



Combined in-gel tryptic digestion and CNBr cleavage for the generation of peptide maps of an integral membrane protein with MALDI-TOF mass spectrometry

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Abstract

A limitation of the in-gel approaches for the generation of peptides of membrane proteins is the size and hydrophobicity of the fragments generated. For membrane proteins like the lactose transporter (LacS) of *Streptococcus thermophilus*, tryptic digestion or CNBr cleavage yields several hydrophobic fragments larger than 3.5 kDa. As a result, the sequence coverage of the membrane domain is low when the in-gel tryptic-digested or CNBr-cleaved fragments are analyzed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS). The combination of tryptic digestion and subsequent CNBr cleavage on the same gel pieces containing LacS approximately doubled the coverage of the hydrophobic membrane domain compared to the individual cleavage methods, while the coverage of the soluble domain remained complete. The fragments formed are predominantly below m/z 2500, which allows accurate mass measurement.

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1. Introduction

The generation of peptide maps of proteins by in-gel approaches is used often in proteomics-type studies [1–4]. This approach combines the high separation capacity of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in one or two dimensions, with the speed, ease, and sensitivity of mass spectrometry (MS) to identify proteins by peptide mass fingerprinting at a high throughput. The in-gel approach can also be used for the generation of peptide maps of membrane proteins [5–7]. However, high sequence coverage is difficult to obtain because of the

lack—or limited accessibility—of proteolytic cleavage sites in the membrane-spanning segments. This results in large fragments that are highly hydrophobic, which limits the use of MS for protein identification or structure–function analysis of membrane proteins. The latter application is of great interest for the analysis of the structure of membrane proteins such as the lactose transporter (LacS) from *Streptococcus thermophilus*. The LacS protein has been recently shown to function as a cooperative dimer in proton motive force-driven lactose uptake [8,9]. Peptide maps of LacS with high sequence coverage would help the identification of the dimer interface when analysed by cross-linking methods. The same holds true for the helix packing models of LacS and other members of the GPH family, which are largely based, thus far, on the analysis of second-site suppressor mutants [10].

Recently, we reported in-gel approaches to generate peptide maps of integral membrane proteins, including LacS, using tryptic digestion and CNBr cleavage [11]. The sequence coverage of membrane domains in tryptic

Abbreviations: MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; MS, mass spectrometry; LacS, lactose transporter of *Streptococcus thermophilus*; OBG, octyl- β -glucopyranoside

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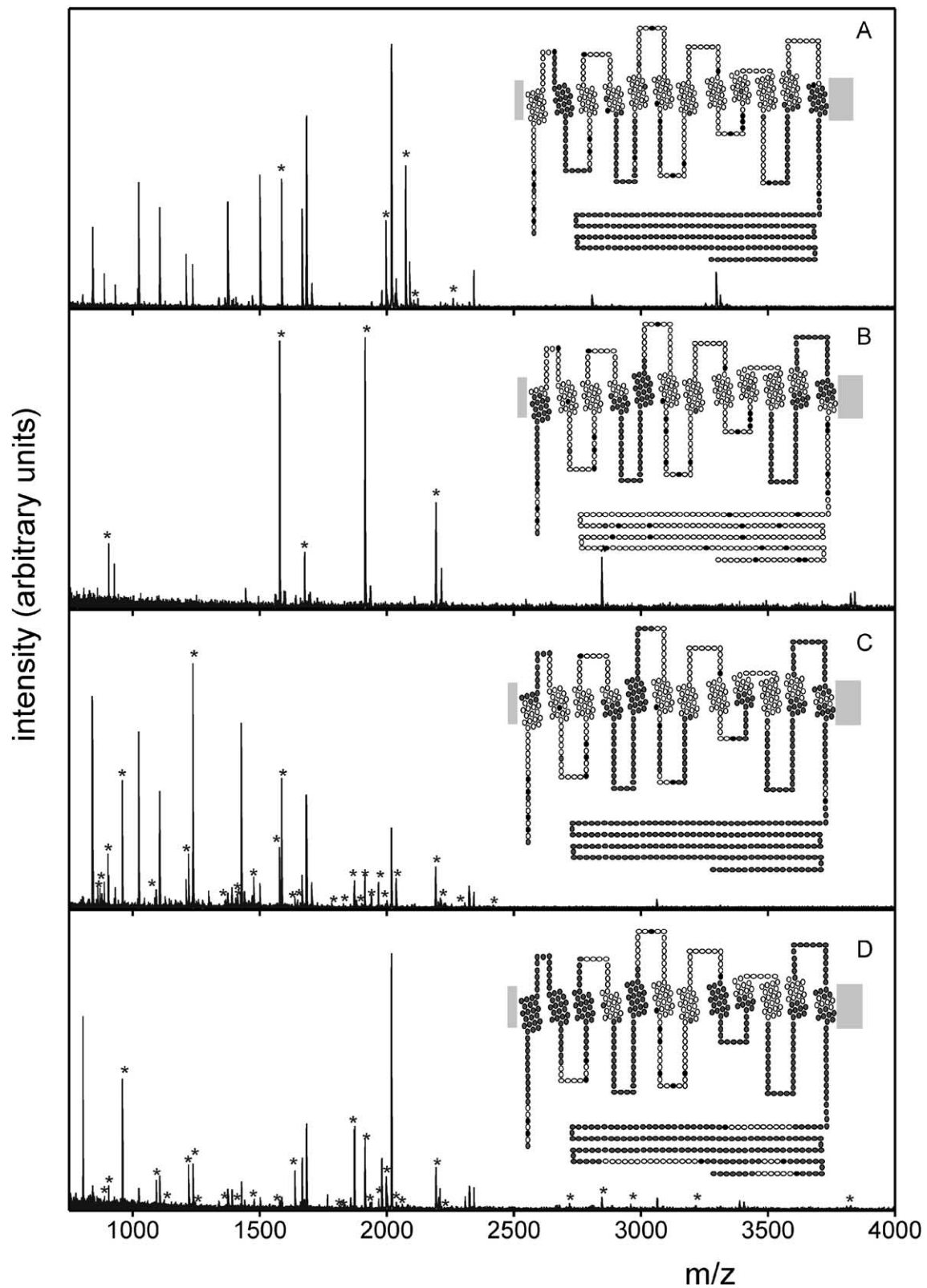


Fig. 2. MALDI-TOF mass spectra of in-gel generated peptides of LacS. (A) Tryptic digest, extracted without OBG; (B) CNBr cleavage, extracted without OBG; (C) combined trypsin/CNBr cleavage, extracted without OBG; (D) combined trypsin/CNBr cleavage, extracted with OBG. (*) Peaks of fragments of the hydrophobic membrane-embedded part of LacS. The insets show the sequence coverage depicted in the topology model of LacS.

acrylamide gels [13] and visualized with Coomassie blue staining.

2.3. In-gel tryptic digestion, CNBr cleavage, or the combination of both

After visualization, the bands containing the protein were excised from SDS-polyacrylamide gels, treated and subjected to digestion with trypsin or CNBr cleavage, following a modified procedure of Hellman et al. [14] as described in Ref. [11]. Tryptic digestion or CNBr cleavage was started by the addition of 5 μ l of 75 ng/ μ l trypsin in 25 mM ammonium bicarbonate, pH 8, or 25 μ l of CNBr in 70% TFA (one small crystal was dissolved in 200–300 μ l of 70% TFA), respectively, to the dried gel pieces. For the tryptic digestions, the gel pieces were covered with an overlay of 25 mM ammonium bicarbonate (approximately 20 μ l) after reswelling and incubated for at least 14 h at 30 °C. The CNBr cleavage was performed for at least 14 h in the dark at room temperature. The combined tryptic and CNBr cleavage was performed as follows. After tryptic digestion, the peptides were not extracted, but the gel pieces were dried in the SpeedVac. These dried gel pieces were washed and dehydrated twice by addition of 100 μ l of acetonitrile followed by SpeedVac drying. Subsequently, CNBr cleavage was started by the addition of 25 μ l of CNBr in 70% TFA as above. After collection of the supernatant, the peptides were extracted twice by sonication for 5 min in 30 μ l of 60% acetonitrile, 1% TFA in the absence or presence of 0.1% OBG. The overlay and extracts were pooled and dried in a SpeedVac. The last traces of ammonium bicarbonate were removed by adding 10 μ l of 1% TFA and subsequent drying in the SpeedVac.

2.4. MS

The samples were dissolved in 5 μ l of 50% acetonitrile, 0.1% TFA, and sonicated for 5 min. Aliquots of 0.75 μ l were applied onto the target and allowed to air dry. Subsequently, 0.75 μ l of 10 mg/ml α -cyano-4-hydroxysuccinamic acid in 50% acetonitrile, 0.1% (v/v) TFA was applied to the dried sample and again allowed to dry. MALDI-TOF mass spectra were recorded with a Micromass Tofspec E MALDI time-of-flight mass spectrometer operated in reflectron mode. The spectra were calibrated externally, but if necessary, the spectra were internally recalibrated on trypsin autodigestion products and a matrix peak.

3. Results and discussion

The lactose transporter of *S. thermophilus* is a 70-kDa integral membrane protein with a 50-kDa N-terminal membrane-embedded part (membrane domain), a 20-res-

Table 1

Relative sequence coverage (%) for the soluble and membrane domain, or the total protein, with in-gel tryptic digestion, CNBr cleavage, or a combination of these two methods

Method	Extraction solvent	Soluble domain	Membrane domain	Total protein
Trypsin	– OBG	98	24	47
CNBr	– OBG	0	33	23
Trypsin/CNBr	– OBG	98	40	58
Trypsin/CNBr	+ OBG	84	57	66
Trypsin/CNBr	both ^a	100	66	77

The absence or presence of the detergent OBG in the extraction solvents is denoted.

^a Since some of the fragments observed are different for extractions with and without OBG, the total sequence of the combined extractions is higher than that of the individual methods.

idue glutamate-rich linker, and a 18-kDa cytoplasmic domain (IIA domain). The linker and IIA domain together are denoted here as soluble domain. Fig. 1 shows the topological organization of LacS and the tryptic and CNBr cleavage sites, clearly indicating the lack of lysines and arginines in the membrane-spanning regions. We reported previously MALDI spectra of in-gel tryptic and CNBr-cleaved LacS [11]. For comparison, in-gel tryptic digestion and CNBr cleavage without OBG extraction were repeated (Fig. 2A and B) and the sequence coverage was determined (Table 1). Tryptic digestion predominantly resulted in peptides of the soluble domain and only few peptides of the membrane-embedded domain, while CNBr cleavage resulted only in peptides of the membrane domain. The latter is caused by the presence of only one methionine in the soluble domain. The low coverage of the membrane domain is, at least partially, caused by the fact that tryptic digestion or CNBr cleavage results in hydrophobic fragments up to 6 and 8 kDa, respectively.

To obtain a good coverage of both the membrane and soluble domain in one sample, fragments were generated by tryptic digestion and subsequent CNBr cleavage on the same gel piece. Fig. 2C and D shows the spectra of the combined procedure, without (C) or with OBG (D) in the extraction solvent. Both samples clearly show many intense peaks from the membrane domain at signal-to-noise ratios comparable to those in Fig. 2A and B. Several peptides were partially cleaved by trypsin, while CNBr cleavage was always complete. Fragments of the soluble domain are present as tryptic fragments as in Fig. 2A and covered the complete soluble domain (Table 1). The sequence coverage of the membrane domain almost doubled compared to individual trypsin digestion or CNBr cleavage. The extraction with OBG has the same effect as reported previously [11]. Several new peptides of the membrane domain at higher m/z values were observed, adding another 17% to the coverage of the membrane domain. Moreover, the size of most fragments generated by the combined method was below 2.5 kDa,

which allowed very accurate mass determination and will simplify sequencing by MS/MS.

We suggested previously that the low efficiency of the tryptic in-gel approach was caused by the limited accessibility of the gel-trapped protein for the protease [11]. Upon removal of SDS from the gel pieces, it is likely that the membrane protein aggregates and becomes inaccessible for the protease, which would explain the low intensity of the spectra of the tryptic peptides of the membrane domain. If the accessibility were the problem, the spectrum of the combined method (Fig. 2C and D) would predominantly contain CNBr-cleaved fragments as in Fig. 2B. Since this is not the case, it suggests that the extraction, the solubility, and/or the MALDI MS analysis of the tryptic peptides form a bottleneck and not the accessibility of the membrane domain for the protease. Control experiments excluded the possibility that solely the presence of 70% TFA used in the CNBr cleavage helped the solubilisation of the hydrophobic fragments, since a subsequent incubation of an in-gel tryptic digest in 70% TFA (as in the CNBr cleavage) did not increase the sequence coverage (not shown).

4. Conclusion

The combined use of in-gel tryptic digestion and CNBr cleavage results in intense spectra with a high sequence coverage of the membrane domain and complete coverage of the soluble domain of a large hydrophobic integral membrane protein. The combined method can be used for the identification of structure–function relations as mentioned above. In addition, this method should, in principle, be applicable for many other membrane proteins and make this class of proteins amenable to proteome analysis. Although conventional 2D electrophoresis with isoelectric focusing in the first dimension is often not possible with hydrophobic proteins, the use of blue native electrophoresis in the first dimensional separation could represent a good alternative [15]. On the assumption that individual membrane proteins constitute 0.1–1% of the total membrane protein fraction, 2–20 pmol of protein should be present per spot on a 2D gel, which should be sufficient to identify the protein. Preliminary experiments indicated that it is possible to obtain a high sequence coverage of LacS using the combined method and only 2–4 pmol of protein.

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