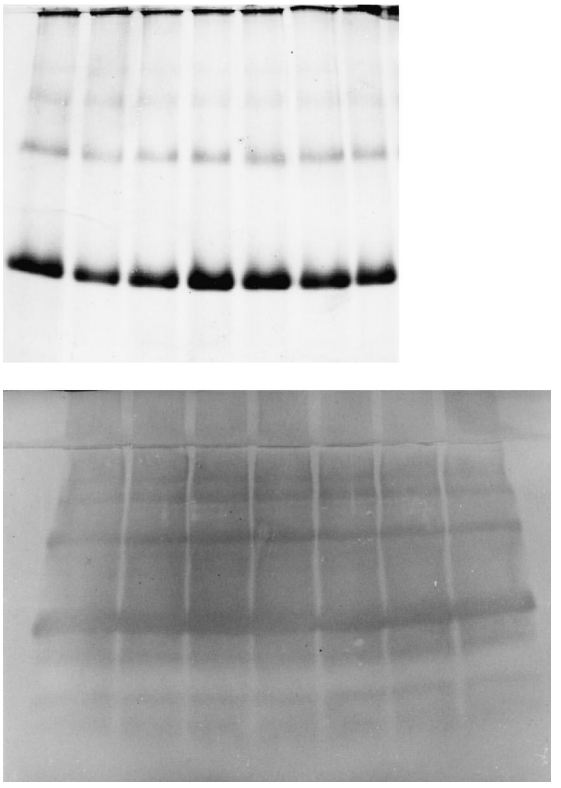

Fig. 3. Analysis of r-HBsAg eluted from the immunosorbents. (a) SDS–PAGE: Samples of r-HBsAg were electrophoresed under reducing conditions (12.5% polyacrylamide gel). (b) Western blot: Samples of r-HBsAg were transferred by a semi-dry electrophoretic transfer onto a nitrocellulose membrane. Line 1: Control sample (r-HBsAg in PBS); Lines 2, 3 and 4: Samples of r-HBsAg eluted using 20 m*M* Tris pH 11.6 from the runs 1, 5 and 15, respectively: Lines 5, 6 and 7: Samples of r-HBsAg eluted using 20 m*M* Tris/3 m*M* EDTA/3 *M* KSCN from the runs 1, 5 and 15, respectively.

We also compared the eluted antigens by Western blot (Fig. 3b) and ELISA. In Western blot analysis, we obtained the same band pattern in all cases, which suggests that the same antigenic population was selected from both matrixes. Likewise the recognition capacity determined by ELISA was higher than 90%. The r-HBsAg eluted from the immunosorbent treated with 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA pH 7.0, showed the same

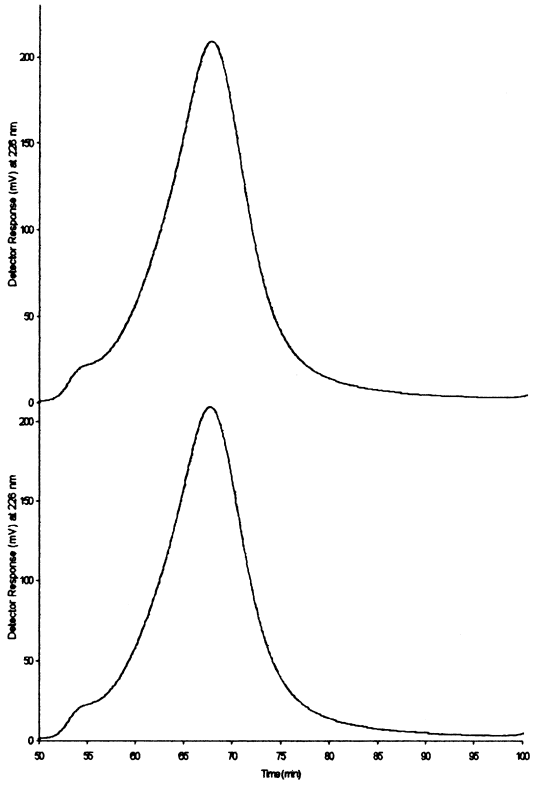

Fig. 4. HPLC-gel filtration analysis of r-HBsAg eluted from the immunogels. (a) r-HBsAg eluted from the matrix treated with 20 m*M* Tris pH 11.6, (b) r-HBsAg eluted from the matrix treated with 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA.

recognition pattern for the CB.Hep-1 mAb, than for r-HBsAg treated with 20 m*M* Tris pH 11.6.

Finally, as shown in Fig. 4 the eluted antigens were analyzed by HPLC-gel filtration. We compared the retention time for every case (antigen obtained from the matrix treated using 20 m*M* Tris/3 *M* KSCN/3 m*M* EDTA pH 7.0 and using 20 m*M* Tris pH 11.6 respectively) the retention times were similar in both cases. This suggests a molecular similarity between both group of antigens.

4. Conclusions

A new method has been developed to elute r-HBsAg from immunosorbents. This opens the possibility to increase the useful immunosorbents half-life and also its yield.

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References

[1] F. Gavilanes, J.M. González-Ros, D.L. Peterson, J. Biol. Chem. 257 (1982) 7770.
[2] E. Pentón, L. Herrera, V. Muzio, V. Ramirez, A. García, C. Duarte, C. Ruiz, M. Izquierdo, L. Pérez, G. Fontirrochi, M. González, M. Nazabal, A. Beldarrain, G. Padrón, J. García, G. de la Riva, A. Santiago, F. Ayan, R. Páez, A. Agraz, R. Díaz, Y. Quiñones, Eur. Pat. Apl. 480 525 (1992).
[3] R.W. Ellis, in: G.C. Woodrow, M.M. Levine (Eds.), New Generation Vaccines, Marcel Dekker, New York, 1990, p. 439.
[4] R.W. Ellis, P.J. Kniskern, in: A. McLachlan (Ed.), Molecular Biology of the Hepatitis B Virus, CRC Press, Boca Raton, 1991, p. 3077.
[5] D.E. Wampler, E.D. Lehman, J. Boger, W.J. McAleer, E.M. Scolnick, Proc. Natl. Acad. Sci. USA 82 (1985) 6830.
[6] F. Hamada, K. Sungahara, K. Shiosaki, S. Adachi, H. Mizikami. US Patent 4738926.
[7] E. Pentón, V. Muzio, M. González, Biotechnol. Aplic. 11 (1994) 1.
[8] A. Agraz, C. Duarte, L. Costa, L. Pérez, R. Páez, V. Pujol, G. Fontirrochi, J. of Chromatogr. A 672 (1994) 25.
[9] P. Mohr, K. Pommerening, Affinity Chromatography, Marcel Dekker, New York, 1985.
[10] G. Köller, C. Milstein, Nature 256 (1975) 495.
[11] J.B. Robins, R. Schneerson, Methods Enzymol. 34 (1974) 703.
[12] T. Kristiansen, in: O. Hoffmann Ostenhot (Ed.), Affinity Chromatography, Pergamon Press, Oxford, 1978, p. 191.
[13] S. Fuchs, M. Sela, in: M. Weir (Ed.), Handbook of Experimental Immunology. I. Immunochemistry, 4th edn. Blackwell Scientific Publications, Oxford, 1986, p. 161.
[14] M.W. Stewart,. in M. Weir (Ed.), Handbook of Experimental Immunology. I. Immunochemistry, 4th edn. Blackwell Scientific Publications, Oxford, 1986, p. 251.
[15] H.A. Chase, Chem. Eng. Sci. 39 (1984) 1099.
[16] J.M. Cregg, J.F. Tschopp, C. Stillman, R. Siegel, M. Akong, W.S. Craig, R.G. Buckholz, K.R. Madden, P.A. Kellaris, G.R. Davis, B.L. Smiley, J. Cruze, R. Torregrossa, G. Velicelebi, G.P. Thill, Biotechnology 5 (1987) 479.
[17] N. Harford, T. Cabezon, B. Colau, A.M. Delisse, T. Rutgers, M. de Wilde, Postgrad Med. J. 63 (Suppl. 2) (1987) 65.
[18] G. Fontirrochi, M. Dueñas, M.E. Fernández de Cossio, P. Fuentes, M. Pérez, D. Mainet, M. Ayala, J.V. Gavilondo, C.A. Duarte, Biotecnol. Appl. 10 (1993) 24.

[19] A. Danielson, A. Ljunglof, H. Linblom, J. Immunol. Methods 115 (1988) 79.
[20] J. Kohn, M. Wilchek, Appl. Biochem. Biotechnol. 9 (1984) 285.
[21] Affinity Chromatography, principles and methods, Pharmacia LKB Biotechnology, 1993.
[22] M.E. Fernández de Cossio, Ph.D. Thesis, CIGB, Havana, 1997.
[23] U.K. Laemmli, Nature 227 (1970) 680.
[24] H. Towbin, Proc. Natl. Acad. Sci. USA 76 (1979) 4350.
[25] W.N. Burnette, Anal. Biochem. 112 (1981) 195.
[26] D. H Lowry, N.J. Rosembrough, A. L Farr, R.J. Randal, J. Biol. Chem. 193 (1951) 265.
[27] C. Staak, F. Salchow, P.H. Clausen, E. Luge, J. Immunol. Methods 194 (1996) 141.
[28] K. Brahimi, J.L. Perignon, M. Bossus, H. Gras, A. Tartar, P. Druilhe, J. Immunol. Methods 162 (1993) 69.
[29] M.L. Yarmush, K.P. Antonsen, A. Sundaram, D.Y. Yarmush, Biotechnol. Prog. 8 (1992) 168.
[30] K.P. Antonsen, C.K. Colton, M.L. Yarmush, Biotechnol. Prog. 7 (1991) 159.
[31] M.L. Yarmush, A.M. Weiss, K.P. Antonsen, D.J. Odde, D.M. Yarmush, Biotechn. Adv. 10 (1992) 413.