An improved non-denaturing method for the purification of spiralin, the main membrane lipoprotein of the pathogenic bacteria *Spiroplasma melliferum.*

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Running title:
High performance spiralin purification

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Highlights

- An improved procedure for the purification of a bacterial lipoprotein is presented.
- The new procedure shortens the purification from 24 hours to 4 hours.
- Detergent screening revealed that spiralin is mostly insoluble in Sarkosyl and Triton X-100.
- Milligram quantities of highly pure spiralin can be obtained.
- The procedure is non-denaturing.

Abstract

Spiralin is the most abundant protein of several species of spiroplasmas, helical, motile bacteria pathogenic for arthropods and plants. This amphiphilic protein is anchored to the outer face of the plasma membrane by a lipoylated *N*-terminal cysteine. Although spiroplasma pathogenicity in mammals is controversial, it was shown that spiralin is highly immunogenic and endowed with immunomodulatory activity. In this paper, we describe a high performance method for the purification of *Spiroplasma melliferum* spiralin under non-denaturing conditions. The protein was selectively extracted with 3-[(3-cholamidopropyl) dimethylammonio]-1-propyl sulfonate (CHAPS) from the membrane pre-treated with sodium dodecyl-*N*-sarcosinate (Sarkosyl), and purified to homogeneity by cation-exchange HPLC with an overall yield of ~ 60%. Detergent-depleted, water-soluble micelles of spiralin displaying a mean diameter of 170 Å, as evidenced by transmission electron microscopy, were obtained by dialysis detergent removal. Circular dichroism spectroscopy and cross immunoprecipitation assay of the purified spiralin strongly suggested that this purification method could retain the structural characteristics of the native spiralin. The strategy developed to purify spiralin (two successive selective extractions of membrane proteins with mild detergents followed by ion-exchange chromatography) should prove useful for the purification of membrane lipoproteins of other bacteria of the class *Mollicutes* including different pathogens for humans, animals and plants.

Keywords: CE-HPLC; Detergents; Membrane lipoprotein; Spiralin; *Spiroplasma melliferum*; Mollicutes

1. Introduction

Bacterial membrane lipoproteins are bound to the lipid bilayer by a di- or a tri-fatty-acylated *N*-terminal cysteine [for a recent review: [1]]. This covalent modification occurs in proteins belonging to different families acting as cell receptors, enzymes, transporters, or adhesins. Some of them, such as *E. coli* Braun's lipoprotein have also a structural function [2-4]. In host-bacteria interactions, the lipidic moiety of lipoproteins modulate the activity of the vertebrate immune system through binding to host Toll-like receptors [5]. Some lipoproteins further display antigen variability within their polypeptide chain enabling the pathogen to evade the host immune defenses [6]. In Mollicutes, murein-less bacteria including *Mycoplasma* spp. and *Spiroplasma* spp., lipoproteins are tightly anchored to the external leaflet of the plasma membrane and face the host environment. A large number of mycoplasmal lipoproteins have been associated to bacterial cytadherence [7], to phase, size, and antigenic variation [8], or to immunomodulatory effects [9-11]. Spiralin is the major membrane lipoprotein of a certain number of *Spiroplasma* species infecting plants and arthropods. This lipoprotein was first discovered in *Spiroplasma citri* [12] and later in *S. melliferum* [13], *S. kunkelii*, and *S. phoeniceum* [14]. Spiralin is one of the most well-studied Mollicute lipoproteins as evidenced by the large range of structural, expression and functional data available [15-20]. Spiralin purified from *S. melliferum* membrane possesses immunostimulating activity on human peripheral blood mononuclear cells and murine splenocytes [16]. In the phytopathogenic *S. citri*, spiralin is essential for an efficient transmission of the bacteria to the plant host by the insect vector [18], is involved in the invasion of the insect cells, and exhibits glycoconjugate binding properties [21]. In addition, given the high abundance of the protein, which is located exclusively on the outer leaflet of the bacterial membrane, a mechanical function for spiralin has been proposed in addition to its putative role as a lectin. However, structural information that could help understanding the function of spiralin is still missing. For instance, no information on the tertiary structure of spiralin is available to date. Similarly, no thorough mass spectrometry analysis of the protein to elucidate whether spiralin is a target of unidentified posttranslational modifications as proposed before [22] has been conducted.

Valuable information about the function of spiralin could thus be obtained from structural studies. However, the availability of efficient purification procedures is still nowadays the bottleneck of lipoprotein studies. The general strategy of recombinant technology consisting of overproducing the protein in a genetically modified host cell has allowed the purification of a large number of proteins. However, in the case of Mollicutes

lipoproteins, many difficulties may be encountered when using the recombinant methodology. The solubilization of the overproduced amphiphilic protein may prove difficult without loss of the native structure. The host cell may also fail to perform the covalent modifications of the naturally produced protein. Incorrect interpretation of the membrane targeting signals and/or toxicity due to the accumulation of the foreign protein in the cell membrane(s) may occur.

In the case of *S. citri* spiralin, a recombinant protein could be expressed in *E. coli* [23]. However, the recombinant product proved to be localized not only in the inner membrane but also in the outer membrane and cytoplasm of *E. coli* [24]. In addition, the recombinant spiralin did not appear to be correctly post-translationally modified as three forms differing in their apparent molecular mass could be detected in *E. coli*, and not in *S. citri* [24]. In the past, we developed different strategies to purify spiralin [12,17,20]. Starting from *S. citri* membranes, the purification process of the native spiralin entailed essentially three steps: (1) removal of extrinsic proteins using Tween 20 or Sarkosyl, (2) selective extraction of spiralin with 0.2 M sodium deoxycholate (DOC) or 100 mM CHAPS, and (3) fractionation of the DOC/CHAPS-soluble material by preparative agarose-suspension electrophoresis (ASE). However, this protocol is time-consuming and the amount of protein obtained is limited to 1 mg per separation. Moreover, the different steps were empirically designed. In this work, we aimed at optimizing the spiralin purification protocol by screening a variety of detergents for the initial extraction step, and establishing a cation exchange chromatography for the final purification. We could thereby shorten the total duration of the process and the final separation step offered a higher adaptability to amount of available starting material.

2. Materials and methods

2.1. Reagents

Acrylamide and *N,N'*-methylene bisacrylamide were from BDH (U. K.). Agarose (Indubiose A37) was from IBF (France). HECAMEG, *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulfonate (SB-12, lauryl sulfobetaine), *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propane sulfonate (SB-14, tetradecyl sulfobetaine), 3-[(3-cholamidopropyl) dimethylammonio]-1-propyl sulfonate (CHAPS), *N*-octyl-ß-D-glucopyranoside (OG), : nonaethyleneglycol octylphenol ether (Triton X-100) and sodium lauroyl-*N*-sarcosinate (Sarkosyl) were obtained from Sigma (U.S.A.). Sodium cholate and sodium deoxycholate (DOC) were from Calbiochem (La Jolla, California, U. S. A.). Sodium dodecylsulfate (SDS) was from Merck (Germany). *N*-dodecyl-*N*,*N*-dimethylammonio butyrate (DDMAB) and *N*-dodecyl-*N*,*N*-dimethylammonio undecanoate (DDMAU) were generous gifts from Y. Chevalier (CNRS

UPR 9031, Vernaison, France). Cholylsarcosine was a generous gift from Diamalt (München, Germany). *N*-octanoyl-beta-D-glucosylamine (NOGA) was synthesized through the procedure described in Brenner-Henaff et al. (1993).

2.2. Protein concentration determination

Protein concentration was determined with the method of Lowry *et al*. [25] modified by Markwell *et al*. [26], using bovine serum albumin as standard. Titration of individual proteins was performed by scanning densitometry after SDS-PAGE and Coomassie brilliant blue R250 staining.

2.3. Preparation of S. melliferum membranes

S. melliferum (strain BC-3^T, ATCC 33219) [27] was grown under microaerobic conditions as previously described [28]. Cells were harvested by centrifugation at 10,000*g* for 15 min and washed once in 50 mM sodium phosphate buffer containing 150 mM NaCl and 550 mM D(-)sorbitol. The cells were then dispersed in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM of 4-(2-aminoethyl)-benzene sulfonyl fluoride (protease inhibitor, AEBSF) and disrupted by sonication (2 x 30 sec) with 500-Watt Ultrasonic Processor equipped with a titanium-tapered microtip. Plasma membranes were centrifuged three times 1 h at 38,000*g* in 50 mM Tris-HCl buffer pH 8.0 to remove cytoplasmic components. They were dispersed and stirred overnight in 5 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 1 mM DTT. They were finally centrifuged at 38,000*g* and dispersed in 10 mM HEPES buffer (pH 7.4). All the operations were performed at 0-4°C and 5 ml membrane suspensions containing 10 mg protein ml^{-1} were stored at –80°C until use.

2.4. Membrane protein extraction with detergents

Proteins were extracted with detergents by mixing one volume of membrane suspension (10 mg of protein per ml of 100 M Na phosphate buffer, pH 7.5) with one volume of detergent solubilized in water. After 1 h with intermittent shaking, the mixture was centrifuged at 260,000*g* for 15 min (Beckman TL-100 ultracentrifuge, TLA 100.1 rotor) to separate solubilized proteins from insoluble material. Operations were performed at 18°C when SDS was used and at 4°C in the case of other detergents.

2.5. Preparation of antibodies

Antibodies against *S. melliferum* membrane proteins were elicited in three rabbits by subcutaneous inoculation, twice a month, of isolated membranes. Each inoculum was composed of 1 mg of membrane protein in 0.5 ml of 150 mM NaCl and emulsified with 0.7 ml of Freund adjuvant. The latter was used complete only for the first inoculation. After 3 months of immunization, the rabbits were bled and the three sera were pooled. The same procedure was used to obtain the antibodies which can recognize spiralin specifically. In that case, the immunogen was composed of 10 µg of pure spiralin detergent-free micelles per inoculum.

2.6. Chromatographic techniques

Preparative protein separations were performed by cation exchange chromatography in a column of methacrylate co-polymer resin beads covalently bonded with propylsulfonic acid functionalities (Waters Protein Pack SP8HR: resin particle diameter, 8 µm; pore size, 0.1 µm; column internal diameter, 1 cm; column length, 10 cm) and a Waters 625 HPLC system. The samples contained membrane proteins solubilized with 100 mM CHAPS in 20 mM sodium citrate buffer (pH 4.5) containing 4 mM DTT. Elution of the proteins adsorbed on the column was performed with a salt concentration gradient using the following buffers: 20 mM sodium citrate buffer (pH 4.5) containing 16 mM CHAPS and 2 mM DTT (Buffer A) and buffer A containing 1 M NaCl (Buffer B). Elution conditions (1 ml min^{-1}): 0 to 5 min, 100% buffer A; 5 to 40 min, linear gradient to 30% buffer B; 40 to 50 min, 30% buffer B; 50 to 60 min, linear gradient up to 100% buffer B; 60 to 75 min, 100% buffer B, and 75 to 80 min, linear gradient down to 0% buffer B. The eluates were monitored by light absorbance at 280 nm and fractions were analyzed by SDS-PAGE.

The homogeneity of spiralin detergent-free micelles was analyzed by size-exclusion HPLC (SEC) in a Superdex 200 HR column (diameter, 1 cm; height, 30 cm) (Pharmacia, Uppsala, Sweden). The buffer used for column equilibration and protein elution (0.4 ml min^{-1}) was 50 mM sodium phosphate buffer pH 7.0 containing 150 mM NaCl. Spiralin and reference proteins (thyroglobulin, ferritin, catalase, apotransferrin, hemoglobin and cytochrome c) were detected by light absorption at 280 nm.

2.7. Preparation of spiralin detergent-depleted micelles

Detergent was removed using the Lipoprep® (Dianorm) flow-through dialyzer [29]. The central compartment of the Teflon cell was loaded with 8 ml of a solution of spiralin (2 mg protein per ml of 10 mM sodium phosphate buffer) containing 16 mM CHAPS. Dialysis was

performed for 36 h at 6°C through two cellulose membranes (diameter, 5 cm; cut-off, 10 kDa). The solution was stirred with a magnet spinning at 60 rev. min^{-1} and the dialysis buffer (10 mM sodium phosphate buffer, pH 7.2) was pumped through the two side compartments at a flow rate of 5 ml min^{-1}.

2.8. SDS-PAGE

Protein extracts were diluted 1:10 in loading buffer (62.5 mM Tris-HCl pH 6.8, 20 mM DTT, 2% SDS, 40% glycerol, and 0.01% bromophenol blue). The proteins were separated in 120 x 120 x 1 mm polyacrylamide gels in the presence of 0.1% SDS with the method of Laemmli [30]. Compositions of the stacking and separating gels were T = 4.8%, C = 2.6% and T = 10%, C = 2.6%, respectively, with %T being the total monomer (acrylamide + bisacrylamide) concentration and %C the percentage of bisacrylamide relative to total monomer. Proteins were silver stained according to the method of Tunón and Johansson [31].

2.9. Immunoelectrophoresis

Crossed immunoelectrophoresis [32] was performed as described previously [33], in the presence of 10 mM SB_{12} to avoid the precipitation of hydrophobic proteins. Antibodies against *S. melliferum* membranes were prepared as described above and the serum was used at a 1/20 dilution. The directions of migration are indicated on Figure 7. After electrophoresis, the gels were washed and dried, and the immunoprecipitates were stained with Coomassie brilliant blue R-250.

Fused-rocket immunoelectrophoresis was performed as described previously [12], using antibodies directed against *S. melliferum* membrane similarly to the crossed immunoelectrophoresis.

2.10. Circular dichroism spectroscopy

Circular dichroism spectra of spiralin were recorded from 190 to 260 nm at 20°C on a Jasco 810 (Jasco, Bouguenais, France) spectropolarimeter equipped with a thermostatically controlled quartz cell with a path length of 2 mm. Spectra were required at a step resolution of 0.1 nm and at a 50 nm min^{-1} speed (bandwidth: 1 nm). The sample was prepared in 10 mM sodium phosphate buffer pH 7.4 at a final protein concentration of 3.8 µM. When needed, 50 mM CHAPS or 35 mM SDS was included in the samples. For each analysis three scans were performed and subsequently averaged. Corrections were made for buffer contribution.

Spectrum deconvolution was performed using the CDPro package. The ContinLL method was chosen to estimate secondary structure of spiralin. Wavelengths from 190 to 240 nm were submitted for analysis and the SMP56 reference set was chosen [34].

2.11. Transmission electron microscopy

Suspensions of detergent-free spiralin micelles (0.02 mg ml^{-1} of 50 mM sodium phosphate buffer pH 7.4) were deposited on glow-discharged carbon-coated 200-mesh copper grids. After one min of contact, the adsorbed micelles were washed three times with distilled water and negatively contrasted with 2% uranyl acetate. Micrographs were taken with a Philips CM12 microscope operating at 120 kV and at a magnification of x 45,000.

3. Results and Discussion

3.1. Selective extraction of spiralin from the spiroplasma membrane

Selective extraction of membrane proteins by detergents has proven to be a very powerful tool to purify proteins [35,36]. Spiralin can be extracted from spiroplasma membranes by sequential action of detergents [12]. In the original protocol describing the purification of spiralin, Tween 20 was used to deplete the whole membranes from proteins leaving most of the spiralin in the insoluble fraction. Then, spiralin was solubilized by DOC, which selectively extracted this protein. Later, Sarkosyl was used in place of Tween 20 to reduce the time required for the extraction, and DOC was replaced by CHAPS [20]. There was no rationale behind the choice of these detergents except that they are non-denaturing detergents. We then decided to screen for other detergents which would have low selectivity for spiralin and could be used to deplete non-spiralin proteins from the membranes.
Fifteen non-denaturing detergents were compared for their ability to extract spiralin from the plasma membrane of *S. melliferum*. In each case, the amount of spiralin *vs.* total protein extracted was determined as a function of detergent concentration. Fig. 1 illustrates the results

obtained with cholylsarcosine, CHAPS, and Sarkosyl, and Table 1 recapitulates the data obtained with the whole series of detergents. Most of the detergents extracted spiralin efficiently and selectively except for Triton X-100 and Sarkosyl. Triton X-100 could extract only 20% of proteins from the membrane and provide low amounts of spiralin. On the other hand, Sarkosyl could extract more proteins and had less selectivity for spiralin than Triton X-100. Sarkosyl at concentrations ≥ 50 mM extracted 55% of total protein but only 15% of spiralin (Fig. 1A & 1B). Among the alkylated ionic detergents, Sarkosyl had the unique property of being unable to efficiently extract spiralin from the plasma membrane even at a concentration ten times higher than its critical micellar concentration of 14 mM (Fig. 1B). On the opposite, bile salt derivatives and alkyl glycosides proved to be very potent detergents to extract selectively spiralin from plasma membranes. In particular, CHAPS and DOC solubilized spiralin with a high enrichment factor (2.0 and 1.8, respectively), and a low optimal concentration (60 and 50 mM, respectively). Detergent concentration has to be kept as low as possible during the purification procedure because it has to be removed in the last step of the purification. This is the reason why we chose not to use detergents that gave higher enrichment factor at higher optimal concentrations such as cholylsarcosine, for example.

Consequently, we chose a sequential detergent extraction procedure as a first step for the purification of spiralin before chromatographic separation. *S. melliferum* membranes were first treated with 20 mM Sarkosyl which solubilized about 40% of proteins including very little amount of spiralin (ca. 8%) and the fraction insoluble in these conditions was subsequently treated with 50 mM CHAPS which permitted to obtain a solution which contains spiralin as a major protein (Fig. 2, lane1, 2, and 3). Using Sarkosyl allowed not only to shorten the step of removing extrinsic proteins, but also to operate at more moderate pH (pH 7.4 instead of 9.5) as reported before [20,33].

3.2. Purification of spiralin by cation-exchange HPLC (CE-HPLC)

The amount of spiralin obtained by preparative ASE is limited to 1 mg per separation. Indeed, an increase of the column diameter aiming at increasing the loading capacity would result in an excessive temperature transverse gradient and a poorer resolution. We aimed at optimizing the spiralin purification protocol by using a final separation step offering a higher adaptability to large amounts of available starting material. Our choice fell on the technique of ion exchange HPLC.

The theoretical titration curve of *S. melliferum* spiralin was used to define the best conditions for purification by ion exchange HPLC. Since this protein has a pI of 8.4, a cation-

exchange method would be more appropriate than the anion-exchange option. Furthermore, a pH of 4.5 appeared to be optimal since in these conditions, the protein would have a net charge ca. +15, in principle a good compromise for adsorption/desorption when using a strong cation exchanger.

The elution profile of CE-HPLC showed that the major single peak was eluted at 0.2 M NaCl as expected (Fig. 3). The isolated major peak was analyzed by SDS-PAGE and provided a single band at the expected size for spiralin (28.7 kDa) (Fig. 2, lane4). It should be noted that the amount of spiralin in the HPLC peak is larger than suggested by its height because spiralin has a low molar extinction coefficient ($\varepsilon \approx 6520\ M^{-1}\ cm^{-1}$ at 280 nm) due to the lack of tryptophan in its amino acid sequence [12].

The time of purification was shortened by choosing the chromatographic procedure instead of the agarose-suspension electrophoresis separation: 12h for the ASE vs. 90 min for the CE-HPLC. Moreover, the capacity of the CE-HPLC column allows recovering up to 6 mg of purified spiralin per chromatography starting from 50 mg of total spiroplasma membranes. The efficiency of the overall purification procedure was estimated to be around 60%. We also successfully used a low-pressure cation exchange chromatography system to purify even larger amounts of spiralin. This permitted to collect 14 mg of purified spiralin per purification starting from 50 mg of total spiroplasma membranes (data not shown).

3.3. Spiralin detergent-free micelles

The final step of the purification procedure consisted in removing both NaCl and CHAPS from the mixed micelle solution obtained by CE-HPLC. This was achieved by controlled dialysis using the Lipoprep equipment. During detergent removal, spiralin molecules aggregated under the form of globular particles as shown by transmission electron microscopy (Fig. 4). The size distribution of these particles revealed that the supra-molecular structures had a mean diameter of 19 nm (n=1359, sd = 4.7 nm). Further analysis by SEC confirmed that the mean diameter was in the same range of magnitude, i.e. 17.0 nm (Fig. 5A). Spiralin was eluted together with thyroglobulin, which has a molecular mass of 669 kDa and a Stokes radius of 8.5 nm. The average mass of spiralin micelles ca. 700 kDa, was similar to the spiralin detergent-depleted micelles obtained when spiralin was purified by preparative agarose suspension electrophoresis and subsequent dialysis [17]. The elution fractions of SEC were analyzed by fused-rocket immunoelectrophoresis (Fig. 5B). This result proved that the purified protein was spiralin. This, as explained below, suggests that the protein kept its

native structure during the SEC step. Consequently, what was measured here is indeed the size of spiralin micelles rather than aggregates of unfolded protein.

3.4. Evidence for the native structure of purified spiralin

Far-UV circular dichroism spectra of spiralin detergent-depleted micelles and of spiralin solubilized with SDS are displayed in Fig. 6A. The spectrum of spiralin micelles is characteristic of a highly organized protein containing a high proportion of periodic secondary structures. Deconvolution of the spectra was realized using the CDPro package (Fig. 6B). Results obtained with the ContinLL method indicate that spiralin has about 32% of ß-sheet structures and 18% of α-helices consistently with previous observations [17]. Moreover spiralin has 22% of turns. The addition of the mild detergent CHAPS did not have any effect on the content of secondary structure in spiralin, supporting the idea that the improved purification procedures could keep the spiralin structure similar to its native structure. Strikingly, the spectrum of SDS-treated spiralin shows that the denatured protein has a high proportion of α-helical structures (36%) and a low ß-sheet content (9%), while the proportion of turns and other structures (including disordered structures) is the same in the native and the denatured protein. Helix formation in the presence of SDS can be attributed to the presence of amphiphilic helices in spiralin [37-39]. Primary structure analysis suggested that spiralin might contain amphiphilic α-helices that could be non-structured in the absence of a hydrophobic environment. This was confirmed experimentally for one α-helix [40,41]. Our preferred hypothesis is that spiralin possesses one or more amphipathic α-helices that have no structure in solution but become ordered in the presence of detergent or another hydrophobic environment. The impact of SDS on the secondary structures of spiralin is thus consistent with the previous results [37]. That this could reflect a physiological function of spiralin still remains to be determined.

Antigenicity of the purified spiralin was tested by crossed immunoelectrophoresis (CIE). Proteins from whole *S. melliferum* membranes or purified spiralin were loaded on separate gels (Fig 7). The second migration was performed in agarose gels containing antibodies directed against total membrane protein extracts *of S. melliferum*. Fig. 7A shows that spiralin is by far the most abundant membrane protein in *S. melliferum* membranes and that it keeps its antigenic properties after extraction by DOC. The high purity of spiralin after CE-HPLC is illustrated in Fig. 7B. No other protein than spiralin could be detected confirming the results obtained by SDS-PAGE. Moreover, the positive signal obtained by CIE

suggests that the purified spiralin retained some original structural conformations during the whole purification processes. Indeed, in-gel immunoprecipitation is possible only if at least three distinct epitopes are available to react with antibodies [42]. It is thought that a vast majority of the epitopes on proteins are conformational epitopes [43,44]. Therefore, given the rather small size of spiralin, the existence of at least three different epitopes strongly suggests that the protein kept its native structure during the purification procedure.

4. Conclusion

Mollicutes include a high number of pathogens for humans, animals and plants [45]. Because many lipoproteins are involved in Mollicutes pathogenicity or virulence, it is essential to have these proteins in a purified native form for functional studies aiming at better understanding Mollicutes-hosts interactions. Currently, most functional studies are carried out using truncated forms of Mollicutes lipoproteins to evaluate their effect on host cells. For example, synthetic lipopeptides corresponding to the *N*-terminal moiety of native lipoproteins, such as MALP-2 and FSL-1 derived from lipoproteins produced in mycoplasmas infecting mammals, initiated the innate immune response in host cells [46,47]; the production of a recombinant protein corresponding to *S. citri* spiralin polypeptidic moiety (thus lacking the acylated part) allowed to uncover the role of this lipoprotein in recognition of host cell receptors [21]. The use of whole detergent extracts containing a mixture of lipoproteins in functional studies proved to be also useful in demonstrating the putative functional role of lipoproteins in the host immune response, as illustrated by the work on the human mycoplasma *Mycoplasma hominis* described in Truchetet et al. [48]. Now the availability of purified, complete lipoproteins in their native form could help validating the putative function in Mollicutes pathogenesis observed when using truncated lipoproteins. This should also enable us to gain more insights into the function of spiralin at the molecular level. Notably, protein-lipid interactions can now be tested in vitro in order to evaluate the importance of such interactions in living bacteria and in the context of the host-pathogen interactions. Moreover, the impact of spiralin on membrane shape and dynamics can be assessed using model systems such as liposomes. The data thus obtained should confirm or rule out the putative mechanical role of spiralin. We previously described a procedure for the purification of spiralin [10][17]. The previous purification process included the selective extraction of the protein by detergents from spiroplasma membranes, and a subsequent separation by agarose-suspension electrophoresis. This previous strategy was used to purify spiralin and show its

immunomodulatory function [16]. In this work, we found that Sarkosyl has a unique property of extracting large amounts of membrane proteins but not spiralin. This property was not obtained with any other detergents and suggests that spiralin is mostly insoluble in Sarkosyl. Moreover, replacing the ASE separation by a CE-HPLC step shortened drastically the time of the purification procedure. The amount of purified spiralin per round was increased from 1 mg (ASE) to > 6 mg (CE-HPLC).

This new purification procedure is based on the poor solubilization of spiralin by Sarkosyl, while this detergent could solubilize a large number of other membrane proteins. This peculiar behavior of spiralin during the extraction step could be due to specific spiralin-lipid interactions. This assumption is based on the existence of preferred interactions of diverse acylated proteins with membrane parts enriched in specific lipids, such as sterols, sphingolipids -enriched membrane domains (rafts) in other biological membrane systems [49]. Following our purification process, both circular dichroism spectroscopy and crossed immunoelectrophoresis strongly suggested that spiralin kept a structure very close to its native structure. The high amount of protein that can be obtained by this new method opens the possibility for structural studies that are usually highly demanding in terms of protein material. We think that the whole strategy, based on a detergent screening for the double extraction step, will also prove to be useful for the purification of other lipoproteins, involved in Mollicutes pathogenesis and for which specific interactions with membrane lipids are suspected. This should also open the way for the analysis of the three dimensional structure of lipoproteins, a procedure that usually requires high amounts of protein. Furthermore, this approach allows to obtain large quantities of proteins from the natural organism making possible the study of posttranslational modifications of surface proteins. The importance of such modifications, such as proteolysis, has been recently put forward and may play an important role in pathogenicity and increase the diversity of the cell surface proteome [50].

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Figure captions

Fig. 1. Examples of *S. melliferum* membrane protein extractions by detergents. Membranes were treated with cholylsarcosine (triangles), deoxycholate (circles), or Sarkosyl (squares) at various concentrations of detergents. Extracted proteins were separated from the insoluble material by centrifugation, and total proteins (A) and spiralin (B) were quantified in the solubilized fraction by densitometry analysis after SDS-PAGE.

Fig. 2. Purity control of spiralin by SDS-PAGE. Lane 1, *S. melliferum* BC3 membrane proteins (10 µg) solubilized with 1% SDS; lanes 2 and 3, membrane proteins (2.5 µg) selectively extracted with 0.1 M CHAPS from membranes treated with 20 mM Sarkosyl; lane 4, spiralin (2 µg) purified by cation-exchange HPLC; lane M, molecular weight markers. S indicates the migration of spiralin. All the samples contained 2 mM DTT. Protein bands were silver stained.

Fig. 3. Chromatographic separation of spiralin. Spiralin purification by cation-exchange HPLC. Sample: 5 mg of protein extracted with 0.1 M CHAPS in 4.2 ml of 20 mM sodium phosphate-citrate buffer (pH 4.5) containing 16 mM CHAPS and 2 mM DTT. Column: cation-exchange Protein Pack (Waters Millipore) SP8HR (∅, 1 cm ; length, 10 cm). Flow rate: 1 ml min^{-1}. Proteins were desorbed from the column using a 0 to 0.6 M NaCl linear gradient from fraction 20 to fraction 100. Elution of the was monitored by recording light absorption at 280 nm.

Fig. 4. Analysis of spiralin detergent-free micelles by electron microscopy. Spiralin micelles were visualized by transmission electron microscopy after negative staining. Representative pictures (left) and the size distribution of the particles (right) are shown. The scale bar represents 200 mm.

Fig. 5. Analysis of spiralin detergent-free micelles by size-exclusion chromatography (SEC). A. Spiralin micelles (dotted line) were eluted from a Superdex 200 HR 10/30 column with 50 mM sodium phosphate pH 7.0 buffer containing 150 mM NaCl as the mobile phase (flow = 0.4 ml min^{-1}). Elution was followed by recording absorbance at 280 nm. Standard proteins (solid line) were: A, thyroglobulin; B, apoferritine; C, catalase; D, apotransferrin; E,

ovalbumin; F, hemoglobin dimer; G, myoglobin; H, cytochrome c; I, vitamin B12. B. Fractions were collected after SEC and analyzed by fused-rocket immunoelectrophoresis.

Fig. 6. Analysis of spiralin by circular dichroism spectroscopy. A. Circular dichroism spectra were obtained in the absence or presence of 50 mM CHAPS or 35 mM SDS in 10 mM phosphate buffer pH 7.4. Spiralin concentration was 3.8 µM. B. The relative abundance of secondary structures was measured using the CDPro package.

Fig. 7. Antigenicity of spiralin. Proteins solubilized with DOC were analyzed by crossed immunoelectrophoresis in veronal buffer pH 8.6 containing 13 mM DOC. The samples were composed of 25 µg of *S. melliferum* membrane proteins (A) and 5 µg of pure spiralin (B). The immunoprecipitates were stained with Coomassie blue R250. S indicates the spiralin immunoprecipitation peak.

Figure 1

single-column fitting figure

Should appear in black and white in the online and the print versions

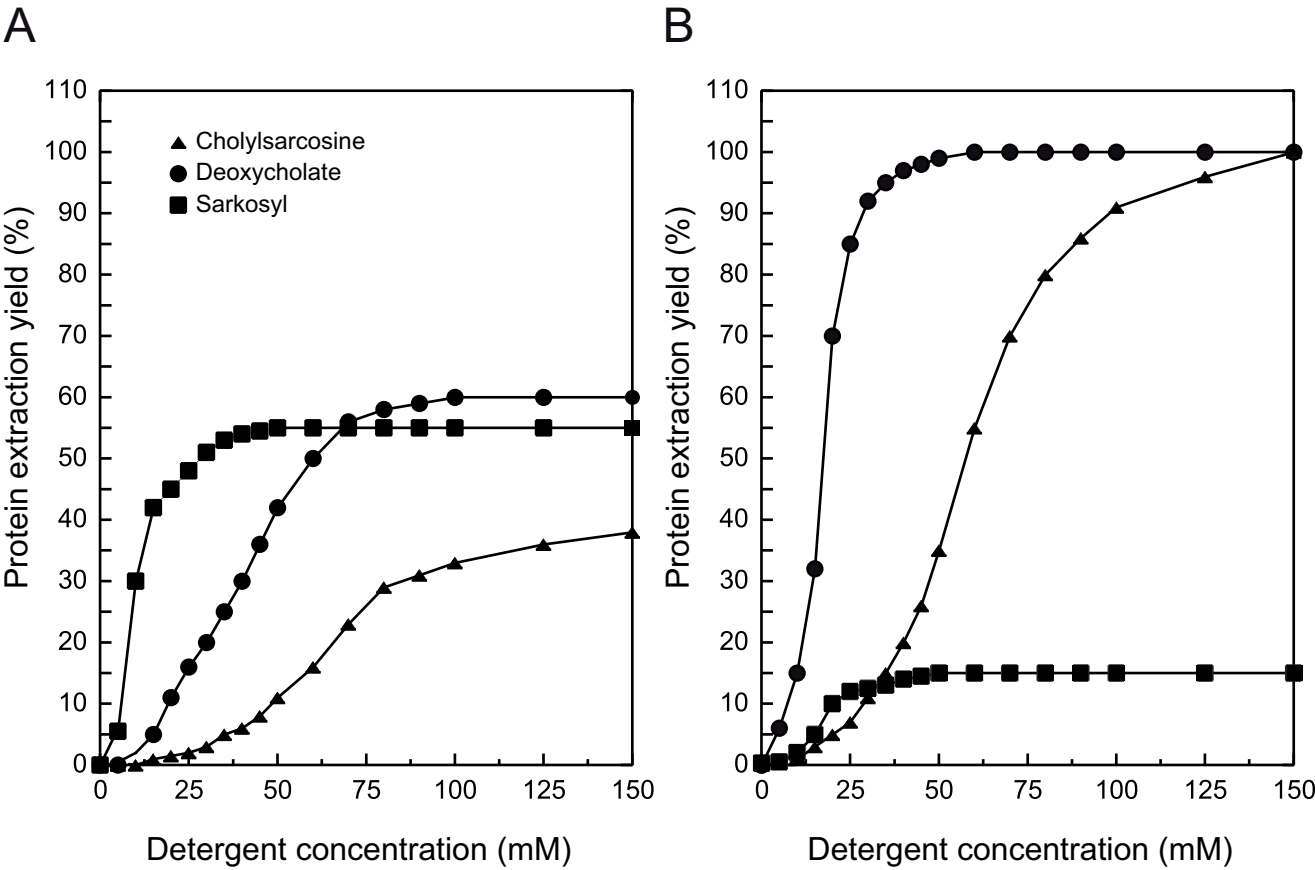


Figure 2

single-column fitting figure

Should appear in black and white in the online and the print versions

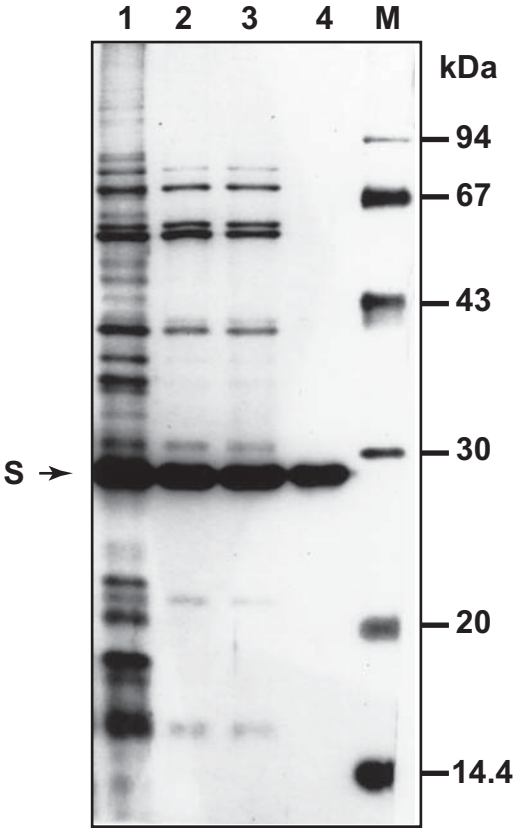


Figure 3

single-column fitting figure

Should appear in black and white in the online and the print versions

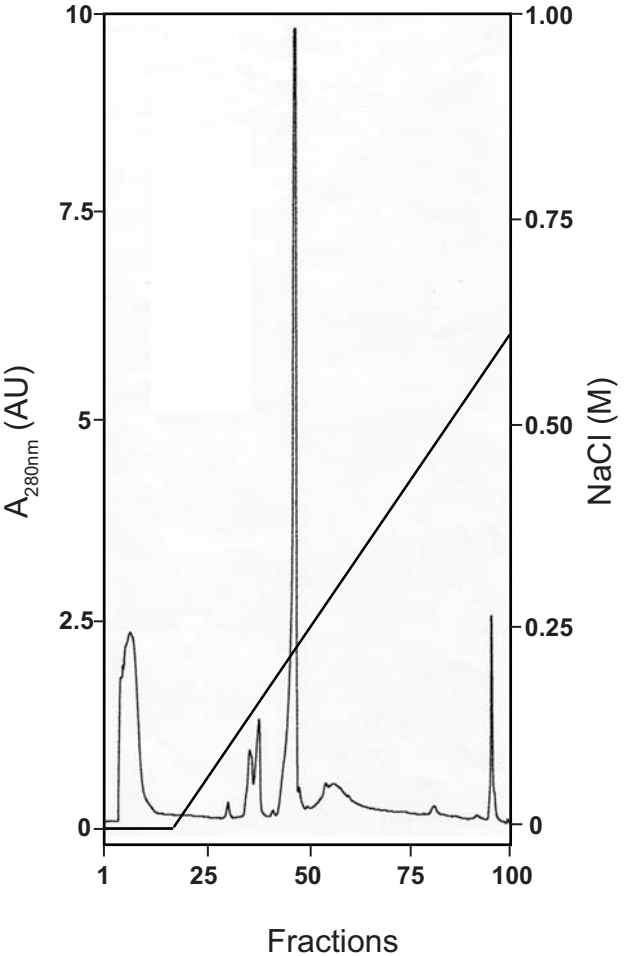
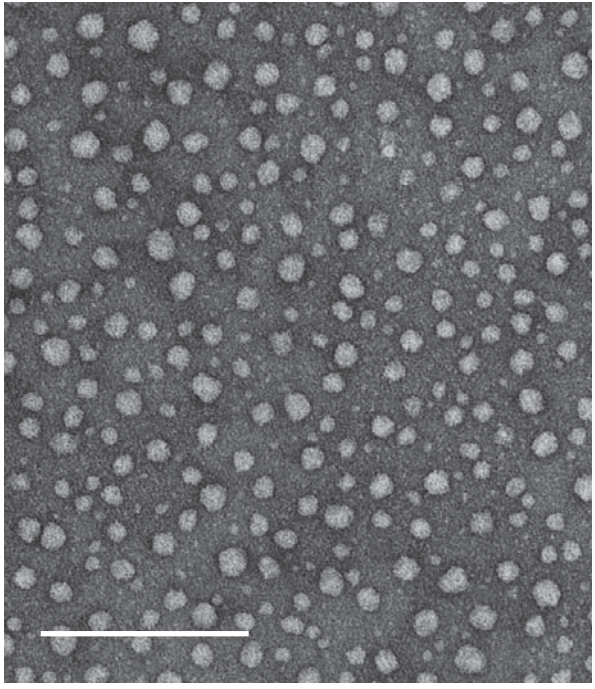


Figure 4

2-column fitting figure

Should appear in black and white in the online and the print versions

A



B

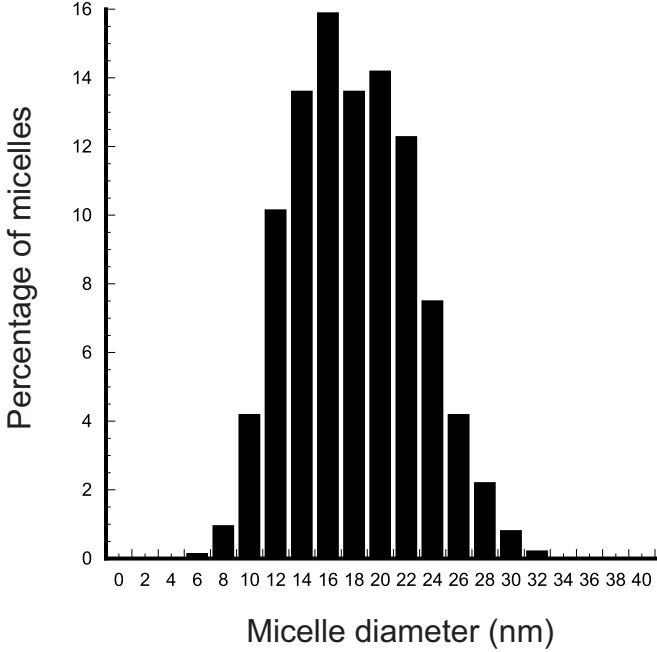


Figure 5

1.5-column fitting figure

Should appear in black and white in the online and the print versions

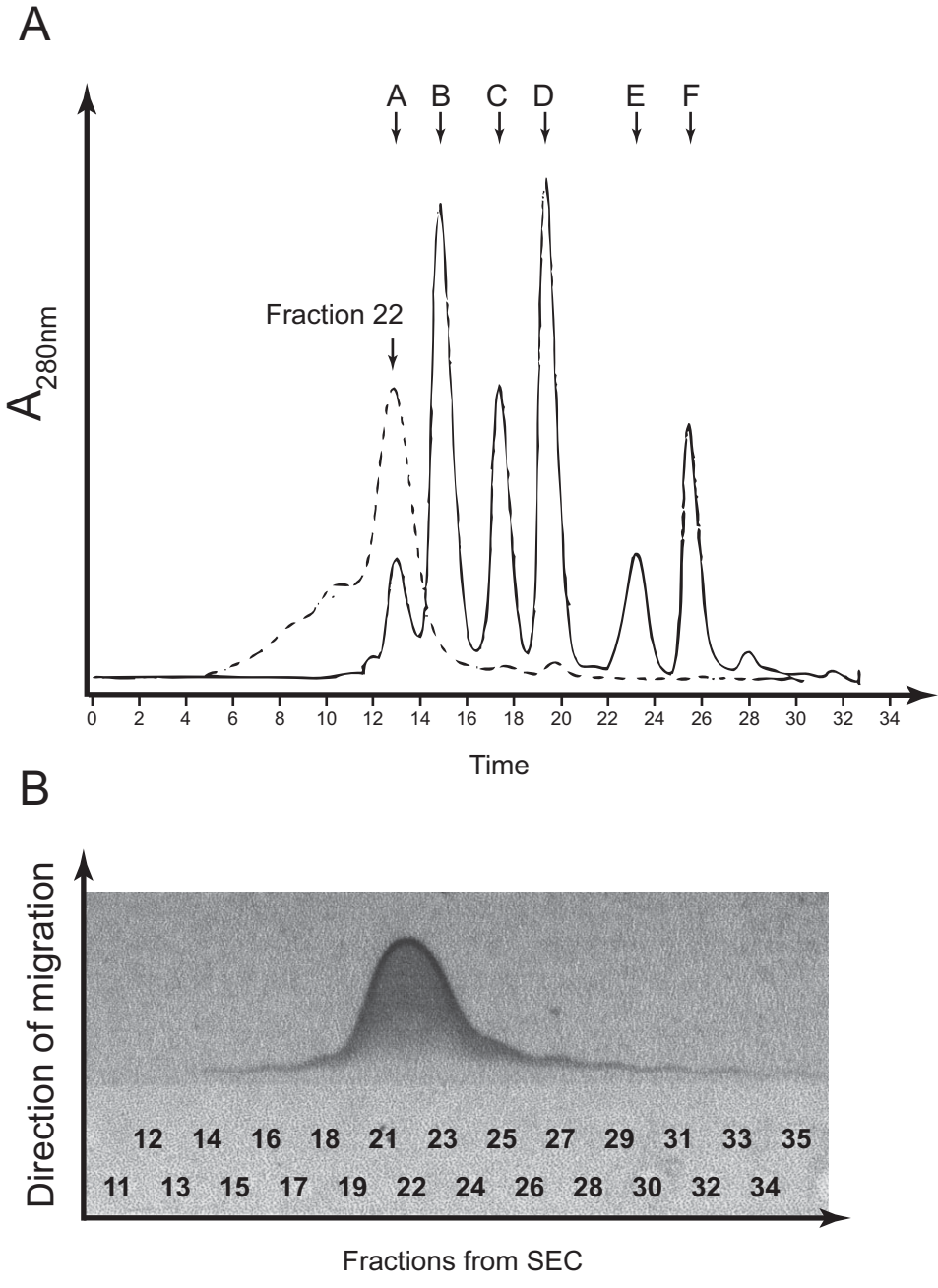
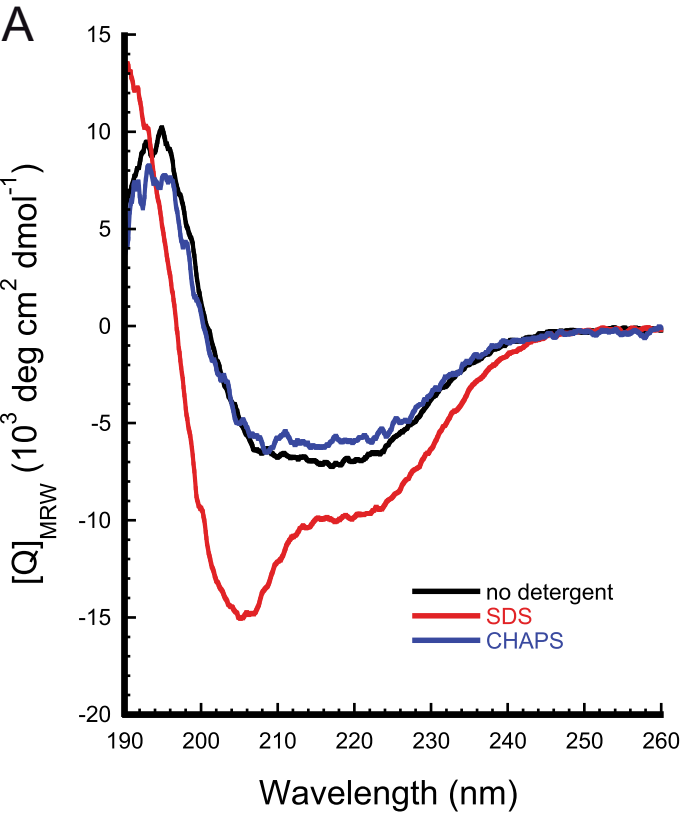


Figure 6

single-column fitting figure

Should appear in color in the online version and in black and white in the print version

A



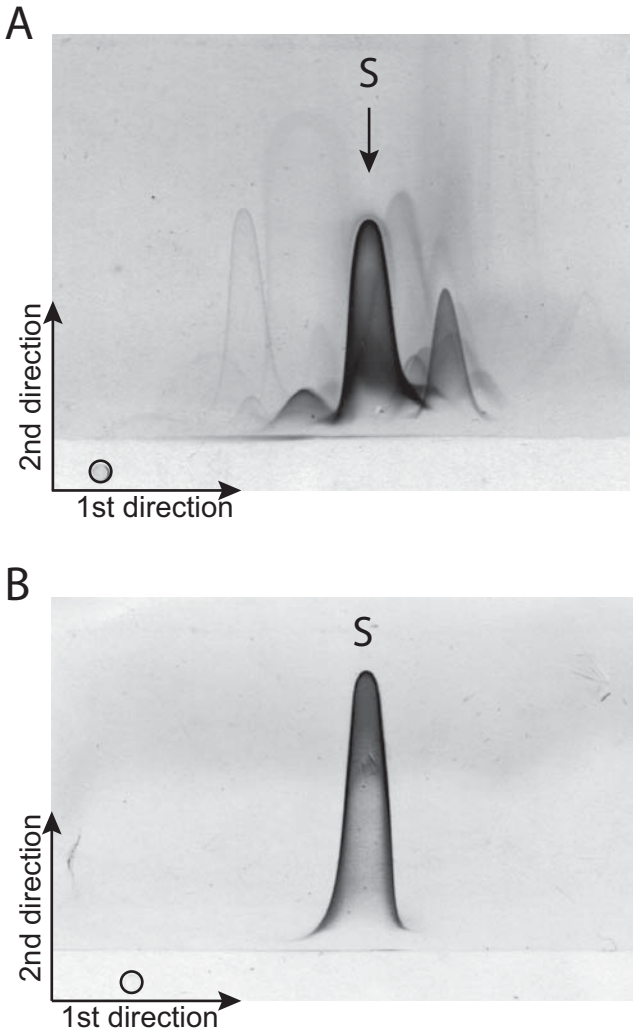
B

	Helices	Sheets	Turns	Other
No detergent	18	32	22	28
50 mM CHAPS	18	30	22	30
35 mM SDS	36	9	23	31

Figure 7

single-column fitting figure

Should appear in black and white in the online and the print versions



Tables

Table 1. Comparison of the efficacies of different detergents for the extraction of *S. melliferum* membrane proteins.

Detergents	$Conc_{opt}$ (mM)[a]	Protein yield (%)[b]	Spiralin yield (%)[c]	EF[d]
Alkylated ionic detergents				
DDMAB	100	86	70	0.8
DDMAU	25	90	65	0.7
Sarkosyl	50	55	15	0.3
SB-12	150	33	85	2.6
SB-14	150	41	95	2.3
Bile salts and derivatives				
CHAPS	60	50	100	2.0
Cholic acid	100	50	90	1.8
Cholylsarcosine	150	38	100	2.6
DOC	50	55	100	1.8
Taurocholic acid	100	55	80	1.5
TauroDOC	100	55	85	1.6
Alkylglycosides				
HECAMEG	60	35	100	2.8
NOGA	100	45	100	2.2
OG	75	73	100	1.4
POE detergents				
Triton X-100	60	25	20	0.8

(a) Lowest detergent concentration giving the highest yield of spiralin extraction.

(b) Percentage of total membrane protein extracted.

(c) Percentage of spiralin extracted.

(d) Spiralin enrichment factor (EF= % spiralin yield / % total protein yield). The fraction solubilized with a given detergent is enriched with spiralin if EF > 1. EF = 4 is the maximum valuable that can be attained since spiralin represents 25% (by mass) of the total membrane protein.