Chapter 4

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

Bart A. van Montfort
Mark K. Doeven
Benito Canas
Liesbeth M. Veenhoff
Bert Poolman
George T. Robillard

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 coverages of the membrane domain are low when the in-gel tryptic-digested or CNBr-cleavaed fragments are analyzed by MALDI-TOF 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 determination.

Introduction

The generation of peptide maps of proteins by in-gel approaches is used very often in proteomics-type studies (*131-134*). This approach combines the high separation capacity of sodium dodecylsulfate polyacrylamide gel electrophoresis, 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 rate. The in-gel approach can also be used for the generation of peptide maps of membrane proteins (*110-112*). However, high sequence coverages are 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 mass spectrometry for proteomics purposes or structure-function analysis of membrane proteins.

Recently, we reported improved in-gel approaches to generate peptide maps of integral membrane proteins using tryptic digestion and CNBr cleavage (*chapter 3*). The sequence coverage of membrane domains in tryptic maps was increased when the MALDI-TOF-compatible detergent octyl-β-glucopyranoside (OBG) was used in the extraction solvents. In-gel CNBr cleavage of the membrane domains resulted in spectra that were 5 to 10-fold more intense than those obtained from in-gel tryptic digestion. A limitation of an in-gel approach is the size of the fragments generated. In general, it is difficult to extract larger fragments from the gel pieces. This disadvantage can be overcome by generating smaller fragments with a combination of different proteases or chemical cleavage methods in one sample.

The combined use of tryptic digestion and CNBr cleavage is presented here for the lactose transporter (LacS) of *Streptococcus thermophilus*. Peptide mapping of this integral membrane protein with our original improved procedures resulted in maps, which missed several, predominantly large and hydrophobic peptides (*chapter 3*). In-gel tryptic digestion followed by CNBr cleavage on the same gel pieces, as presented here, resulted in intense spectra from peptides that were mostly below m/z 2500. The method approximately doubled the sequence coverage of the membrane domain. Moreover, the addition of OBG to the extraction solvents resulted in the recovery of peptides at higher m/z values, which increased the sequence coverage even more.

Materials and methods

Materials

Chemicals and solvents used in this study were of analytical grade. The purification of His-tagged lactose transporter of *Streptococcus thermophilus*, in which Cys^{320} was replaced with an alanine (LacS-C320A), was done as described (*136*). Trypsin for in-gel tryptic digestions was sequencing grade modified trypsin from Promega. Octyl-β-glucopyranoside (OBG) was purchased from Sigma. CNBr was from Fluka.

SDS-polyacrylamide gel electrophoresis

Approximately 3 μg of LacS (40 pmol) was solubilized in Laemmli sample buffer and heated for 10 minutes at 50 °C. The samples were separated on Laemmli 10% SDS-polyacrylamide gels (*117*) and visualized with coomassie blue staining.

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 (*109*) as described (*chapter 3*). Briefly, tryptic digestion or CNBr cleavage were started by the addition of 5 μl 75 ng/μL trypsin in 25 mM ammonium bicarbonate buffer or 25 μl CNBr in 70% TFA, respectively, to the dried gel pieces. For the tryptic digestions, these were covered with an overlay of 25 mM ammonium bicarbonate after reswelling and incubated for at least 14 hours at 30 °C. The CNBr cleavage was performed for at least 14 hours 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 μl of acetonitrile followed by SpeedVac drying. Subsequently, CNBr cleavage was started. The peptides were extracted twice by sonication for 5 minutes in 30 μl 60% acetonitrile, 1% TFA in the absence or presence of 0.1% OBG. The last traces of ammonium bicarbonate were removed by adding 10 μl of 1% TFA and subsequent drying in the SpeedVac.

Mass spectrometry

The samples were dissolved in 5 μl of 50% acetonitrile, 0.1% TFA and sonicated for 5 minutes. Aliquots of 0.75 μl were applied onto the target and allowed to air dry. Subsequently, 0.75 μl of 10 mg/ml α-cyano-4-hydroxysuccinnamic 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.

Results and discussion

The lactose transporter of *Streptococcus thermophilus* is a 70 kDa integral membrane protein with a 50 kDa N-terminal membrane-embedded part (membrane domain), a 20 residue glutamate-rich linker, and a 20 kDa cytoplasmic domain (IIA domain). The linker and IIA domain together are denoted here as soluble domain.

Figure 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 (*chapter 3*). For comparison, in-gel tryptic digestion and CNBr cleavage without OBG extraction were repeated (fig. 2A and B) and sequence coverages were 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 coverages of the membrane domain are, at least partially, caused by the fact that tryptic digestion or CNBr cleavage results in hydrophobic fragments up to 6 and 8 kDa, respectively.

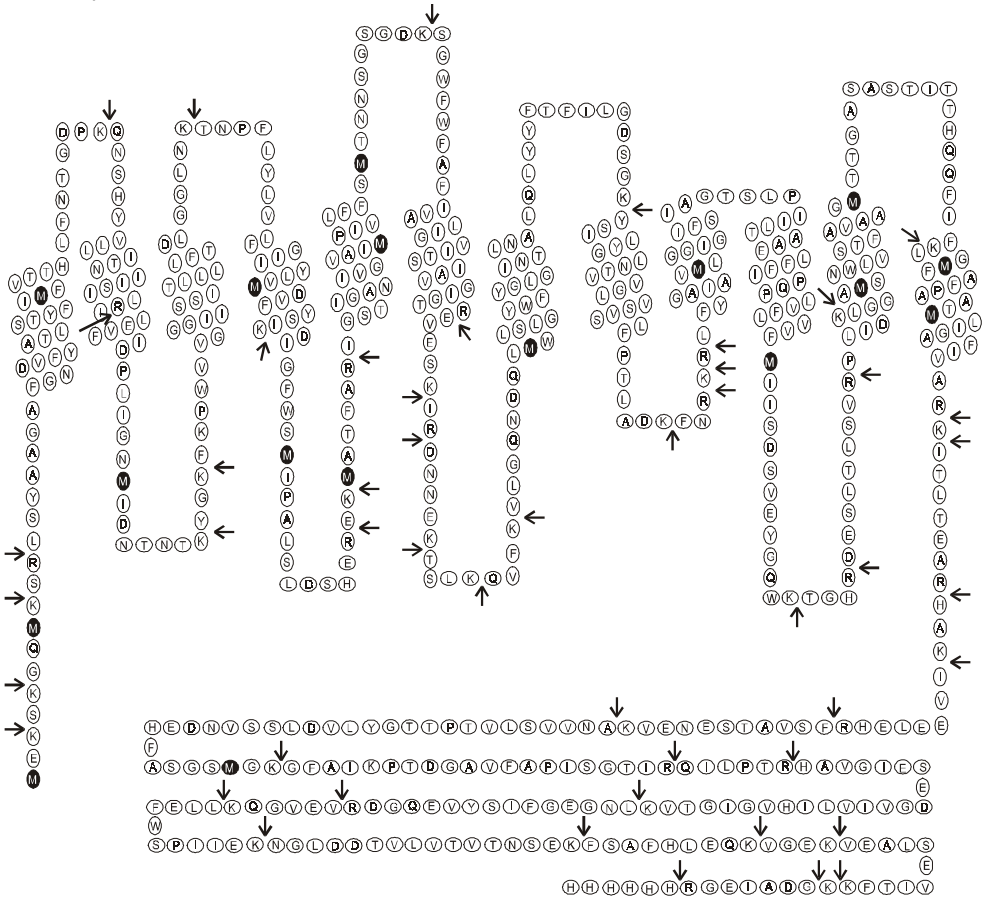

Figure 1. The cleavage sites of trypsin and CNBr are indicated in the topology model of LacS. Trypsin cleaves at the C-terminal side of lysines and arginines as indicated by the arrows. CNBr cleavage sites at the C-terminal side of methionines are shown in black.

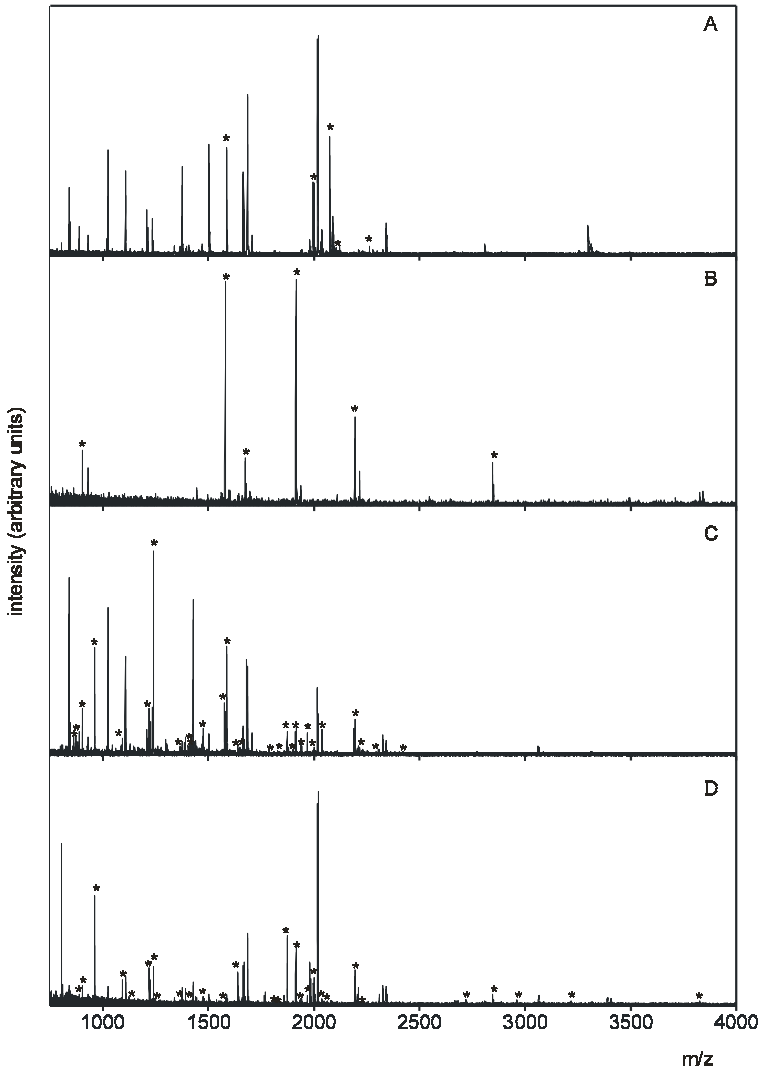

Figure 2. MALDI-TOF mass spectra of in-gel generated peptides of LacS. **(A)** Tryptic digest, extracted without OBG. **(B)** CNBr cleavage, extracted without OBG. **(C)** Combined trypsin/CNBr cleavage, extracted without OBG. **(D)** Combined trypsin/CNBr cleavage, extracted with OBG. (*) Peaks of fragments of the hydrophobic membrane-embedded part of LacS.

Table 1. Relative sequence coverages (%) for the soluble domain, the membrane domain, or the total protein, with in-gel tryptic digestion, CNBr cleavage, or a combination of these two methods. The absence or presence of the detergent octyl-β-glucopyranoside (OBG) in the extraction solvents is denoted.

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

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. Figure 2C and D show 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 figure 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 figure 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 (*chapter 3*). 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.

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 (*chapter 3*). 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 figure 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 an incubation of an in-gel tryptic digest in 70% TFA did not increase the sequence coverage (not shown).

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 should, in principle, be applicable for many other membrane proteins and make this class of proteins amenable for proteome analysis. 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 high sequence coverage of 2-4 pmol of LacS.