0273-2289/05/125/0041/$30.00

Application of Recombinant Phage Display Antibody System in Study of *Codakia orbicularis* Gill Proteins

Jean-Philippe Gourdine,[1] Pamela Greenwell,[2] and Emilie Smith-Ravin*,[1]

[1]Département de Biologie, Equipe DYNECAR EA 926, UFR Sciences Exactes et Naturelles, Université des Antilles et de la Guyane, 97 159 Pointe-à-Pitre, Guadeloupe, E-mail: jsmithra@univ-ag.fr; and [2]Department of Biomedical Sciences, University of Westminster, 115 New Cavendish Street, London W1W 6UW, United Kingdom

Received July 27, 2004; Revised October 22, 2004; Accepted December 6, 2004

Abstract

We used the recombinant phage display antibody system (RPAS) to obtain chimeric single-chain fragment variable (ScFv) antibodies to gill proteins of the white clam *Codakia orbicularis* (Linné, 1758). After three rounds of selection on immunotubes loaded with total gill protein extract, recombinant phages exhibiting antibodies to gill proteins were isolated and tested by enzyme-linked immunosorbent assay (ELISA). Clones exhibiting a high affinity for the mollusk proteins were selected for production of soluble ScFv antibodies, which were purified for subsequent analysis. ScFv antibodies exhibited a reaction specific for a protein whose molecular mass was about 15,000 Daltons and that was detected by the antigen capture technique followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting.

Index Entries: Antigen capture; *Codakia orbicularis*; gill proteins; phage antibodies; single-chain fragment variable.

Introduction

Following the monoclonal antibody (MAb) technique developed by Kohler and Milstein in 1975 *(1)*, a new method for obtaining recombinant antibodies was developed by McCafferty et al. *(2)*. This technique is carried

*Author to whom all correspondence and reprint requests should be addressed.

out using genes for the variable regions of immunoglobulins derived from hybridomas or B-lymphocytes; these genes are amplified by polymerase chain reaction (PCR) and cloned into expression vectors of viral origin. These vectors allow the expression of antibodies at the surface of the virus. This constitutes the recombinant phage display antibody system (RPAS), which makes it possible to obtain single-chain fragment variable (ScFv) antibodies. The major advantage of this technological advance is the expression of antibodies both at the surface of the phage (antibodies linked to the phage) and in the medium (soluble antibodies). Since this biotechnological discovery, numerous genomic libraries of ScFv have been produced. These libraries are generally of three types: *immune*, from B-lymphocytes of animals such as mice or human donors immunized against the desired antigen *(3)*; *naive*, from B-lymphocytes of animals or human donors not immunized against the desired antigen *(4,5)*; and *synthetic*, from the random assembly of the genes of the variable fragments *(6)*. Numerous strategies for producing novel repertoires of recombinant phage display antibodies or antibodies directly produced in bacteria have been developed *(7,8)*. The recombinant antibodies may be composed of only variable parts joined by a linking peptide (ScFv) or by a disulfide bridge (disulfide-linked ScFv), or they may be composed of variable and constant parts (ScFv-Fc or ScFv-IgG) on the phage or in bacteria *(9,10)*.

This technique for the production of recombinant antibodies has several advantages: rapid culture of phage clones; easy handling and detection of secreted antibodies; genetic stability; lower production costs compared with MAbs; use of unpurified antigens from organs or of antigens blotted on membrane *(11,12)*; and use of the recombinant antibodies in traditional immunologic techniques such as enzyme-linked immunosorbent assay (ELISA), Western blotting, and immunohistochemistry *(13)*. The RPAS technique has been exploited in various fields of biology including the biology of invertebrates, such as scorpion toxins *(14)*; plant virology *(15)*; and human virology, such as the acquired immunodeficiency syndrome virus *(16)*. In this article, we present the results obtained using the RPAS method applied to the detection of gill proteins of the white clam *Codakia orbicularis*.

This tropical bivalve inhabits phanerogam seagrass beds of high hydrogen sulfide concentrations. It is readily accessible in shallow waters around the Caribbean island of Guadeloupe. It possesses bacterial chemoautotrophic endosymbionts, which are contained in its gills *(17)*. These microorganisms allow it to withstand the acidity of its environment. The mollusk, by filtering seawater, can obtain oxygen and nutrients such as plankton, and it also absorbs molecules of hydrogen sulfide and sulfuric acid, which without the action of the symbiont would have a destructive action on the gills. It has been shown that the mode of transmission of this sulfur-oxidizing symbiosis is horizontal *(18)*. Histologic studies on the gills of *C. orbicularis* at the adult stage have shown that they are composed of bacteriocytes, basal and ciliated cells, mucocytes, intercalated cells, and

granule cells *(19)*. The latter cells secrete compounds of a protein nature that are thought to be involved in the storage or metabolism of sulfur. Indeed, the presence of sulfur-containing amino acids were revealed in high concentration in the secretory region of the gills. To understand the mechanisms for adapting to a high-sulfur environment and the mechanisms for symbiosis, it is necessary to characterize the gill proteins. The electrophoretic profiles of *C. orbicularis* gill proteins and glycoproteins on acrylamide gel in the presence of sodium dodecyl sulfate (SDS) were recently established *(20)*. They have made it possible to demonstrate disulfide bridges and sugar residues such as *N*-acetylgalactosamine, *N*-acetylglucosamine, α-glucose, and α-mannose in the gill glycoproteins.

In the present study, we used the vector phagemid pHEN2 from the Griffin.1 genomic library containing the variable regions of the heavy chains (V_H) and light chains (V_L) artificially joined by a linking peptide *(6)*. The soluble ScFv antibodies isolated were tested by ELISA, Western blotting, and antigen capture in order to determine the specificity of these antibodies for the gill proteins.

Materials and Methods

Protein Extraction

The *C. orbicularis* coat and gill proteins were extracted according to a protocol described by elsewhere *(20)*.

Biopanning

The vector phagemid (pHEN2) from the Griffin.1 genomic library was used, and three rounds of selection of the phage were carried out according to the Griffin protocol (available on the web site of the Medical Research Council, UK, http://www.mrc-cpe.cam.ac.uk). Immunotubes were loaded with 200 µg/mL of total native protein extract of *C. orbicularis* gills in 50 m*M* $NaHCO_3$, pH 9.6, overnight at room temperature. After washing the immunotubes with PBS, pH 7.0, and blocking them with 2% Marvel Milk PBS for 2 h at room temperature, the antigens were exposed to the phage obtained from the library at a concentration of 10^{12}–10^{13} transforming units for 30 min while rotating the tubes. After allowing the tubes to stand at room temperature for 1 h, 30 min, nonspecific phage was removed by washing 10 times with 0.1% PBS-Tween, and then 10 times with PBS. Specific phage was eluted with 1 mL of 100 m*M* triethylamine; the eluate was neutralized with 0.5 mL of 1 *M* Tris, pH 7.4; and the phage populations were then enriched. The *Escherichia coli* strain TG1 was infected with the eluted phage with the aid of the helper phage M13K07. Three rounds of selection were used, and the phage from the final selection was used to infect the *E. coli* strains TG1 and HB2151. The titer of the phage populations selected was recorded after each round of selection.

Propagation of Phage-ScFv for ELISA

The *E. coli* strain TG1 was used for the propagation of the phage-ScFv, and the cultures were carried out in 96-well plates. The infected TG1 bacteria were cultured overnight at 37°C, with shaking (100 rpm), in 100 µL of 2TY medium containing 100 µg/mL of ampicillin and 1% glucose. A 2-µL aliquot of this culture was added to 200 µL of fresh 2TY medium containing 100 µg/mL of ampicillin and 1% glucose, and then the suspension was left for 1 h with shaking. Helper virus M13KO7 at 10^9 plaque-forming units was added, and the medium was kept for 30 min without shaking, and then for 1 h with shaking. The suspension was centrifuged at 1800*g* for 10 min; the pellet was resuspended in 200 µL of 2TY medium containing 100 µg/mL of ampicillin, 50 µg/mL of kanamycin, and 1% glucose; and the medium was left overnight at 37°C with shaking. After centrifugation at 1800*g* for 10 min, 100 µL of supernatant was used directly as immunological reagent.

Production of Soluble ScFv

The *E. coli* strain HB2151 was used for the production of soluble ScFv for ELISA, and the culture volumes were identical to those for the propagation of the phage-ScFv. For the production of ScFv for Western blotting and antigen capture on plates, the culture volume was 10 mL. The infected HB2151 bacteria were cultured overnight in 2TY medium containing 100 µg/mL of ampicillin and 1% glucose with shaking at 100 rpm. A 100-µL aliquot of this culture was added to 2TY medium containing 0.1% glucose, and the suspension was kept shaking until the optical density (OD) at 600 nm reached 0.9 (about 3 h). A volume of 1.25 mL of 2TY medium containing 100 µg/mL of ampicillin and 9 m*M* isopropyl-β-D-thiogalactoside was added, and the suspension was kept shaking (100 rpm) at 30°C overnight. After centrifugation at 1800*g* for 10 min, the supernatant was used directly as immunologic reagent or purified either by filtration on 0.45-µm filters or by ammonium sulfate precipitation (75% saturation) as indicated next.

Ammonium Sulfate Precipitation

The supernatant containing ScFv was subjected to ammonium sulfate precipitation (75% saturation) overnight at +4°C and was then centrifuged at 12,400*g*. The pellet was resuspended in PBS and dialyzed, with several changes, against PBS overnight at +4°C. The dialysate, supplemented with 0.02% sodium azide, was stored at +4°C for subsequent use.

Enzyme-Linked Immunosorbent Assay

For the selection of phage clones exhibiting high affinity for the gill proteins, ELISA reactions were carried out using phage-ScFv and soluble ScFv. ELISA plate wells were coated with 100 µL of nondenatured *C. orbicularis* protein extracts per well at a concentration of 100 µg/mL in 50 m*M* $NaHCO_3$, pH 9.6. The plate was incubated overnight at +4°C. After wash-

ing three times with PBS, the nonspecific sites were blocked with PBS-3% bovine serum albumin (BSA) for 2 h at 37°C. After washing with PBS, 100 µL of phage supernatant or supernatant containing the soluble ScFv antibodies was added. The plate was incubated for 1 h at room temperature. The wells were washed as just described. The procedure was as follows in the case of indirect ELISA: Fifty microliters of supernatant of anti-c-myc antibody (9E10) and 50 µL of peroxidase-conjugated antimouse antibody diluted 1/500 in PBS-1% BSA were added, and the plate was incubated for 1 h at room temperature. For direct ELISA, peroxidase-conjugated anti-M13 antibody diluted 1/5000 in PBS-3% BSA was added, and the plate was incubated for 1 h at room temperature. In both cases, three washes were carried out with PBS-Tween-20 and then three washes with PBS, and, finally, the reaction was visualized with tetramethylbenzidine (TMB)/H_2O_2. Fifty microliters of 3 *M* H_2SO_4 was used to stop the colored reaction. The intensity of the reaction was read on an ELISA reader at $OD_{450nm.}$

Antigen Capture and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

ELISA plate wells were saturated with 100 µL of ScFv overnight at +4°C. After washing three times with PBS, the plates were incubated for 2 h at 37°C with 100 µL of gill proteins diluted 1/10 in 50 m*M* $NaHCO_3$. After washing three times with PBS, 5 µL of Laemmli buffer containing SDS and 1 *M* dithiotreitol (DTT) was added per well and allowed to act for 5–10 min at 37°C. The solutions were recovered and loaded onto a 12% polyacrylamide-SDS gel for electrophoresis. A control was prepared by replacing gill protein antigens with coat protein extract of *C. orbicularis*. Figure 1 presents the antigen capture procedure used.

Western Blotting

The clam gill proteins were transferred onto polyvinylidene difluoride by electrophoresis as previously described *(20)*. After blocking for 1 h at 37°C with PBS-1% BSA containing 0.1% Tween-20, the supernatant containing ScFv antibodies was added and the medium was incubated for 1 h at room temperature with shaking. The secondary antibody anti-c-myc (9E10) diluted 1/100 in PBS-1% BSA was added for 1 h with shaking, and then horseradish peroxidase (HRP)-conjugated tertiary antibody (Sigma, St. Louis, MO) diluted 1/100 in PBS-1% BSA was added for 1 h with shaking. After each incubation, three 5-min washes with TBS-0.1% Tween-20 and three washes with TBS were carried out. Visualization was made with diaminobenzidine (DAB) and hydrogen peroxide.

Immobilized Metal Affinity Chromatography

Purification of ScFv containing the histidine tag was carried out by immobilized metal affinity chromatography (IMAC) with a Qiagen Ni-NTA Spin kit using bacterial pellets from infected HB2151 bacterial

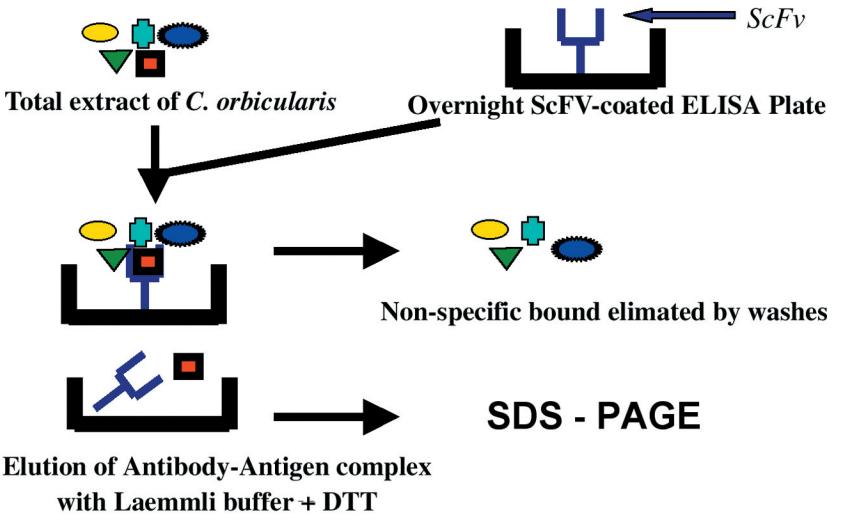

Fig. 1. Schematic representation of antigen capture procedure. Gill or coat proteins were used as antigens.

cells (the same conditions as described under Production of Soluble ScFv). The bacterial cells were lysed with urea buffer, pH 8.0 (8 *M* urea, 0.1 *M* NaH_2PO_4, 0.01 *M* Tris-HCl), for 1 h at room temperature with shaking. The lysate was centrifuged at 10,000*g* for 20 min and then loaded onto a nickel column previously equilibrated with urea buffer, pH 8.0. The column was centrifuged for 4 min at 700*g*. After two washes by centrifuging for 4 min at 700*g* with urea buffer, pH 6.3 (8 *M* urea, 0.1 *M* NaH_2PO_4, 0.01 *M* Tris-HCl), the column was eluted twice by centrifuging with 200 µL of urea buffer, pH 4.5 (8 *M* urea, 0.1 *M* NaH_2PO_4, 0.01 *M* Tris-HCl), and an aliquot of each eluate fraction was loaded onto an SDS-polyacrylamide gel. ScFv concentrations were estimated by densitometric analysis of gels scanned with a Bio-Rad ChemiDoc image acquisition device equipped with Quantity One software according to the manufacturer's instructions and using glyceraldehyde-3-phosphate dehydrogenase as reference marker.

Results and Discussion

Phage Antibody and ScFv Isolation

The recombinant phage display antibody technique developed by McCafferty et al. *(2)* was applied, for the first time, to the production of antibodies to *C. orbicularis* gill proteins. Of the phage clones selected and analyzed, 87.5% were positive and 12.5% were negative in the presence of native protein antigens from *C. orbicularis* gills (Table 1). All the clones that were positive for the gill proteins were negative in the presence of coat proteins (negative control). They were selected for the production of soluble antibodies and tested by ELISA against gill antigens and coat antigens.

Table 1
ELISA Reactions Using Clones Selected for Antibodies to Gill and Coat Proteins[a]

	No. of reactions	
	+	–
Gill proteins	14	2
Coat proteins	0	16

[a]The intensity of the colored reactions was assessed visually and compared with the negative control. +, positive reaction; –, negative reaction.

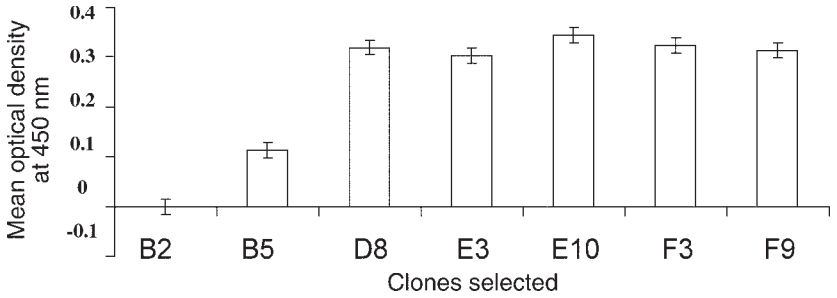

Fig. 2. ELISA reactions using selected clones. The reactions were performed with soluble antibodies. Five experiments were carried out using seven clones. Vertical bars represent standard deviations.

Figure 2 presents the results of five experiments for seven clones. None of the clones exhibited positive staining with coat proteins. Five clones had mean OD_{450nm} values ≥0.3, one had a value of 0.114, and another had a value of practically zero.

We were able to isolate clones capable of expressing antibodies specific for *C. orbicularis* gill protein antigens. The novelty of our work lies in the use of the RPAS against a total pool of native antigens. Some investigators have reported the selection of antigens by Western blotting *(12)* on tissue sections *(11)* or at the surface of cells in culture *(21)*. In our experiments, a high level of positive clones of recombinant antibodies to gill proteins of *C. orbicularis* was obtained in three rounds of selection, which were sufficient to greatly amplify the initial phage population (Table 2). This high level of positive clones is comparable with that obtained by the traditional MAb technique *(1)*. Analysis of the ELISA results obtained for the phage antibodies and the soluble antibodies shows that some strongly positive phage antibody clones can, in some instances, give weakly positive soluble antibodies (Table 3). This phenomenon could reflect, on the one hand, a change in affinity between the phage antibodies and the soluble antibodies and, on the other hand, a decrease in their production associated

Table 2
Enrichment of Specific Phages Presenting Antibodies to Gill Proteins[a]

	Round		
	1st	2nd	3rd
Titer (phage/mL)	12×10^3	41×10^3	38×10^5
OD_{450nm}	0.155	0.285	1.620

[a]The titer of the phages selected and the OD_{450nm} of the ELISA reactions against gill proteins were measured after the different rounds of selection.

Table 3
ELISA Reactions Using Phage Antibodies and Soluble ScFv for Three Clones[a]

	Clones					
	D8		E3		E10	
Antibody	Phage	ScFv	Phage	ScFv	Phage	ScFv
OD_{450nm}	0.200	0.147	0.250	0.167	0.300	0.180

[a]Clones were tested against gill proteins. The intensity of the colored reactions was read at OD_{450}nm.

or otherwise with a loss of the *c-myc* gene *(22)*. This variability in the production of the ScFv antibodies may be owing to changes in bacterial metabolism. McCafferty et al. *(2)* showed that for a given clone, the production of soluble ScFv antibodies varies between 2 µg and 1000 µg/mL of culture (Greenwell, P., personal communication, 2003). Densitometric analysis with the Bio-Rad ChemiDoc image acquisition device following IMAC purification allowed us to estimate the yield of ScFv antibodies at about 200 µg/mL of culture. This could also be explained by the way the antigen-antibody complex is detected in the ELISA reaction. On the one hand, the detection occurs via the pIII protein that is present in several copies on the phage antibody and, on the other hand, it occurs via the c-*myc* oncogene that is present in a single copy in the ScFv antibody. However, ELISA tests using phage antibody and ScFv antibody showed mean OD_{450nm} values of 0.048 and 0, respectively, with coat proteins as controls. These results demonstrate the specificity of the selected antibodies toward the gill proteins. In our results, the mean OD_{450nm} values of between 0.30 and 0.34 of the five positive clones indicate a relative homogeneity in the metabolism of these clones (Fig. 2). We plan to carry out PCR and sequencing experiments in the future in order to establish whether these clones are unique or are multiple representations of the same clones.

Antigen Capture, Western Blotting, and Purification of Antibody

The concentrated ScFv antibody preparations obtained under Materials and Methods were used in antigen capture. SDS-polyacrylamide gel electrophoresis (PAGE) for this experiment and that for *C. orbicularis* gill

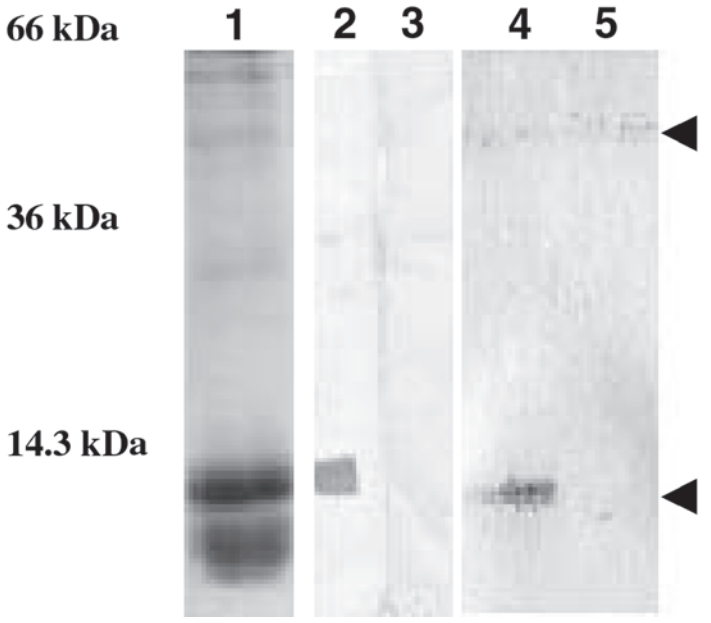

Fig. 3. SDS-PAGE of antigens obtained from antigen capture and Western blotting. All the samples were treated with DTT. Arrowheads indicate protein bands at 60,000 and 15,000 Daltons. Molecular weights are indicated on the left. Lane 1, SDS-PAGE of *C. orbicularis* gill proteins; lane 2, SDS-PAGE of F9-ScFv complexes on *C. orbicularis* gill protein extracts; lane 3, SDS-PAGE of F9-ScFv complexes on *C. orbicularis* coat protein extracts; lane 4, Western blot of *C. orbicularis* gill proteins visualized by means of B5-ScFv; lane 5, Western blot of *C. orbicularis* coat proteins visualized by means of B5-ScFv.

proteins revealed a similar band of about 15,000 Daltons, which was also found in Western blotting (Fig. 3). The F9 clone, which was selected for the production of ScFv, proved to be specific for the same 15,000-Dalton protein that constitutes one of the major proteins in this clam gill *(20)*. Another band of 60,000 Daltons, of much weaker intensity, was detected by antigen capture with gill or coat proteins. It may correspond to the ScFv-pIII fusion protein resulting from the expression of the amber codon *(23)*. The control for the Western blotting, prepared with *C. orbicularis* coat proteins, showed no bands. Some of the clones selected proved to be specific for a glycoprotein band of high molecular weight as previously reported *(20)*. The high glycosylation pattern of these *C. orbicularis* proteins would explain the increased tendency to obtain numerous ScFv clones specific for the same type of antigens. The sugar motifs are thought to increase competition for the selection of ScFv *(12)*.

The culture supernatants containing ScFv antibodies of clones E3 and F9 were subjected to denaturing gel electrophoresis on 18% polyacrylamide gel, in the presence of DTT, without further purification. Figure 4 shows a band of 30,000 Daltons characteristic of the ScFv antibodies for

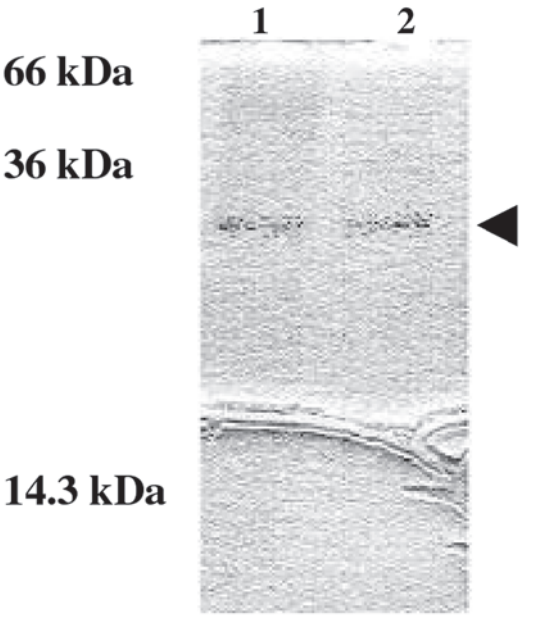

Fig. 4. Denaturing gel electrophoresis. Culture supernatants of clones E3 and F9 were analyzed as described under Materials and Methods without further purification. Molecular weights are indicated on the left. Lane 1, E3; lane 2, E9. Arrowheads indicate ScFv at 30,000 Daltons.

both clones E3 and F9. Lanes 1 and 2 represent E3 and F9, respectively. The culture supernatants of several other clones containing ScFv antibodies were subjected to electrophoresis as just described after purification on IMAC columns. Figure 5 shows the results obtained for clone B5. Lanes 1 and 2 represent the cellular lysate and flow-through respectively; lanes 3–5, the washings; and lanes 6 and 7, the eluates. The flow-through was comparable to the cellular lysate and contained proteins that did not remain attached to the nickel column. The different washings did not reveal the presence of any major proteins. A protein of about 30,000 Daltons, characteristic of ScFv antibodies and present in the cellular lysate and the flow-through, was observed in the eluates but was absent from the washings. This eluted protein corresponds to the ScFv antibody possessing the hexahistidine tag. It has been reported that the molecular weight of the ScFv antibodies varies between 26,000 and 30,000 Daltons *(24)*. We observed the presence of a faint band of similar size from SDS-PAGE following antigen capture. This observation may be attributed to the difference in the purification of ScFv between IMAC and ammonium sulfate precipitation followed by antigen capture. The IMAC purification is based on the affinity between the histidine peptide of the ScFv antibody and the nickel column and has a better yield than that obtained from ammonium sulfate precipitation and filtration *(25)*. These results can also be explained by the use of culture supernatants containing a low concentration of ScFv compared with the periplasm *(10)*.

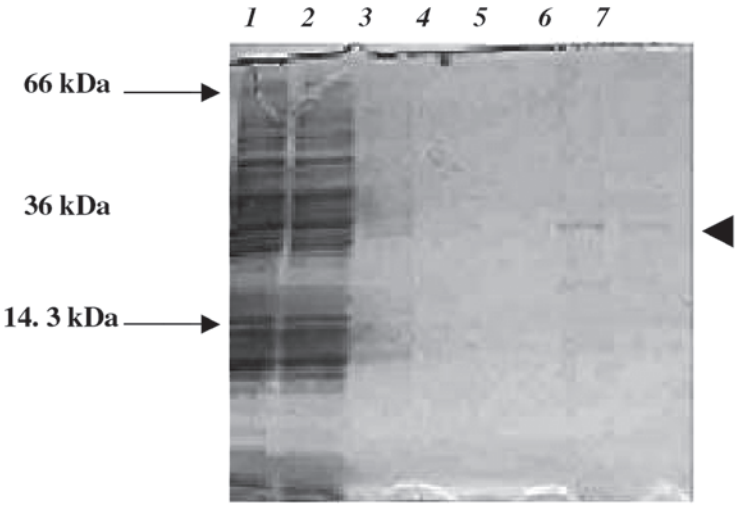

Fig. 5. Denaturing gel electrophoresis. Culture supernatants of the B5 clone were purified on IMAC columns, and the IMAC samples were separated by electrophoresis on an 18% polyacrylamide gel as described under Materials and Methods. Molecular weights are indicated on the left. Lane 1, cellular lysate; lane 2, flow-through; lanes 3–5, washings; lanes 6 and 7, eluates. Arrowheads indicate ScFv at 30,000 Daltons.

Conclusion

Our work shows the application of the RPAS technique to the production of phage-ScFv antibodies to clam proteins. It has indeed been possible to isolate several recombinant antibodies to a major gill protein using a native antigen extract of the bivalve *C. orbicularis*. These antibodies will be valuable in carrying out further immunologic studies to elucidate the location of this gill protein and its role in this clam, which inhabits a high-stress environment.

Acknowledgments

We wish to thank La Région Guadeloupe and La Société Anonyme de la Raffinerie des Antilles for funding this work and for a grant to J.P.G. We also thank La Société Karulabo for providing reagents.

References

1. Kohler, G. and Milstein, C. (1975), *Nature* **256,** 495–497.
2. McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990), *Nature* **38,** 552–554.
3. Barbas, C. F. 3rd, Collet, T. A., Amberg, W., et al. (1993), *J. Mol. Biol.* **230,** 812–823.
4. Gram, H., Marconi, L. A., Barbas, C. F. 3rd, Collet, T. A., Lerner, R. A., and Kang, A. S. (1992), *Proc. Natl. Acad. Sci. USA* **89,** 3576–3580.
5. Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J., and Johnson, K. S. (1996), *Nat. Biotechnol.* **14,** 309–314.

6. Griffiths, A. D., Williams, S. C., Hartley, O., et al. (1994), *EMBO J.* **13,** 3245–3260.
7. Winter, G. (1998), *FEBS Lett.* **430,** 92–94.
8. Griffiths, A. D. and Duncan, A. R. (1998), *Curr. Opin. Biotechnol.* **9,** 102–108.
9. Shan, D., Press, O. W., Tsu, T. T., Hayden, M. S., and Ledbetter, J. A. (1999), *J. Immunol.* **162,** 6589–6595.
10. Kontermann, R. and Dubel, S. (2001), *Antibody Engineering*, Springer Lab Manual, Heidelberg.
11. Johns, M., Georges, A. J. T., and Ritter, M. A. (2000), *J. Immunol. Methods* **239,** 137–151.
12. Foy, B. D., Killeen, G. F., Frohn, R. H., Impoinvil, D., Williams, A., and Beier, J. C. (2002), *J. Immunol. Methods* **261,** 73–83.
13. Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D., and Winter, G. (1994), *EMBO J.* **13,** 692–698.
14. Mousli, M., Devaux, C., Rochat, H., Goyffon, M., and Billiald, P. (1999), *FEBS Lett.* **442,** 183–188.
15. Chae, J.-S., Choi, J.-K., Lim, H.-T., and Cha, S.-H. (2000), *Mol. Cell* **11,** 7–12.
16. Herschhorn, A., Admon, A., and Hizi, A. (2003), *Biochim. Biophys. Acta* **1648,** 154–163.
17. Berg, C. J. and Alatalo, P. (1984), *Aquaculture* **39,** 165–179.
18. Gros, O., Darasse, A., Frenkiel, L., and Mouëza, M. (1996), *Appl. Environ. Microbiol.* **62,** 2324–2330.
19. Frenkiel, L. and Mouëza, M. (1995), *Zoomorphology* **115,** 57–61.
20. Gourdine, J.-P. and Smith-Ravin, E. J. (2002), *Prep. Biochem. Biotechnol.* **32,** 341–353.
21. Hombach, A., Pohl, C., Heuser, C., Sircar, R., Diehl, V., and Abken, H. (1998), *J. Immunol. Methods* **218,** 53–61.
22. Harper, K., Toth, R. L., Mayo, M. A., and Torrance, L. (1999), *J. Virol. Methods* **81,** 159–168.
23. Zavala, A. G., Lancaster, T., Groopman, J. D., Strickland, P. T., and Chandrasegaran, S. (2000), *Nucleic Acids Res.* **28,** E24.
24. McCafferty, J. and Johnson, K. S. (1996), in *Phage Display of Peptides and Proteins: A Laboratory Manual*, Kay, B. K., Winter, J., and McAfferty, J., eds., Academic, San Diego, pp. 227–276.
25. Muller, K. M., Arndt, K. M., Strittmatter, W., and Pluckthun, A. (1998), *FEBS Lett.* **442,** 259–264.