Screening of expression libraries using ELISA: identification of immunogenic proteins from *Tityus bahiensis* and *Tityus serrulatus* venom

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Abstract

The present report describes the use of ELISA with cDNA expression libraries in the identification of immunogenic proteins. The methodology described was applied using libraries constructed with mRNA isolated from *Tityus serrulatus* and *Tityus bahiensis* venom glands. In addition we describe for the first time the sequence of a neurotoxin from *Tityus bahiensis* venom gland named TbTx5 whose amino acid sequencing showed 93% similarity with the *Tityus bahiensis* TbTx IV-5 neurotoxin. The methodology described can be used for the generation of an immunogenic bank in order to contribute to genome and proteome projects. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Screening; Expression library; ELISA; Genome; Proteome; *Tityus bahiensis*; *Tityus serrulatus*

1. Introduction

The identification of antigenic proteins from a specific tissue or even from the whole organism is very important in areas such as toxinology and parasitology. To this end, specific antibodies have been successfully used to isolate genes from expression libraries (Young and Davis, 1983;

Sambrook et al., 1989). The protocol used involves antibodies that must be as pure as possible in terms of immunological activity. However, a major problem with this technique is the cross-reactivity with proteins produced by different host cells leading to a difficult interpretation of the results obtained on nitrocellulose or nylon membranes. In addition, antibodies that do not react strongly with an antigen are normally difficult to use. In the present report we describe the use of an enzyme-linked immunosorbent assay — ELISA, (Engvall and Perlmann, 1971) to screen expression libraries. The rationale behind this approach involves the specificity of ELISA and the possibility to quantify the immunological signal on ELISA microtitration plates. In addition, we have found that this methodology can generate a large amount of information within a short period of time and can contribute to genome and proteome projects by the identification of proteins and toxins important for the development of antivenoms for clinical use. The system has been established using *Tityus bahiensis* and *Tityus serrulatus* venom gland cDNA libraries.

Abbreviations: Bp, base pair(s); Da, daltons; EDTA, ethylenediamine tetra acetic acid; Ig, immunoglobulin; IPTG, isopropyl- β -D-thiogalactoside; Kb, kilobase(s); PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TAE, tris-acetate-EDTA buffer; TBS, tris-buffered saline; Ts, *Tityus serrulatus* scorpion; Tb, *Tityus bahiensis* scorpion; TstFG50, *Tityus serrulatus* toxic fraction purified through Sephadex G50; Xgal, 5-bromo-4-chloro-3-indolyl- β -D-galactoside

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2. Materials and methods

2.1. General molecular biology

Standard recombinant DNA techniques (plasmid purification, phenol extraction, ethanol precipitation, electrophoresis, etc.) were carried out as described by Sambrook et al. (1989).

2.1.1. Construction and analysis of the venom-gland cDNA library

Venom glands from 50 *Tityus bahiensis* scorpions were removed 2 days after venom extraction. The glands were frozen immediately, pulverized with a mortar and pestle in liquid nitrogen and lysed in the presence of guanidinium thiocyanate according to the published procedures (Chomczynsky and Sacchi, 1987). Total RNA was passed through an oligo(dT)-cellulose column as described by Sambrook et al. (1989). Complementary DNA synthesis was performed using the ZAP-cDNA synthesis kit (Stratagene). The cDNA molecules were prepared from 5 µg of *Tityus bahiensis* venom gland mRNA and inserted directionally into a Uni-Zap™ XR vector at the *Xho*I and *Eco*R I sites. The vector was subsequently packaged into viable lambda ZAP phage particles using the Gigapack II Packaging Extracts Kit (Stratagene). The insert sizes were analyzed by PCR (Saiki et al., 1988) using the primers M13R (5' GGAAACAGCTATGACCATG 3') and M13U (5' GTAAACGACGGCCAGT 3'). PCR was carried out using plasmids isolated from the total library. The *Tityus serrulatus* venom gland cDNA library has been described by Guatimosim et al. (1999) and was also constructed as described above.

2.1.2. Animals and venoms

Adult female New Zealand white rabbits (2.0–2.5 kg) were used and maintained at the Fundação Ezequiel Dias (FUNED), Belo Horizonte, Brazil. All animals received water and food under controlled environmental conditions. *T. serrulatus* scorpions were collected near the town of Santa Bárbara, Minas Gerais, Brazil. *T. bahiensis* were collected in the Town of Ouro Preto, Minas Gerais, Brazil. The venom from mature animals was obtained by electric stimulation, pooled, lyophilized and stored at –20°C in the dark before use.

2.1.3. Preparation of antibodies

The immunization protocol was carried out in rabbits as described by Chávez-Olórtegui et al. (1993) using 100 µg of total venom (*Tityus serrulatus* or *Tityus bahiensis*) or 20 µg of purified toxins from the venom of *Tityus serrulatus*: α-type toxin tityustoxin (TsTx) (Gomez and Diniz, 1966; also called toxin IV-5 by Possani et al. (1981) or Ts IV by Martin-Eauclaire et al. (1994)), and the β-type toxin TsTx I (Toledo and Neves, 1976; also called Ts VII by Bechis et al. (1984) and toxin-γ by Possani et al. (1985)). Booster

injections of 20 µg of toxin in incomplete Freund's adjuvant were made 1, 2, 3 and 4 weeks later. Immune sera were collected 10 days after the last injection and analysed by Western blotting and ELISA. Anti-TsTx and anti-TsTxI sera were further purified as described by Chávez-Olórtegui et al. (1994). The chromatographic profile obtained by reverse-phase high-performance liquid chromatography showed a single peak, suggesting that TsTx and TsTxI were homogeneous, in good agreement with the results of SDS-PAGE electrophoresis for basic proteins. The specific activity and titration of serum from rabbits immunized was carried out as described by Chávez-Olórtegui et al. (1997).

2.1.4. Western blotting

Electrophoresis for basic proteins (Reisfeld et al., 1962) was performed using the BIO-RAD, Mini-Protein II system with 15% polyacrylamide gels and 30 µg total protein per lane as determined by protein assay (Lowry et al., 1951). Gels were electrophoretically blotted as follows: nitrocellulose membranes (0.45 µm, Schleicher and Schuell) containing the venom transferred from the polyacrylamide gels were blocked for 1 h in 0.3% Tween-20/PBS and then exposed to the first antibody diluted 1:100 in 0.05% Tween-20/PBS, for 1 h at 37°C or overnight at 4°C. After 6 washes in 0.05% Tween-20/PBS the membranes were incubated in 0.05% Tween-20/PBS containing anti-rabbit-IgG conjugated for 1 h at room temperature. The membranes were washed 3 times in 0.05% Tween-20/PBS and twice in PBS and then stained using 1 mg DAB/ml (3,3'-diaminobenzidine, Sigma Chemical Co., St Louis, MO, USA) in PBS with 0.04% H₂O₂ and 8.3% methanol.

2.1.5. cDNA library screening using ELISA

In vivo excision of the pBluescript phagemid from the Uni-Zap XR vector was carried out using the ExAssist helper phage according to manufacturer's specifications (Zap-cDNA synthesis manual, Stratagene). After excision, bacteria (*E. coli* strain SOLR) containing the pBluescript vector were plated onto agar L broth containing 100 µg ml⁻¹ ampicillin, 200 µg ml⁻¹ X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 250 µg ml⁻¹ IPTG (isopropylthiogalactoside) and incubated at 37°C for 10 h. Individual white colonies were picked with sterile 200 µl tips and transferred to 2 ml of L broth containing 100 µg ml⁻¹ ampicillin and 0.5 mM IPTG in a sterile 15 ml tube. The collected material was allowed to grow at 37°C for 8–10 h and harvested by centrifugation (3000g for 5 min) at 4°C. The pellet was resuspended in 0.5 ml of carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6 and 0.5 mM PMSF) and cells were disrupted at 4°C by sonication (2 pulses of 15 s at a power setting of 3.5). The sonicated material was centrifuged at 4000g for 5 min. The supernatant (100 µl per well) was then applied to the microtitration plate (Falcon 3912, Becton Dickinson) and incubated for 8–12 h at 4°C. After blocking with 1% casein in PBS for at least 1 h at

Fig. 1. Western blotting analysis of (1) *Tityus serrulatus* fraction TsFG50, (2) *Tityus serrulatus* whole venom and (3) *Tityus bahiensis* whole venom. The venoms were solubilized and analysed by electrophoresis on 15% polyacrylamide gels for basic proteins using 30 μ g total protein per lane. After transfer to nitrocellulose they were probed with a specific antibody to *T. serrulatus* whole venom (A); specific antibody to *T. bahiensis* whole venom (B); rabbit antiserum against β -type toxin TsTx I (C); rabbit antiserum against the α -type toxin TsTx (D). Anti-rabbit-IgG conjugate and DAB were used to visualize the bands. TsFG50 is a *Tityus serrulatus* toxic fraction purified in sephadex G50 containing the major toxic components of the venom (Chávez-Olórtegui et al., 1994).

37°C and washing four times with 0.15 M NaCl and 0.05% Tween 20, the plates were incubated for 2 h at 37°C with specific antibodies diluted 1:250 in incubation buffer (0.2% casein in PBS + 0.05% Tween 20). Antibodies against the whole venom of *Tityus serrulatus* and *Tityus bahiensis* were used in the first round of screening. The plates were washed as described above and incubated for 1 h at 37°C with peroxidase-coupled anti-rabbit IgG (Sigma) diluted 1:1000 in incubation buffer. The wells were washed and the assay was completed with citrate buffer (50 mM Na₂HPO₄, 24 mM citric acid, pH 5.0), 0.2 mg/ml of OPD (*o*-phenylenediamine) and 0.2 μ l/ml of H₂O₂. After incubation at 25°C for 20 min in the dark the reaction was stopped by the addition of 20 μ l/well of a 1:20 dilution of sulphuric acid. Absorbance values were determined at 492 nm with a Titertek Multi-scan plate spectrophotometer. Results were confirmed by a second round of ELISA, and the positive clones were grown with and without IPTG. Total venom of *Tityus serrulatus* and *Tityus bahiensis* (100 ng/well), purified TsTx and TsTxI toxins from *Tityus serrulatus* (50 ng/well) were used as positive control. As an alternative to disrupted bacteria, we used glass beads (3 mm diameter) and vortexing at high speed for 30 s. We also found that

lysozyme (0.1 mg/ml for 2–5 min at 20–25°C) can improve (10–20%) the result when used before the glass bead treatment. After cell disruption the supernatant was immediately used or stored at –80°C in an ultra-low freezer before use.

2.1.6. DNA sequencing and computer analysis

Large scale plasmid isolations from *E. coli* were carried out according to the alkaline lysis SDS method and purified on CsCl–ethidium bromide gradients as described by Sambrook et al. (1989). DNA sequencing reactions were performed on both strands using the chain termination method of Sanger et al. (1977). All reactions were carried out using the Automated Laser Fluorescent ALF DNA Sequencer (Pharmacia, LKB Biotechnology Inc.). Nucleic acid sequences were analysed using the MacVector™ 5.0 program (Oxford Molecular Group PLC). After editing, the sequences were compared to sequences in the GenBank databases (Altschul et al., 1997).

3. Results and discussion

Scorpion toxins form a family of structurally related proteins and it is expected that several of these proteins share common epitopes. In this work, the immunological cross-reactivity between *Tityus serrulatus* and *Tityus bahiensis* was demonstrated by Western blotting and also by ELISA and the results illustrated the possibility to use antibodies generated against the venom or purified toxins from one species of scorpions to isolate similar toxins in the venom of a second species (Fig. 1). In reality, toxins from both the species have shown high level of similarity at the amino acid and DNA levels (Becerril et al., 1996; Guatimosim et al., 1999).

We report here the results of a screening carried out using ELISA and expression libraries constructed with venom glands from the scorpions *Tityus serrulatus* and *Tityus bahiensis*. The *Tityus bahiensis* cDNA library, containing approximately 1.1×10^6 independent recombinants showed the presence of cDNAs corresponding to molecules of approximately 350 to 1,100 bp (data not shown). The *Tityus serrulatus* cDNA library has been used by our group and contains 6.7×10^6 recombinants and one major class of cDNA (300–650 bp). After an initial screening of 350 colonies we found approximately 10% positive clones that showed different levels of immunoreactivity. In contrast, 90% of the clones did not show higher reactivity than the negative control (clones not induced by IPTG). The positive clones were used for further immunological characterization using purified antibodies against the TsTx and TsTxI toxins. Since the best results were obtained using the specific anti-TsTx and anti-TsTxI antibodies, we also used a pool of antibodies in the first screening, followed by the use of individual antibodies on a second plate containing positive clones and a few negative clones. The sera from immunized rabbit displayed consistent immunoreactivity with the antigens

Table 1

ELISA results. ELISA was carried out as described in Section 2. The absorbance of pre-immune serum (control) was subtracted. Clones for which no sequencing data is available or no significant similarity has been found using GenBank databases and Fasta program are labelled nd or ns, respectively

<i>Tityus bahiensis</i> cDNA library	ELISA results			Toxin name
	Anti-Tb venom	Anti-TsTx	Anti-TsTx I	
Negative control	0.059	0.067	0.084	–
<i>T. bahiensis</i> venom	2.275	0.950	2.497	–
Clone 7.1	0.128	0.647	0.387	TbTx5 ^a
Clone 7.10	0.100	0.721	0.508	TbTx5 ^a
7.19	0.103	0.570	0.395	nd
7.58	0.096	0.856	0.481	nd
7.75	0.143	0.541	0.348	nd
7.82	0.172	0.624	0.401	nd
24.2	0.369	0.411	0.304	ns
24.33	0.268	0.309	0.328	ns
<i>Tityus serrulatus</i> cDNA library				
	Elisa results			Toxin name
	Anti-Ts venom	Anti-TsTx	Anti-TsTx I	
Negative control	0.052	0.050	0.063	–
<i>T. serrulatus</i> venom	1.292	0.482	1.560	–
Clone 13.33	0.783	0.572	0.757	TsNTxP ^b
Clone 13.49	0.319	0.319	0.303	TsTx I ^c
Clone 13.52	0.156	0.223	0.221	ns
Clone 28	0.163	0.334	0.283	ns
Clone 64	0.121	0.353	0.348	c-TsTxK β ^a
Clone 68	0.105	0.350	0.382	nd

^a The present work.

^b TsNTxP (Guatimosim et al., 1999).

^c TsTx I (Toledo and Neves, 1976; Bechis et al., 1984; Possani et al., 1985).

coated on the microtitration plate, while sera from control mice did not react (data not shown).

We did not expect to detect all immunogenic proteins expressed in the venom gland since some proteins may be post-translationally modified and the *E. coli* system used in the production of the recombinant protein may not be able to modify correctly the protein. In addition, the cDNA must be in frame with the fusion protein (β -galactosidase) and finally, the expression level is different from clone to clone and some clones could express very low levels of recombinant protein. However, several clones were identified as potential immunogenic proteins and the knowledge of these clones may be important to improve anti-serum production and quality (Table 1). This procedure does not allow to screen as many clones as membrane-lifting within a short period of time and good libraries and purified antibodies are recommended. However, this approach gives more quantitative results than the standard visual inspection of antibody signal and approximately 300 clones can be analysed by one person per week. In addition, it is specifically useful for cDNA libraries constructed with RNA isolated from tissues containing a limited number of specific clones such as venom glands. We found that the best time to

induce the cells varies from clone to clone, but 8–10 h yielded recombinant protein detectable by ELISA for most clones. A second round of ELISA was carried out to confirm the positive signal and approximately 90% of the positive clones showed the same result (positive or negative).

Isolated clones were characterized by DNA sequencing, and several were shown to code for previously described proteins, supporting the validity of the process described (see Table 1). To illustrate the methodology, we also showed the complete sequencing of one clone isolated from the *Tityus bahiensis* cDNA library. In reality, two independent clones encoding for the same toxin were isolated (clone 7.1 and 7.10). The toxin described in the present study has been designated TbTx5 (*Tityus bahiensis* Toxin 5) since Becerril et al. (1996) has already reported the sequence of four different toxins isolated from the venom of *Tityus bahiensis* scorpion. The clones encode for a toxin very similar to TbTx IV-5 (93% identity at the amino acid level). Fig. 2 shows the nucleotide sequence of the cDNA encoding the precursor of TbTx5 and also the comparison of amino acid sequence among TbTx5, TbTxIV-5 and TsTx. Sequence analysis showed that the cDNA contained a

A

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ggcagcagattgcc  ATG AAT GAC TTC GTT TTC TTG GTC GTC GCA TGC 46
                  M  N  D  F  V  F  L  V  V  A  C
                  -19                               -10

TTA TTG ACT GCG GGT ACG GAG GGC AAG AAA GAC GGA TAT CCG GTG 91
L  L  T  A  G  T  E  G  K  K  D  G  Y  P  V
                  +1

GAA GGC GAC AAC TGC GCC TTC GTT TGC TTC GGC TAC GAC AAC GCT 136
E  G  D  N  C  A  F  V  C  F  G  Y  D  N  A
          +10                               +20

TAC TGC GAT AAG CTG TGC AAG GAC AAG AAA GCC GAC AGC GGG TAT 191
Y  C  D  K  L  C  K  D  K  K  A  D  S  G  Y
                  +30

TGT TAC TGG GTT CAC ATC CTC TGC TAC TGC TAC GGG CTT CCC GAC 236
C  Y  W  V  H  I  L  C  Y  C  Y  G  L  P  D
          +40                               +50

AAG GAG CCG ACC AAG ACC AAC GGA AGA TGC AAA CCG GGT AAG AAG 281
K  E  P  T  K  T  N  G  R  C  K  P  G  K  K
                  +60

TGA tcccagatccgtcctcgacaatgaatgtttctgaaaaccattcccgaataaatc 339

ccacgcctacaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 385

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B

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TbTx5:  MNDFVFLVVACLLTAGTEGKKDGYFVEGDNCAFCVCFGYDNAYCDKLCCKDKADSGYCYWVHILCYCYGLPDKEPTKTNGRCKPGKK
TbTxIV-5: -----A-----G-----N-----K-----
TsTx:  -----V-----Y---YI.WN-----S-----K...S...

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Fig. 2. (A) Nucleotide sequence of cDNA encoding TbTx5. The coding region is shown in uppercase with the 5' and 3' untranslated regions in lowercase. The signal peptide is indicated in bold. The segment that corresponds to the amino acid sequence of TbTx5 is underlined. (B) Comparison of amino acid sequence of TbTx5 with TbTx IV-5 and TsTx. TbTx IV-5 is a neurotoxin isolated from *Tityus bahiensis* and described by Becerril et al. (1996). TsTx (Tityustoxin) a neurotoxin from *Tityus serrulatus* has been described by different authors (Gomez and Diniz 1966; also called toxin IV-5 by Possani et al. (1981) or Ts IV by Martin-Eauclaire et al. (1994)). Numbering corresponding to the signal peptide is negatively numbered. When there is no similarity the corresponding change is indicated.

short 5'-untranslated region and an open reading frame encoding a peptide of 86 amino acids. The sequence of nucleotides 14 to 70 encodes for a signal peptide of 19 amino acids containing an initiating Met and a series of highly hydrophobic amino acids residues (Fig. 2). TbTx5 and TbTx IV-5 differ by only four residues at positions 9 (G for A), 29 (K for G) 53 (K for N) and position 61 (R for K) and also showed 5 extra residues at the C-terminal peptide. TbTx5 also showed an important similarity with TsTx (also called toxin IV of *Tityus serrulatus* by Martin-Eauclaire et al. (1994)) showing 87% identity at the amino acid level.

Using the *Tityus serrulatus* cDNA library, clones containing the nucleotide sequence encoding for TsNTxP and TsTxI were found. TsNTxP is a protein

described by our group (Guatimosim et al., 1999) that proved to be highly immunogenic, and TsTx I is a neurotoxin active on sodium channel which has been described by several groups (Toledo and Neves, 1976; also called Ts VII by Bechis et al. (1984) and toxin-γ by Possani et al. (1985)). Of interest is clone 64 called here cTsTx-Kβ (cDNA of TsTx-Kβ), whose deduced amino acid sequence (Fig. 3) showed 64% identity with the long chain potassium ion channel blocker BmTxKβ2 isolated from the scorpion *Buthus martensii* (Zhu et al., 1999), approximately 91% with the toxin TsTx-Kβ described by Rogowski et al. (1994) and its sequence is almost identical to the partial sequence of clones Ts-5'Cl.14 and Ts-3'Cl.3 published by Legros et al. (1998). However, a deletion of a guanine base at

A

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ggcacgaggtggaa  ATG GAA AGG AAA TTG GCT CTT CTC TTA 41
                  M  E  R  K  L  A  L  L  L
                  -27                      -20

ATT CTC GGA ATG GTT ACC TTG GCA TCC TGC GGA CTC CGA 80
  I  L  G  M  V  T  L  A  S  C  G  L  R
                        -10

GAA AAG CAC GTT CAG AAG TTG GTG GCT TTA ATT CCC AAT 119
E  K  H  V  Q  K  L  V  A  L  I  P  N
                        +1

GAT CAA TTG AGA TCC ATT CTG AAG GCG GTC GTC CAC AAA 158
  D  O  L  R  S  I  L  K  A  V  V  H  K
      +10                      +20

GTC GCG AAG ACG CAG TTC GGC TGT CCA GCC TAC GAA GGC 197
  V  A  K  T  O  F  G  C  P  A  Y  E  G
                        +30

TAC TGT AAT GAT CAC TGT AAC GAC ATA GAA CGA AAA GAC 236
  Y  C  N  D  H  C  N  D  I  E  R  K  D
                        +40

GGC GAA TGT CAC GGA TTC AAA TGC AAG TGC GCG AAA GAT 275
  G  E  C  H  G  F  K  C  K  C  A  K  D
      +50                      +60

TGA ggtctccgcccgtctattttaagaatggttcctttattccatgtacca 325
-
cgaaccgagccttaataaaaagtcgagtgctatgtcaaaaaaaaaaaaaaa 376

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B

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cTsTx-Kβ:  MERKLALLLILGMVTLASGLREKHVQKLVAL-IPNDQLRSILKAVVHKVAKTQFGCPAYEGYCNDHCNDIERKDGECHGFKCKCAKD
TsTx-Kβ:   -----a.....X.....X..h.....
BmTxKβ2:   .Q.N.VV..F....A.S.....F....KYAV.EGT..T.IQTA...LG.....Q...D...Q..KKEE.F.....
Ts5'Cl.14: -----
Ts3'Cl.3:  -----

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Fig. 3. (A) Nucleotide sequence of cDNA encoding cTsTxKβ. The hydrophobic region (signal peptide) is indicated (bold), the putative propeptide is underlined twice and the segment that corresponds to the amino acid sequence of cTsTxKβ is underlined. (B) Comparison of amino acid sequence of cTsTxKβ with BmTxKβ2 (a putative potassium ion channel blocker from *Buthus martensii*, Zhu et al., 1999); TsTx-Kβ (potassium ion channel blocker from *Titus serrulatus*, Rogowski et al., 1994) and clones Ts5'Cl.14 and Ts3'Cl.3 (Legros et al., 1998). Numbering corresponding to the signal peptide is negatively numbered. When there is no similarity the corresponding change is indicated.

position 102 of this clone was observed and the most likely conclusion is that this deletion is probably a cloning artefact. The toxin encoded by clone cTsTx-Kβ also showed 48% identity with a defensin isolated from the scorpion *Leiurus quinquestratus hebraeus* (Cociancich

et al., 1988). Finally, no significant similarity was found for some other isolated clones after DNA sequence analysis using GenBank databases and the Fasta program of Pearson and Lipman (1988).

In conclusion, we described the use of ELISA in the

screening of expression libraries. The methodology shows good potential for the identification of antigenic proteins and could be very useful for groups interested in information from a specific tissue or organism.

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