Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **26** (2): 165-70 (2007)

Trabajos originales

Recibido el 28 de julio de 2006
Aceptado el 7 de octubrede 2006

Strategy for the Purification of Soluble Fibronectin from Human Plasma, as Ligand for Affinity Chromatography

Giselle REYES ALVAREZ, Ileana MARTÍNEZ CABRERA, Andrés GONZÁLEZ RODRÍGUEZ, Reinier BORRERO MAURA, Aniel MOYA TORRES, María de los A. PADRÓN COLLAZO, Michel ACOSTA SÁNCHEZ, Rubén CABRERA ARIAS & Luis GARCÍA IMÍAS

Instituto Nacional de Investigaciones y Producción de Vacunas y Sueros "Instituto Finlay", Ave 27, N° 19805. La Lisa. Ciudad de La Habana, Cuba.

SUMMARY. Soluble fibronectin from human plasma was purified and characterized in order to be applied in the purification of adhesion protein. Gelatin was immobilized in a chromatographic bed as Sepharose 2B CL to prepare a column. Different alternatives of elution with 0.5 M and 1 M arginin, glicin-NaCl, sodium acetate - NaCl and urea - NaCl buffers, confirmed the advantages of the arginin as elution solution in this column. Second step of purification in a column of Heparin - Sepharose 4B CNBr was included to obtain the native fibronectin with high purity degree, verified by SDS-PAGE. Purified protein was immobilized in a matrix of Sepharose with a substitution grade of 0.98 mg fibronectin/mL of gel that permited an adsorption capacity of free gelatin to the column of 8 mg (0.64 mg gelatin/mL of gel). This matrix will allow its preliminary application in the purification of adhesion proteins which could be used as antigens in vaccines againt leptospirosis.

RESUMEN. "Estrategia para la Purificación de Fibronectina Soluble a partir de Plasma Humano, como Ligando para Cromatografía de Afinidad". El presente trabajo se desarrolló con el objetivo de purificar y caracterizar fibronectina soluble procedente de plasma para su posterior aplicación en la purificación de proteínas de adhesión. Se inmovilizó gelatina en Sefarosa 2BCL como soporte cromatográfico para la preparación de una columna. Se estudiaron diferentes alternativas de elución con arginina 0.5 M y 1M, con glicina -NaCl, acetato de sodio - NaCl y urea - NaCl, lo cual confirmó las ventajas en el uso de la arginina como agente eluyente. Se incorporó una segunda etapa de purificación en una columna de Heparina - Sefarosa 4B CNBr a partir de la que se obtuvo fibronectina con alto grado de pureza en estado nativo, comprobado por SDS-PAGE. La proteína purificada se inmovilizó en una matriz de Sefarosa con un grado de sustitución de 0,98 mg fibronectina/mL de gel lo cual permitió una capacidad de adsorción de gelatina libre con una recuperación de 8 mg (0,64 mg gelatina/mL de gel). La utilización de esta matriz permitirá su aplicación preliminar en la purificación de proteínas de adhesión que podrían ser usadas como antígenos en vacunas contra leptospirosis.

INTRODUCTION

Fibronectin (Fn) is a glycoprotein secreted by a great variety of cells including the endotellial and epithellial cells and the macrophages. It is a protein of high molecular weight (~450kDa), constituted by two subunits between 200-250 kDa joined by disulphide bonds. Soluble form of fibronectin is located in plasma (300-500 µg/mL) and in other physiological fluids as amniotic, seminal, and spinal fluid in smaller concentrations. Its insoluble form occurs in the surface of several types of cells and in the extracellular matrix. Plasma fibronectins of smaller molecular weight also exist, possibly due to proteolitic activity of enzymes on the native molecule of 450 kDa. These fibronectins have the same functional activity than the original molecules [1].

The purification strategies of human plasma fibronectin, based in molecular exclusion chromatography and ionic exchange chromatography have not showed succesful results in terms of purity and recovery [2,3]. Some authors proposed the use of chromatography in Gelatin - Sepharose due to affinity interactions between fibronectin and gelatin [4-6], but the purity is afected by some fibronectin fragments and a seric gelatinase (with proteolitic activity) eluted with

KEY WORDS: Fibronectin, chromatography, colagen, adhesion, vaccine.
PALABRAS CLAVE: Fibronectina, cromatografía, colágeno, adhesión, vacuna.

* Author to whom correspondence should be addressed. *E-mail:* greyes@finlay.edu.cu

the native protein [4,6]. As second step, there are two alternatives: the affinity chromatography in Arginin-Sepharose [4], but the recovery of fibronectins is smaller and the proteolitic fragments are not eliminated, or affinity chromatography in Heparin- Sepharose with better results [6].

The capacity of pathogenic microorganism to penetrate, diseminate and persist in mammals host seems to be related to the capacity of these organisms to adhere at the components of the extracellular matrix (CEM) in the eucaryotic cells [3,7-10]. For example, variety of cellular types affected during the infection by *Leptospira* suggests the presence of several adhesins or the recognition of present CEM in most of the eucaryotics cells, such as colagen and fibronectin. Two possible types of adhesins from *Leptospira* have been only identified, whose expression is correlated with the virulence of the microorganism: fibronectin-binding protein of 36 kDa, identified in serovar *Icterohaemorrhagiae* [3] and a protein family from serovar *Grippotyphosa* with globulin domain ("Ig-like proteins"), whose structure resembles adhesins described in other gram-negative pathogens [11,12]. Nevertheless, the studies directed to the identification and evaluation of adhesins of *Leptospira* that mediate the adhesion and colonization are relatively scarce.

In the present paper a strategy to purify human plasma fibronectin for using as a chromatography ligand to possible purification of bacterial adhesins is described.

MATERIALS AND METODS

Human plasma

Fresh human plasma from the Institute of Blood Derivateds of Havana was used as source of soluble fibronectina and was conserved at -20 °C.

Purification of human fibronectin

Native soluble fibronectin from human plasma was purified, following essentially the principle of double chromatography of gelatina/ heparina affinity, proposed by Poulouin [6].

Immobilization of gelatin in Sepharose 2B CL

Gelatin (Merck, Germany) was immobilized in matrix of Sepharose 2B-CL (Pharmacia, Sweden) following the method proposed by Gemeiner & Breier [13] with slight modifications. Sepharose 2B-CL (200 mL sedimented) was resuspended in 1700 mL of 50 mM NaIO4 and the mixture was incubated during 2 h in the dark, with slow agitation and then, it was filtered. Gel was washed with distilled water and solution 50 mM sodium borate pH 8.9. Activated Sepharose was mixed with a solution of gelatin previously incubated at 50 °C (1.4 g dissolved in 50 mM sodium borate pH 8.9) and it was stirried overnigth at room temperature. After that, 1.4 g of sodium borohydride was added and the mixture incubated, during 80 minutes. Gel was washed and blocked with solution 50 mM sodium borate 1 M glycin 220 mM NaCl, pH 8.9; during 2 h at 18-20°C. Matrix was filtered and washed with solution 50 mM sodium borate 220 mM NaCl, pH 8.9, and then, with solution 100 mM sodium acetate 220 mM NaCl, pH 5 and solution 20 mM Tris-HCl 500 mM NaCl, pH 8 (three times with each solution). Gelatin-Sepharose was washed with balance solution 20 mM Tris-HCl 0.9% NaCl, pH 7.4. Protein concentration was determined in the washings by Lowry method [14] and it was verified the immobilization efficiency, comparing the amount of gelatin released in the washings with the amount applied at the begining. Gel was packed in a column of 26 x 400 mm (Pharmacia, Sweden) and conserved to 4 °C, until use.

Affinity chromatography in Gelatin Sepharose 2B CL

Human plasma was filtered through filter paper and dialized against a balance solution 20 mM Tris HCl 0.9% NaCl, pH 7.4, at 4 °C. Sixty mL were applied in Gelatin-Sepharose 2B with a volumetric flow of 0.8 mL/min, using a FPLC system (Pharmacia, Sweden). Fractions were meassured to 280 nm by a detector SII (Pharmacia, Sweden). It was made first washing with three volumes of column with balance solution and second washing with equal volume of 20 mM Tris-HCl 5.8% NaCl, pH 7.4. Finally, it was eluted with 20 mM Tris-HCl 0.9% NaCl 1M arginin, pH 7.4. Fractions were analyzed by SDS-PAGE method (gel 7.5%). Protein concentration was considered by means of absorbance to 280 nm, considering a molar extinction coefficient as 1.3 for the human fibronectin [4]. Several purification variants under the same conditions previously described were made with different solutions for elution: a) 20 mM Tris-HCl 0.9% NaCl 1 M arginin pH 7.4, b) 20 mM Tris-HCl 0.9% NaCl 0.5 M arginin pH 7.4, c) 20 mM Tris-HCl 1 M NaCl 2 M urea pH 7.4, d) 20 mM Tris-HCl 1 M NaCl 300mM glycin pH 3, and e) 20 mM sodium acetate1 M NaCl pH 5.4.

Immobilization of heparin

Immobilization of heparin in Sepharose 4B-CNBr (Pharmacia, Sweden) was made according to recommended by Delfin *et al.* [15]. Sepharose 4B-CNBr (10 g) was resuspended in 200 mL of 1 mM HCl, during 15 min and it was washed with 800 mL of this solution. In the second step, gel was washed with distilled water and 200 mL of solution 50 mM sodium borate pH 8.9, and then, was resuspended in a solution of 117 mg of heparin (dissolved in 20 mL of the same solution of washing) with slow starring, overnigt at room temperature.

Free reactive sites of the matrix were blocked with solution 50 mM sodium borate 1 M glycin 220 mM NaCl, pH 8.9; during 2 hours at 18-20 °C. Gel was washed with solution 50 mM sodium borate 220 mM NaCl pH 8.9, with solution 100 mM sodium acetate 220 mM NaCl pH 5 and solution 20 mM Tris HCl 500 mM NaCl pH 8 (three times with each solution). Protein concentration was determined to the washings by the Lowry method [14]. Efficiency of immobilization was evaluated comparing the amount of heparin released in the washings with the amount applied at the begining. Matrix was equilibred with a solution 50 mM Tris HCl 1 mM EDTA pH 7.4 and was packed in a column of 16 x 120 mm and conserved to 4 °C, until use.

Affinity chromatography in Heparin - Sepharose 4B CNBr

Fraction purified by the Gelatin - Sepharose chromatography was concentrated and dialized against a solution of 50 mM Tris HCl 1 mM EDTA, pH 7.4. Ten mL were applied in the column of Heparin-Sepharose 4B-CNBr with a volumetric flow equivalent to 0.5 mL/min. After the application of the sample the flow was increased to 1 mL/min and washings with three volumes of column of the balance solution were made. Material bound to the column was eluted with a solution 50 mM Tris HCl 1 mM EDTA 150 mM NaCl, pH 7.4 and it was analyzed by SDS-PAGE (separating gel 7.5%). Protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 1.3 for fibronectin [4].

Affinity chromatography with fibronectin as ligand

Immobilization of fibronectin in Sepharose 4B-CNBr (Pharmacia, Sweden) was made according to recommended by Delfin *et al.* [15]. Immobilization process was carried out in the same way for heparin in Sepharose 4B-CNBr; 12.5 mL of the gel were incubated with 1.45 mg/mL of fibronectin and it was packed in a column 16 x 100 mm, which was balanced with the solution 20 mM Tris HCl-0.9% NaCl pH 7.4. The capacity of the column was evaluated applying a solution of 4 mL and 7.5 mL of gelatin 2 mg/mL in 20 mm Tris HCl-0.9% NaCl pH 7,4. The amount of gelatin applied and eluted of the column was determined using the method of Lowry [14].

RESULTS

The First step of the purification strategy requeried of an affinity column of Gelatin-Sepharose 2B CL preparation. The selected process for gelatin immobilization in this gel guaranteed a binding density of 5.86 mg gelatin/mL of gel. Manufacturer of commercial column recommends this value as acceptable if it is between 4.5 and 8 mg of gelatin/mL of gel [15].

Human plasma was evaluated previously by unidimensional SDS-PAGE, in order to identify the components in the protein mixture. Electrophoretic pattern shown three majority bands, one corresponding to 66 kDa, another one between 116 and 170 kDa and a third band with a high molecular weight that as soon as it migrates of the application point (Fig. 1).

These bands could be seric albumin, immunoglobulins and fibronectin, respectively [17]. Fibronectin subunits, with a molecular size be-

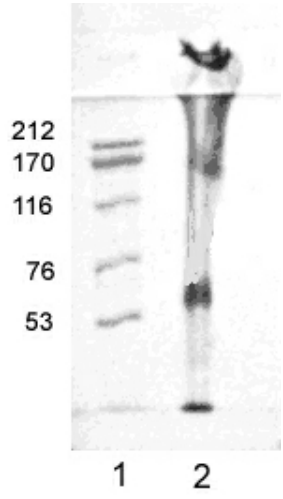

Figure 1. SDS- Polyacrylamide gel electrophoresis of human plasma (1.8 mg/mL) on 7,5% gel. The gel was stained with Coomassie blue. (**1**) Molecular mass standard markers (in kDa). (**2**) Human Plasma.

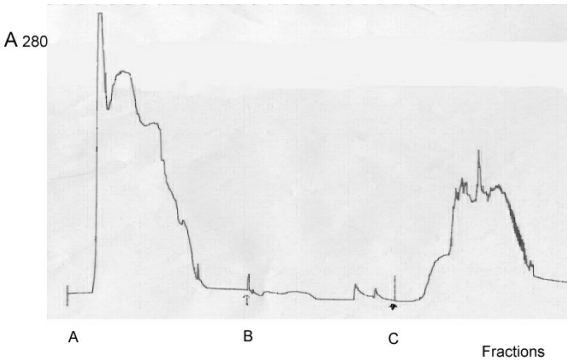

Figure 2. Chromatography profile of human plasma on Gelatin-Sepharose 2B CL column (26 x 400 mm). (**A**) At 20 mM Tris HCl 0,9% NaCl pH 7.4 (Fraction I). (**B**) Washing: 20 mM Tris HCl 5.8% NaCl pH 7.4 (Fraction II). (**C**) Elution: 20 mM Tris HCl 0.9% NaCl 1 M arginin pH 7.4 (Fraction III). Flow: 0.8 mL/min.

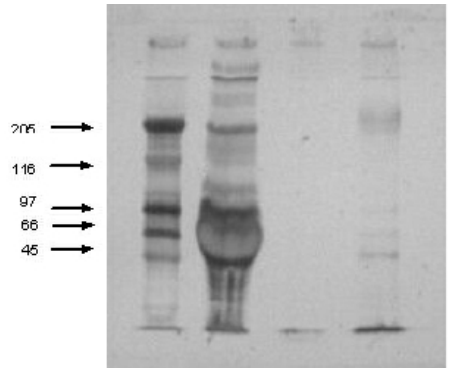

Figure 3. SDS- Polyacrylamide gel electrophoresis of chromatographic fractions from Gelatin - Sepharose 2B CL, on 7.5% gel. The gel was stained with silver staining. (**1**) Molecular mass standard markers (in kDa). (**2**) Fraction I. (**3**) Fraction II. (**4**) Fraction III.

tween 200 and 250 kDa [5,6] could be interacting with other plasma proteins, forming a complex of high molecular weight and slow migration. Chromatographic profile obtained after the application of the human plasma in Gelatin-Sepharose 2B CL (Fig. 2) demonstrated the presence of a protein fraction without affinity by gelatin (Fraction I).

Electrophoretic pattern of this fraction (Fig. 3) indicated the presence of contaminant proteins and a band with a similar size to fibronectin, probably, because all the fibronectin available is not bound to gelatin. The results indicated the absence of proteins in this fraction (Fraction II) by SDS-PAGE (Fig. 3) and by absorbance to 280 nm measure. We obtained a third fraction (Fraction III) applying different elution conditions (Table 1), being the best recovery with 0.5 M and 1 M arginin. Third fraction consisted of four bands of different molecular weighs: a mayority band with 205 kDa (as fibronectin) and three bands (100, 70 and 53 kDa) (Figure 3), which could correspond with fragments of proteolytic degradation of the fibronectin in the human plasma with affinity sites to the gelatin [4-6].

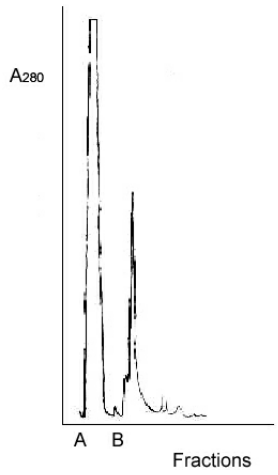

Figure 4. Chromatography profile of Fraction III (from Gelatin - Sepharose 2B CL) on Heparin-Sepharose 4B-CNBr column (16 x 100 mm). (**A**) At 50 mM Tris HCl 1mM EDTA pH 7.4. (**B**): Elution 50 mM Tris HCl 1mM EDTA 150 mM NaCl pH 7.4. Flow: 1 mL/min.

Therefore, we included a second step based on the interation of binding sites to heparin in the native fibronectin, absent in proteolytic fragments of fibronectin. Fraccion III (from Gelatin - Sepharose) was analized by Heparin-Sepharose 4B-CNBr column (4.68 mg of heparin/mL), ob-

Elution conditions	Recovery of protein (mg)
20 mM Tris HCl 0.9 % NaCl 1 M Arginin pH 7.4	24.00
20 mM Tris HCl 0.9 % NaCl 0.5 M Arginin pH 7.4	28.30
20 mM Tris HCl 1M NaCl 300 mM Glicin pH 3	4.26
20 mM Tris HCl 1M NaCl 2 M Urea pH 7.4	2.38
20 mM sodium acetate 1M NaCl pH 5.4	1.08

Table 1. Protein recovery from Gelatin - Sepharose 4B CNBr column, with different elution conditions.

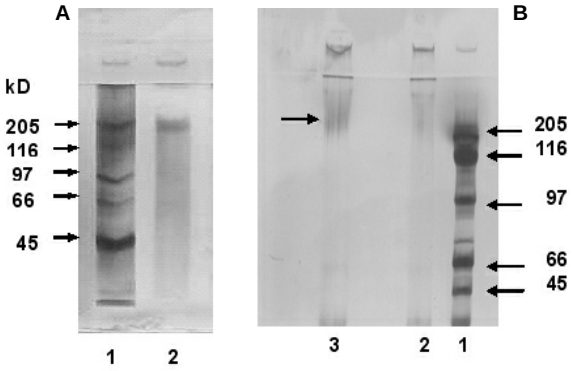

Figure 5. SDS- Polyacrylamide gel electrophoresis of chromatographic fractions from Heparin -Sepharose 4B-CNBr. The gel was stained with Coomassie blue. **A.** (**1**) Molecular mass standard markers (in kDa). (**2**) Fraction eluted with 50 mM Tris HCl- 150 mM NaCl-1 mM EDTA pH 7.4 from Heparin-Sepharose 4B-CNBr, treated with β-mercaptoetanol. **B.** (**1**) Molecular mass standard markers (in kDa). (**2**) Fraction eluted with 50 mM Tris HCl- 150 mM NaCl-1 mM EDTA pH 7.4 from Heparin-Sepharose 4B-CNBr without β-mercaptoetanol. (**3**) treated with β-mercaptoetanol.

taining the chromatographic profile showed in Fig. 4.

Electrophoretic characterization of the fraction corresponding to the elution demonstrated a homogenous band of 205 kDa characteristic of a fibronectin subunit from the treatment with buffer sample with β-mercaptoethanol, free of contaminant fragments (Fig. 5).

Identity of fibronectin was determined by that comparison when the sample was treated with and without reductive conditions. Fig. 5 B shows absence of a band in native fibronectin sample (without β-mercaptoethanol) and a band of 205 kDa when the sample was treated with β-mercaptoethanol. The recovery of the propossed strategy for purification was 0.11 mg of fibronectina/mL of plasma. Similar results were obtained by Poulouin *et al.* [6], after applicating of the double affinity principle of gelatin/heparin for the purification of native fibronectin.

Immobilization of native fibronectin in Sepharosa 4B-CNBr provided a sustitution degree of 0.98 mg fibronectin/mL of gel. Since the binding site of the fibronectin to adhesins is the same one related with the binding site to the gelatin [3], it turned out interesting to verify the binding capacity of the column. Results indicated that the column allowed the retention of at least 8 mg of gelatin.

DISCUSSION

Fibronectin is a protein component of soluble fluids in the human plasma and as associated form to the extracellular matrix of multiple cellular types, could be related to the mechanism that mediate the adhesion and colonization of numerous pathogenic microorganisms [18]. Both types of fibronectin are similar in the structure but extracellular matrix fibronectin purification is more expensive and complex process. Therefore, the soluble fibronectin of plasma is the most used as ligand in the affinity chromatography to purify fibronectin - binding proteins [18].

Immobilization of gelatin in Sepharose according our strategy allowed to prepare a big column to purify a sufficient amount of protein, in one step by affinity chromatography. We evaluated some elution conditions in order to sustitute arginin because it is very expensive. It is evident, arginin is the best elution agent and when we reduced the amount of arginin to 0.5 M we obtained similar results (Table 1). Presence of protein with similar size to fibronectin in the first fraction (Fig. 3) could be due to both proteins (gelatin and fibronectin) present a great molecular size, which can cause a marked steric impediment that disables the suitable interaction ligand - protein and as result low yields can be obtained. Vuento & Vaheri [4] proposed a second solution of washing corresponding to an increase of the ionic strength, allowing the elimination of some polypeptides and small fragments of fibronectin with slow affinity by the gelatin.

In fact, presence of an heparin-binding site in fibronectin structure allowed the purification of native molecules with both sites (for gelatin/ heparin) and the elimination of smaller fragments. We evaluated fibronectin concentration by absorbance to 280 nm because there was interference by Lowry methods, probably because the fibronectin precipitated with alcalin medium in the assays. We obtained similar recovery (0.11 mg fibronectin/mL plasma) according to the results of Poulouin *et al.* [6] (0,16 mg fibronectin /mL plasma).

Gelatin recouvered by Fibronectin-Sepharose 4B-CNBr (8 mg), demonstrated the existence of sufficient sites for available adhesin binding that would allow an appropriate recovery of the interest protein (microbial adhesin).

CONCLUSION

Human plasma fibronectin was obtained by two affinity chromatography steps using our immobilized matrix, with suitable purity and a recovery like Poulouin *et al.* strategy.

REFERENCES

1. Mosher, D.F. (1989) *Fibronectin*. Academic Press. San Diego. pp. 111-41.
2. Johansson, S. & B. Smedsrod (1986) *J. Biol. Chem*. **261**: 4363-6.
3. Merien, F., J. Truccolo, G. Baranton & P. Perolat (2000) *FEMS Microbiol. Lett.* **185**: 17-22.
4. Vuento, M.. & A. Vaheri (1979) *Biochem. J.* **183**: 331-7.
5. García-Pardo, A. & L.I. Gold (1993) *Arch. Biochem. Bioph.* **304**: 181-8.
6. Poulouin, L., O.Gallet, M. Rouahi & J.-M. Imhoff (1999) *Protein. Express. Purific*. **17**: 146-52.
7. Tsuchimoto, M., M. Niikura, E. Ono, H. Kida & R. Yanagawa (1984) *Zent. Bakteriol. Mikrobiol. Hyg.* **258**: 268-74.
8. Vinh, T., S. Faine & B. Adler (1984) *J. Med. Microbiol.* **18**: 73-85.
9. Ballard S.A., M.Williamson, B. Adler, T. Vinh & S. Faine (1986) *J. Med. Microbiol.* **21**: 59-67.
10. Thomas, D.D. & L.M. Higbie (1990) *Infect. Immun*. **58**: 581-5.
11. Palaniappan, R.U., Y.F.Chang, S.S. Jusuf, S.Artiushin, J.F. Timoney, S.P. McDonough, S.C. Barr, T.J.Divers, K.W. Simpson, P.L. McDonough & H.O. Mohammed (2002) *Infect. Immun*. **70**: 5924-30.
12. Matzunaga, J., M.A Barocchi, J. Croda, T.A. Young, Y. Sanchéz, I. Siqueira, C.A. Bolin, M.G. Reis, L.W. Riley, D.A. Haake & A.I. Ko (2003) *Mol. Microbiol.* **49**: 929-45.
13. Gemeiner, P. & A. Breier (1982) *Biotechnol. Bioeng.* **24**: 2573-83.
14. Lowry, O.H, N.J. Rosebrough, A.L. Farr& R.J. Randall (1951) *J. Biol. Chem*. **193**: 265-75.
15. Delfin, J, I. Martínez, W. Antuch, V. Morera, Y. Gonzalez, R. Rodriguez, M. Marquez, A. Saroyan, N. Larionova, J. Diaz, G. Padron & M. Chavez (1996) *Toxicon*. **34**: 1367-76.
16. Amersham Biosciences (2003) BioDirectory. www.amershambiosciences.com.
17. Janeway, C. A., P.Travers, M. Walport& M. Shlomchik (2001) *Inmunobiology* 5th ed. Garland Publishing. New York.
18. Probert, W.S. & B.J.B. Johnson (1998) *Mol. Microbiol.* **30**: 1003-15.