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Letter to the Editor

To the Editor-in-Chief
Sir,

A complete peptide mapping of membrane proteins: a novel surfactant aiding the enzymatic digestion of bacteriorhodopsin

Recent developments in the field of mass spectrometry, namely matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), have enabled the characterization of biomolecules including integral membrane proteins.[1–4] Integral membrane proteins are of high interest since they play vital roles in many cellular and physiological processes. Due to their inherent hydrophobic nature, characterization of these proteins is challenging. A typical approach for characterization of proteins is to cleave them enzymatically or chemically into smaller peptides and analyze them by MALDI-MS or liquid chromatography/mass spectrometry (LC/MS) methods. However, the hydrophobic region (transmembrane) of the tightly folded membrane proteins may not be accessible to enzymes. As a result, the digestion is typically incomplete and few hydrophobic peptides are generated and/or recovered.[3–5]

There have been many efforts on method development to improve the digestion and mass spectrometric analysis of membrane proteins. The membrane protein bacteriorhodopsin (BR, Halobacteria halobium) has been frequently used as a model protein substrate for this purpose. In these methods, detergents are typically used to solubilize and denature the proteins prior to their enzymatic digestion.[1–5] However, the addition of detergents can have a negative impact on the proteolytic activity of the enzymes. Hence, sample dilution prior to proteolysis (to minimize the inhibition of proteolytic enzymes caused by the detergents), increasing amounts of enzymes, and extended digestion times are typically prescribed by the current digestion protocols.

From earlier published reports it is evident that, even in the case of successful proteolysis, interference by detergents of the efficiency of MALDI-MS and LC/MS complicates the sample analysis.[6–8] The sequence coverage and detection sensitivity were limited.[2–9] However, a recent publication by Hixson et al.[3] described a complete peptide mapping of BR by using a combination of denaturants typically used in 2D gel electrophoresis such as SDS, CHAPS, urea and thiourea. Overnight dialysis against Tris-buffer was necessary to remove the excess of SDS prior to tryptic digestion.

Since membrane proteins are rich in methionine, cyanogen bromide (CNBr) has been utilized instead of enzymes to cleave membrane proteins.[1] The drawback of using CNBr is its toxicity; additional steps are required to ensure the safety of the operators. Also, the high concentration of acid employed leads to undesirable modifications.

Here we report a simplified method for tryptic digestion of membrane protein BR using RapiGest™ SF, an acid-labile surfactant (ALS). This surfactant is known to facilitate rapid in-solution enzymatic digestion of protein substrates without inhibiting endopeptidase activity.[10] We demonstrate a complete peptide mapping of BR utilizing ALS solutions for membrane protein digestion. As little as 0.7 μg of sample was used for the LC/MS analysis. The sample preparation is fast, simple, and efficient for both LC/MS and MALDI-TOFMS analyses.

BR and other chemicals used in this study were purchased from Sigma (St. Louis, MO, USA), if not specified otherwise. The purity of BR was approximately 70%. The Sigma recombinant BR protein is modified; the complete amino acid sequence has been published elsewhere.[3] The denaturant ALS was supplied by Waters Corporation (Milford, MA, USA) under the trade name of RapiGest™ SF.

Trypsin digestion of BR was straightforward: a 0.7 mg sample of BR protein was dissolved in 0.5 mL of 0.1% (w/v) ALS solution. The sample solution was boiled at 100°C for 5 min and then cooled to room temperature followed by dilution with 50 mM NH_4HCO_3 (0.5 mL), adjusting the sample pH to 8.0. The final surfactant concentration was 0.05% (w/v). Sequencing-grade trypsin (20 μg, Promega) was added and the BR sample was digested overnight at 37°C. Since BR does not have any disulfide bonds, sample reduction and alkylation were not necessary.

Analysis was performed using a capillary HPLC instrument (CapLC®, Waters Corporation) equipped with a capillary reverse-phase high-performance liquid chromatography (RP-HPLC) column (320 μm × 100 mm, Symmetry 300® C_{18}, 3.5 μm; Waters Corporation). Sample (1 μL, ~0.7 μg) was injected onto the LC column. Mobile phase A was made of 0.1% aqueous formic acid and mobile phase B was made of a 50:50 (v/v) mixture of acetonitrile (ACN) and isopropanol with 0.065% formic acid. A linear gradient was run from 0 to 90% B in 60 min (1.5% B per min). Separation was conducted at 10 μL/min flow rate with the column temperature at 40°C. The LC effluent was directly coupled to an orthogonal time-of-flight (TOF) mass spectrometer (Waters Micromass Q-Tof micro™), interfaced via ESI. The system was controlled by MassLynx software version 3.5.

MALDI analyses were performed using a Micromass M@LDI™ LR TOF mass spectrometer (Waters Corporation). The matrix used for MALDI-TOF analysis was ultrapure α-cyano-4-hydroxycinnamic acid (MassPREP™ MALDI matrix CHCA; Waters Corporation). CHCA was dissolved in 70% ACN with 0.1% trifluoroacetic acid (TFA) to 10 mg/mL. The analyte and matrix solution were mixed 1:1 and 1 μL of the mixture was spotted onto a MALDI target. The quantity loaded onto the MALDI target was about 0.35 μg (~12 pmol). Smaller peptides (800–3000 Da) were detected in TOF reflectron mode, while larger peptides were analyzed in the linear mode (m/z 2500–7000).

Our initial approach was to analyze the BR digest directly without removing the surfactant (Fig. 1(A)). We found that HPLC tolerates small amounts of

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intact ALS (2 μL were injected on a 50 × 1.0 mm column, which represents a mass load of ~1.0 μg of ALS and 1.4 μg of intact BR). Some peak broadening could be attributed to the surfactant injected on-column. The peptide peak widths range from 0.2–1.5 min. Greater amounts (>2 μg) of surfactant injected on-column caused a severe peak tailing, and the peaks shift in the chromatogram towards longer retention times (ALS acts as a strong ion-pairing agent). Since the intact ALS elutes from the RP column at high organic content (Fig. 1(A)), even a heavily contaminated HPLC column can be successfully regenerated prior to the next analysis; no evidence of peak distortion or retention time shift was found in subsequent injections of standard peptides or the BR digest (with surfactant removed from the sample). The trace amounts of intact ALS still present in the HPLC system after column regeneration (as detected by MS) are quickly degraded by the acidic mobile phase.

Figure 1(B) shows extracted ion chromatograms for the selected peptides and their amino acid sequences (only the tryptic peptides that have no missed cleavages are shown). The sequence coverage was 97%; the only undetected parts were short peptides (1 and 2 amino acids). All observed peptides, including those with missed cleavages, are listed in Table 1. The hydrophobicity of the peptide dictates the elution time, the more hydrophobic peptides being more strongly retained in RP-HPLC. The retention times for all observed peptides are compared with their hydrophobicity index values derived using the method of Hopp and Woods.[11] All peptides that elute after T13 are known transmembrane peptides.

Alternatively, the surfactant was removed from the digest by acidic hydrolysis prior to the sample analysis. We found that addition of TFA (final

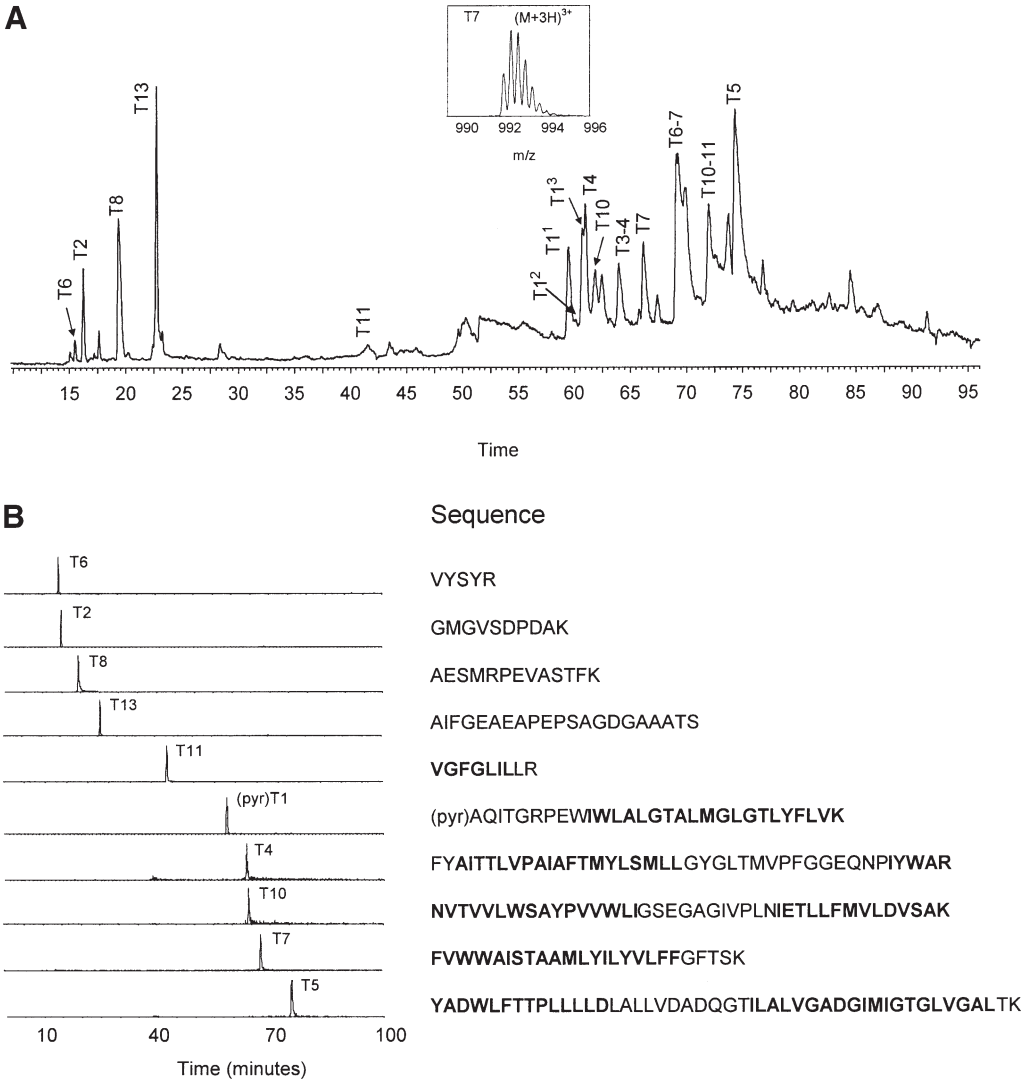
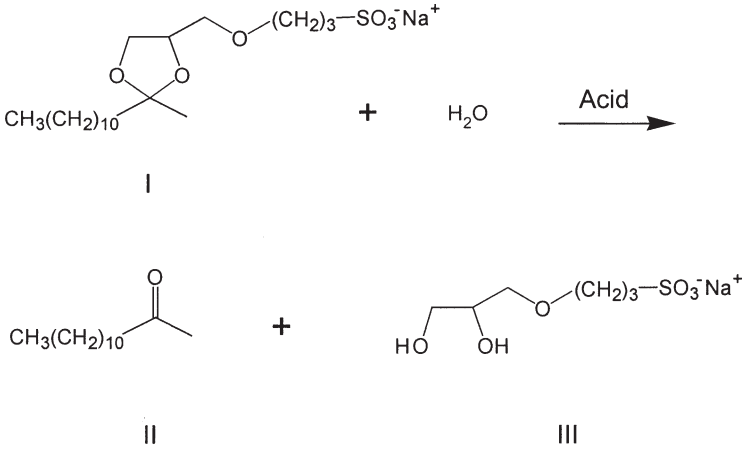


Figure 1. Trypsin-digested BR separated and mass analyzed by LC/MS. (A) Total ion curent (TIC) chromatogram shows the separation of all trypsin-digested bacteriorhodopsin peptides. The identified tryptic peptides were labeled as shown in the figure. (B) Extracted SIR chromatograms for the tryptic peptides are shown with their amino acid sequences. The identified peptides cover about 97% of the amino acid sequence of BR. Amino acids from the transmembrane region are in bold.

Table 1. Tryptic BR peptides identified by LC/MS

Peptide fragment	Residues	Dominant charge	Actual mass (*m*/*z*) (dominant charge)	Theoretical mass (*m*/*z*) (dominant charge)	Retention time (min)	Hydrophobicity index
T6	143–147	+1	687.36	687.35	15.48	14.2
T2	44–53	+2	488.73	488.72	16.22	12.1
T8	173–185	+2	726.87	726.86	19.35	25.7
T13	241–261	+2	959.94	959.94	22.71	58.5
T11	230–238	+2	494.33	494.32	41.53	89.9
T1[1a]	8–43	+4	973.04	973.03	59.47	218.1
T1[2b]	3–43	+4	1111.35	1111.36	60.11	256.9
T1[3c]	15–43	+3	1110.60	1110.61	60.70	216.5
T4	55–95	+4	1154.84	1154.84	60.98	269.8
T10	189–229	+4	1104.10	1104.11	61.84	223.3
T3–4	54–95	+4	1186.87	1186.87	63.97	266.1
T7	148–172	+3	992.18	992.19	66.17	224
T6–7	143–172	+3	1214.96	1214.97	69.21	231.6
T10–11	189–238	+4	1346.33	1346.26	71.98	306.6
T5	96–142	+4	1211.17	1211.17	74.31	351.1

[a] $T1^1$ is a translationally modified T1 peptide. The amino acid sequence of T1 is AQITGRPEWIWLALGTALMGLGTLYFLVK. The sequence of $T1^1$ is AVEGVSQAQITGRPEWIWLALGTALMGLGTLYFLVK.
[b] $T1^2$ may result from chyomtryptic activity of the enzyme. It has the sequence ELLPTAVEGVSQAQITGRPEWIWLALGTALMGLGTLYFLVK.
[c] $T1^3$ is the pyroglutamic acid modified T1 peptide.



Scheme 1. ALS (**I**) rapidly decomposes into two products, **II** and **III**, under low-pH conditions. The $t_{1/2}$ at pH 2 is about 8 min. Product **II** is water-immiscible, and hence can be removed by centrifugation. The aqueous fraction can be directly analyzed by HPLC, LC/MS or MALDI-TOFMS.

concentration 0.5%, v/v) to the sample and incubation at 37°C for 30 min ensured the complete degradation of ALS. The degradation product **II** (Scheme 1) is water-immiscible and forms aggregates; hence it was removed by centrifugation. The supernatant was analyzed by LC/MS. Peptides ($m/z > 400$) were identified based on the observed masses from ESI-TOFMS detection. The ionic degradation product **III** does not interfere with LC/MS or MALDI-MS analysis.

Figure 2 suggests that the presence of the water-insoluble degradation product **II** caused the partition of the hydrophilic/hydrophobic peptides between aqueous supernatant and the organic pellet. LC/MS analysis of supernatant detected mainly the hydrophilic peptides (Fig. 2(A)). The isolated pellet was dissolved with addition of isopropanol (70% in water) and analyzed by LC/MS (Fig. 2(B)). In this way the majority of the hydrophobic peptides were found. The fractionation appears to be rather efficient since only limited amounts of hydrophobic peptides were observed in the first chromatogram (Fig. 2(A)) and no hydrophilic peptides were seen in Fig. 2(B). The quantity of sample used for LC/MS analysis was the same as for Fig. 1.

All expected tryptic BR transmembrane peptides were observed in Figs. 1 and 2(B). Degradation of the ALS and extraction of the hydrophobic peptides helped to concentrate the sample and improve MS signals in terms of higher ion counts. The peak tailing of the peptides in Fig. 2(B) is in part caused by the injection of the sample in a high isopropanol concentration. However, the peptides were correctly identified by MS. ALS degradation was observed to have little effect on the hydrophilic peptides in terms of LC elution profile or the MS signal intensity.

BR digest samples were also analyzed by MALDI-TOFMS. The peptides observed in the MALDI-TOF experiments are listed in Table 2. One challenge of using MALDI-TOF to characterize hydrophobic peptides is their reduced ionization efficiency, often caused by low solubility and a lack of basic amino acid residues.[12] Solubilization agents such as SDS are known to cause severe ion suppression in MS, and have limited utility.[13] However, our results demonstrate that MALDI-TOFMS data can be obtained in the presence of intact ALS (Fig. 3(A)). The mass spectra were acquired in both reflectron and linear TOF modes (Figs. 3(A) and 3(B)) to cover the broad range of the peptides' molecular masses. It should be noted that peptide T11 is the only transmembrane peptide with molecular mass less than 2000 Da (987.64 Da). Despite the signal sup-

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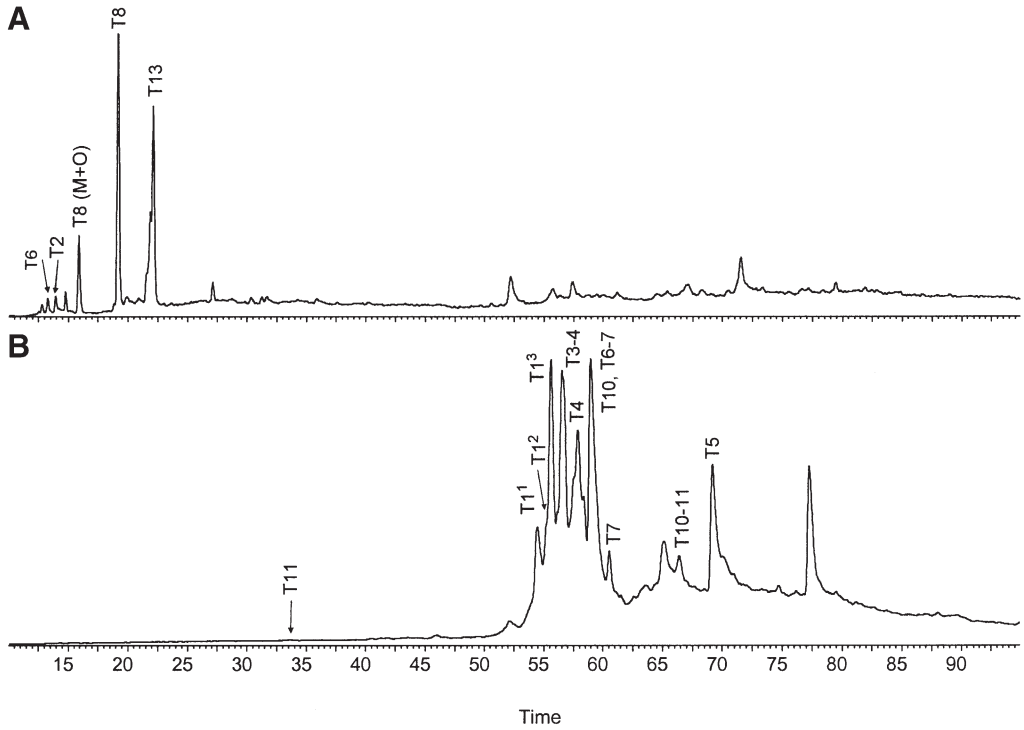

Figure 2. LC/MS analysis of the fractionated BR peptides. Peptides from (A) the hydrophilic aqueous fraction and (B) those extracted by isopropanol from the hydrophobic pellet.

Table 2. Tryptic peptides of BR identified by MALDI-TOFMS

Peptides	m/z (Theoretical)	m/z (Measured)	Mass accuracy (ppm)	
T2	976.44	976.50	60	$M+H^+$
T11	987.64	987.67	30	$M+H^+$
T8	1452.72	1452.77	34	$M+H^+$
T13	1918.87	1918.79	41	$M+H^+$
T7	2976.60	2976.84	81	$M+H^+$
$T1^3$	3331.99	3331.73	78	$M+H^+$
T6–7	3645.35	3645.00	27	$M+H^+$
$T1^1$	3891.61	3891.31	77	M+H
$T1^3$-2	4290.05	4289.88	40	$M+H^+$
$T1^2$	4445.26	4444.92	76	$M+H^+$
T4	4619.52	4619.05	102	$M+H^+$
T3–4	4747.69	4747.37	67	$M+H^+$
T10–11	5403.51	5403.32	35	$M+H_2O+H^+$

pression, all transmembrane peptides were observed (Fig. 3(A)) from the sample containing ALS. When ALS was acid-degraded prior to analysis, no transmembrane peptide signals were observed in the aqueous portion of the sample. Presumably, the water-insoluble peptides are adsorbed on degradation product **II** (data not shown). In order to analyze insoluble peptides, the sample was centrifuged and the isolated pellet was extracted with isopropanol. MALDI analysis of these extracted hydrophobic peptides is shown in Fig. 3(B). Substantially better signals for the transmembrane peptides were observed compared with those in Fig. 3(A). Apparently, a similar fractionation of hydrophobic peptides between aqueous and organic solvent portions of the sample again occurs, as detected earlier by LC/MS experiments (Figs. 1 and 2).

Two factors are probably contributing to the peptide signal enhancement (Fig. 3(B)). First, the transmembrane peptides were greatly concentrated in the pellet prior to isopropanol extraction. Second, the ALS degradation minimized the ion suppression of surfactant in MALDI-TOFMS analysis. Our observations suggest that signal suppression related to ALS is more pronounced for transmembrane peptides than for hydrophilic ones. Since the transmembrane peptides are rather large, the suppression may be more generally related to the molecular masses of the analytes.

In summary, we have developed a method for tryptic digestion of membrane protein BR using a novel acid-labile surfactant (RapiGest^{TM} SF). This surfactant facilitates the tryptic digestion of membrane protein BR by effectively solubilizing and unfolding the protein. Complete peptide mapping of BR via LC/MS was achieved using less than 1 μg of protein. Small amounts of the surfactant did not interfere with LC/MS and MALDI-MS analysis of the BR peptides. At larger amounts ALS may interfere with sample analysis, and it is recommended to degrade it with acid prior to LC or MS analysis. Upon surfactant degradation the peptides were fractionated between aqueous and water-immiscible layers according to their hydrophobicity. The fractionation was used with advantage to reduce sample complexity, and to isolate (concentrate) the highly hydrophobic peptides such as the transmembrane peptides. Peptides from both fractions identified by

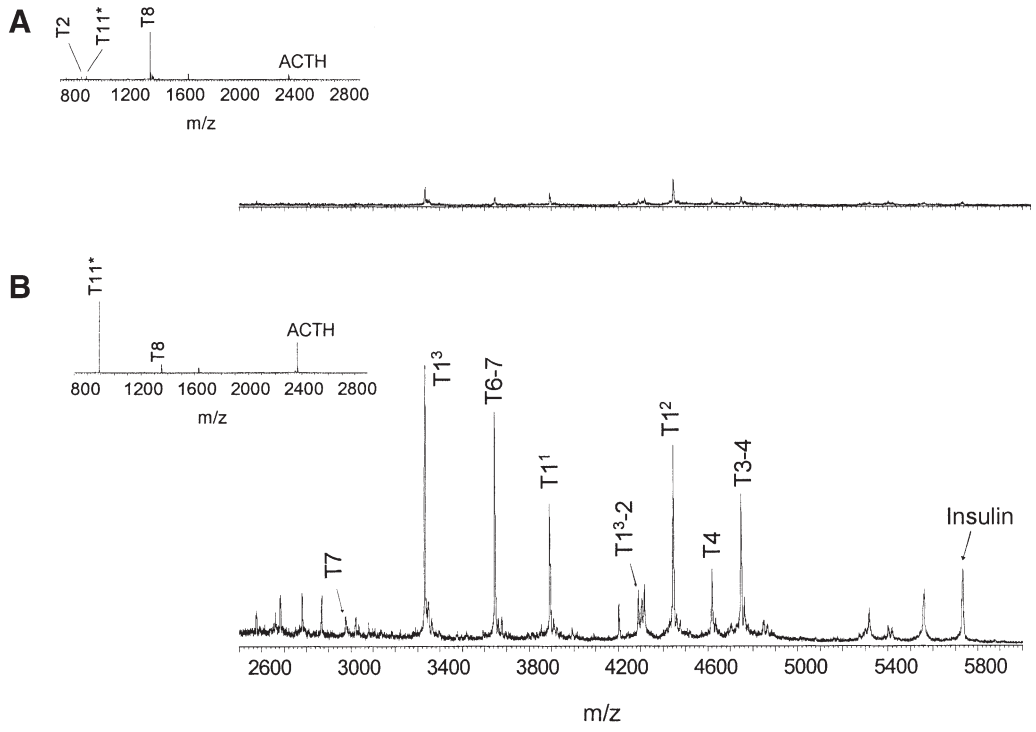

Figure 3. Normalized MALDI-TOF spectra of bacteriorhodopsin tryptic peptides. (A) ALS was not degraded, surfactant is present in the sample. (B) ALS was degraded, pellet isolated and the hydrophobic peptides extracted by isopropanol. The inset mass spectra were acquired in reflectron mode; ACTH was used for internal mass calibration. The mass spectra in the range *m/z* 2500–6000 were acquired in linear mode; insulin was used as internal calibrant.

MALDI-TOFMS cover 79% of the bacteriorhodopsin sequence. The method enables an efficient tryptic digestion of hydrophobic protein, easy sample cleanup, and concentration of transmembrane peptides. The developed method represents a general tool for analysis of integral membrane proteins.

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