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Efficient in-gel digestion procedure using 5-cyclohexyl-1-pentyl-β-D-maltoside as an additive for gel-based membrane proteomics

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A cycloalkyl aliphatic saccharide, 5-cyclohexyl-1-pentyl-β-D-maltoside (CYMAL-5), was evaluated as a novel additive in a high-throughput in-gel protein digestion system using 96-well plates. Addition of 0.1% CYMAL-5 (final concentration) during trypsin treatment was compatible with both matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis, and gave a better digestion efficiency than n-octylglucoside, which we previously reported. In-gel reduction and alkylation of Cys residues under denaturing conditions also improved the sequence coverage of peptides. In-gel tryptic digestion with the optimum combination of 0.5 mm thick gels, negative staining, alkylation under denaturing conditions (6 M guanidine hydrochloride), and digestion in the presence of CYMAL-5, gave excellent performance especially for membrane protein analysis, where recovery of hydrophobic peptides was markedly enhanced. The new protocol is simple and convenient, and should be widely applicable to gel-based proteomics. Copyright © 2004 John Wiley & Sons, Ltd.

In the field of gel-based proteomics, improvement of the in-gel digestion efficiency is important to increase the sequence coverage and signal intensity of mass spectrometry (MS) in order to give greater confidence in protein identification and post-translational modification analysis. The standard in-gel digestion protocol developed by Shevchenko *et al*. enabled low-level sequencing by MS in combination with silver staining and trypsin digestion.[1] However, silver-stained bands clearly observable on the gel can sometimes not be identified by MS because of inadequate peptide recovery from the gels. This often occurs when the target samples are membrane proteins.

In order to overcome those difficulties, we previously developed a modified procedure in which trypsin treatment is conducted in the presence of n-octylglucoside (OG).[2] Saccharide-based non-ionic detergents such as OG do not interfere with matrix-assisted laser desorption/ionization (MALDI) mass spectra,[3] and actually increase the intensity of high-MW peptide signals.[4] Such an MS-compatible detergent can help to dissolve precipitated proteins in the gel during the digestion process;[2] we obtained improved peptide recovery of yeast proteins separated by two-dimensional electrophoresis, and OG also helped to prevent adsorption of the peptides on the tube wall or pipette tip.[2,5] Combining a negative staining procedure such as Zn-imidazole staining and the use of a thinner gel (below 1 mm thickness) with addition of OG further improved the outcome, because negative staining allows better peptide recovery than silver staining, the use of thinner gel aids efficient entry of trypsin into the gel, and the resultant tryptic peptides are more easily extracted. Although we could identify membrane proteins of the post-synaptic density fractions as well as soluble proteins by using OG in-gel digestion,[5] we were aware that we might still have missed some interesting membrane proteins. Therefore, it is important to optimize recovery of proteins from gels.

Reduction and alkylation steps are very important in detection of peptides containing Cys residues, and it would also be valuable to perform this step more efficiently.[6] Although 8 M urea is often used during the reduction and alkylation steps for in-solution digestion,[7] most in-gel digestion protocols do not adopt denaturing reagents for in-gel reduction and alkylation. Jeno *et al*. used 0.1% sodium dodecyl sulfate (SDS) in their in-gel reduction and alkylation steps, followed by lysyl endopeptidase (Lys-C) digestion.[8] Lys-C is still active in the presence of low concentrations of SDS, though there is the risk that the MS sensitivity will be reduced by residual SDS. One of the advantages of our previous in-gel digestion protocol[2,5] arises from careful clean-up of salts by effective shaking before protease treatment; hence, denaturing reagents can be used during reduction and alkylation steps to increase the recovery of Cys-peptides without impairing MS sensitivity.

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Here, we describe a superior protocol using 5-cyclohexyl-1-pentyl-β-D-maltoside (CYMAL-5) as an additive for trypsin treatment. CYMAL-5 is a non-ionic detergent that has a ten-fold lower critical micelle concentration (CMC) in comparison with OG, and has previously been used for membrane protein extraction[9,10] or crystallization of membrane proteins for X-ray crystallographic studies.[11] Here, we evaluated the utility of CYMAL-5 as an additive for in-gel reduction and alkylation under denaturing conditions; the analyses were performed by liquid chromatography/mass spectrometry (LC/MS) after in-gel tryptic digestion of cells of human colon cancer cell line HCT116.[12]

EXPERIMENTAL

Materials

Standard peptides, angioteinsin III and insulin B chain (oxidized form), were obtained from Sigma (St. Louis, MO, USA). For analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS), 2,5-dihydroxybenzoic acid (DHB) (Wako Pure Chemical, Osaka, Japan) was used as a matrix. The water used for all experiments was obtained from a MilliQ UV plus water purification system (Millipore, Bedford, MA, USA). Bacteriorhodopsin (*Halobacterium halobium* (gi/3659944) and phospholipase A2 (*Naja mossambica mossambica*) (gi/129410) were obtained from Sigma. TPCK-treated sequencing-grade modified trypsin was obtained from Promega (Madison, WI, USA). n-Octylglucoside (OG) was obtained from Dojin (Kumamoto, Japan) and 5-cyclohexyl-1-pentyl-β-D-maltoside (CYMAL-5) was obtained from Anatrace (Maumee, OH, USA). The negative gel stain MS kit and other reagents for silver staining were obtained from Wako.

SDS-PAGE and in-gel digestion

Gels with thicknesses of 0.5, 0.8 and 1.0 mm (Tris-HCl, 5–20 %T) were obtained from DRC (Tokyo, Japan). After analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE), silver staining was carried out using the protocol of Shevchenko *et al.*,[1] and negative staining according to the Zn-imidazole method was performed according to the instructions with the negative gel stain MS kit. The in-gel digestion procedure was based on the protocol which we previously reported, using a 96-well plate.[2,5] The bands were cut out, and the gel pieces were transferred to an 800-μL scale polypropylene 96-well plate (Nippon Genetics). The silver-stained bands were destained by soaking the gel pieces in a mixture of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. The negatively stained bands were treated with destaining solution containing EDTA (Wako). The plate was sealed with film (UniSeal, Whatman Clifton, NJ, USA) and was sonicated with a 350-W ultrasonic cleaner (Toho, Tokyo, Japan). After destaining, conventional reduction and alkylation steps were performed under 100 mM ammonium bicarbonate buffer. After addition of 10 mM DTT, followed by 10-min sonication, the solvent was discarded, then 500 mM acrylamide was added and the mixture was sonicated for 10 min. Reduction and alkylation under denaturing conditions were done in the same manner except for the use of 6 M guanidine hydrochloride in 100 mM ammonium bicarbonate buffer. Then the gel pieces were washed with 400 μL of methanol/water/acetic acid 50:40:10 (MWA) and sonicated for 30 min. After four replacements of MWA, the gel pieces were sonicated in 400 μL of 100 mM ammonium bicarbonate buffer for 5 min, then in 400 μL of acetonitrile for 5 min, and dried thoroughly in a Speedvac evaporator (Thermo Savant, NY, USA). The dried small gel pieces (ca. 1 mm band width) were re-swollen in 2 μL of 100 mM ammonium bicarbonate containing 0.02 μg of trypsin and 0.1% w/v (3.4 mM) OG or 0.1% w/v (2.0 mM) CYMAL-5 (final concentration), and then 20 μL of 100 mM ammonium bicarbonate were added. In the case of sample preparation for larger gel pieces (ca. 10 mm band width), the dried gel pieces were re-swollen in 20 μL of 100 mM ammonium bicarbonate containing 0.2 μg of trypsin and 0.01% w/v (0.34 mM) OG or 0.01% w/v (0.2 mM) CYMAL-5. Then, 70 μL of 100 mM ammonium bicarbonate were added. The plate was incubated at 37°C for 4 h. Trypsin-digested peptides were extracted twice by 15 min sonication in acetonitrile/water/trifluoroacetic acid (TFA) 75:25:0.1. The combined extracts were dried in a Speedvac and desalted using StageTips.[13]

Preparation of HCT116 samples

HCT116 cells were grown to about 1×10^7/15-cm dish in culture medium. The cultured cells were collected, homogenized and centrifuged at 500 *g* in order to remove the precipitated nuclear fraction. The supernatant was again centrifuged at 100 000 *g* for 1 h at 4°C, and the supernatant and the pellet were collected as the soluble fraction and membrane fraction, respectively. Proteins from the membrane fraction were extracted with Tris-Gly buffer (pH 7.4) containing 1% SDS. For LC/MS analysis, approximately 500 μg of protein were separated by SDS-PAGE. After electrophoresis, each lane was cut into six equal pieces, and in-gel digestion was performed.

MS analysis

After in-gel digestion, the dried samples were redissolved in 5 μL of acetonitrile/water/TFA (5:95:0.1) and a 0.5-μL aliquot was used for MALDI-TOFMS analysis. Matrix solution was prepared by saturating DHB in acetonitrile/water/TFA (33:67:0.1) and was four-fold diluted before mixing with the peptides solution. Co-crystallization was done by depositing 0.5 μL of the peptide solution and 0.5 μL of the diluted matrix solution together at the same place on a MALDI plate, followed by drying at room temperature. MALDI-TOFMS spectra were obtained using a Voyager Linear DE (Applied Biosystems, USA). The Voyager was operated in a delayed-extraction mode and the spectra were calibrated using internal standards, angiotensin III (average MW 931.1 Da) and oxidized insulin B chain (average MW 3495.9). MASCOT (Matrix Sciences) was used as the search engine for peptide mass fingerprinting.

The LC/MS/MS system consisted of an HPLC LC10A-VP system (Shimadzu, Kyoto, Japan), a QSTAR PULSARi quadrupole-time-of-flight instrument (Applied Biosystems) with a lab-made nanospray ion source, and a PAL cycle composer (CTC Analytics, Zwingen, Switzerland) as an autosampler. To prepare the nano-LC column, C18 modified 3 μm particles were packed in a 150 μm i.d. × 10 cm tapered

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Table 1. Classification of the in-gel digestion protocols compared in this work

	Gel (mm)	Stain	Alkylation condition	Detergent
Procedure 1[a]	1.0	Silver	Normal	—[d]
Procedure 2[b]	1.0	Silver	Normal	OG
Procedure 3[c]	0.8	Zn-imidazole	Normal	OG
Procedure 4	0.5	Zn-imidazole	Normal	OG
Procedure 5	0.5	Zn-imidazole	Normal	CYMAL-5
Procedure 6	0.5	Zn-imidazole	Guanidine	OG
Procedure 7	0.5	Zn-imidazole	Guanidine	CYMAL-5

[a] Based on Shevchenko's method.
[b] Based on our method (trypsin (OG)).[2]
[c] Based on our method (negative staining+thinner gel separation +trypsin (OG)).[5]
[d] Detergent not added.

capillary by utilizing the stone bridge effect[14] and the flow rate was adjusted to 300 nL/min. A linear gradient from 5 to 30% of B in 65 min was run, using a mobile phase A of 0.5% acetic acid and mobile phase B of 0.5% acetic acid/acetonitrile (20:80). MASCOT (Matrix Sciences) was used as the search engine for MS/MS analysis. MASCOT search parameters were the following: allowing one missed cleavage of trypsin, NCBI database with *Homo sapiens* taxonomy, fixed modification of propionamide (Cys) and variable modification of oxidation (Met), peptide charge of 2+ and 3+, and 0.25 Da tolerance in MS and MS/MS; a score above 35 ($p < 0.05$) was accepted for identification of peptides.

Classification of in-gel digestion protocols

The various in-gel digestion protocols compared in this study were classified as shown in Table 1. Procedure 1 was a standard protocol based on Shevchenko's method in combination with 1 mm gel separation and silver staining, and procedure 2 was that previously modified by us using OG with trypsin treatment.[2] Procedures 3 and 4 were further modified using thinner separation gels, 0.5 or 0.8 mm, and negative staining.[5] Procedure 5 was the new trial in this study using CYMAL-5 instead of OG. Procedure 6 and 7 were also new approaches using 6 M guanidine solution for Cys alkylation.

RESULTS AND DISCUSSION

In-gel digestion of test samples

First, the utility of CYMAL-5 was checked using the membrane protein bacteriorhodopsin as a test sample. Bacteriorhodopsin (gi/3659944) is a G protein coupled receptor that has seven transmembrane domains. The digestion efficiency was compared using several combinations of conditions with negative staining and a thinner gel of 0.8 mm, as we previously reported.[5] Figure 1 shows the MALDI mass spectra of tryptic digests of bacteriorhodopsin. Only one peak (S/N = 3) was observed when detergent was not added with the trypsin (Fig. 1(A)). On the other hand, several peaks were detected when OG was added with trypsin (Fig. 1(B)), and even more were observed when CYMAL-5 was added with trypsin (Fig. 1(C)) and clearly identified (gi/3659944). The numbers of assigned peptides after digestion in the presence of OG and CYMAL-5 were 3 and 5, respectively, and the signal intensity, as well as sequence coverage, was apparently improved by CYMAL-5. In addition, CYMAL-5 at this concentration did not interfere with the MALDI-MS spectra; no adduct peaks of detergent and no increase in background were observed. Another group has reported an improved in-gel digestion of membrane proteins using a combination of CNBr cleavage and trypsin digestion in the presence of OG.[15] Although this combination was effective for membrane protein digestion, the procedure was quite time-consuming since CNBr cleavage required 12 h and several more hours were needed for the subsequent trypsin treatment. In addition, CNBr sometimes causes side reactions, and is quite toxic. Therefore, our simple procedure of changing the detergent from OG to CYMAL-5 should be more convenient for membrane protein analysis.

Next, the efficiency of in-gel reduction and alkylation of Cys residues under denaturing conditions was studied. Although many proteins such as bovine serum albumin are easily alkylated at Cys residues, we have found that some proteins are not efficiently alkylated in-gel, under conventional conditions in the absence of denaturing reagents. When we in-gel-digested phospholipase A2 (*Naja mossambica mossambica*) (gi/129410), which contains seven disulfide bonds, under conventional in-gel reducing and alkylating conditions, the yield of tryptic peptides was quite low, as shown in Fig. 2(A). The use of CYMAL-5 slightly increased

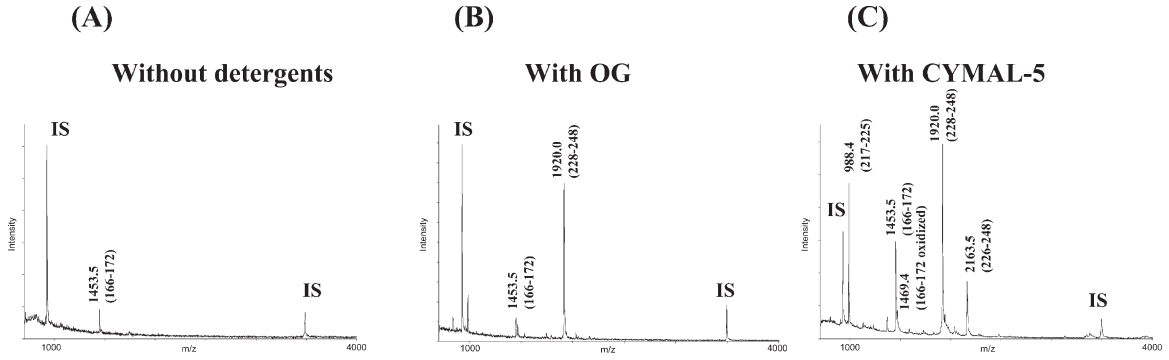

Figure 1. MALDI-TOFMS spectra of in-gel digestion products of bacteriorhodopsin. Conditions: Zn-imidazole staining with 0.8 mm gel; bacteriorhodopsin 1 μg. (A) Without detergent, (B) with OG, and (C) with CYMAL-5. Other conditions are described in the text.

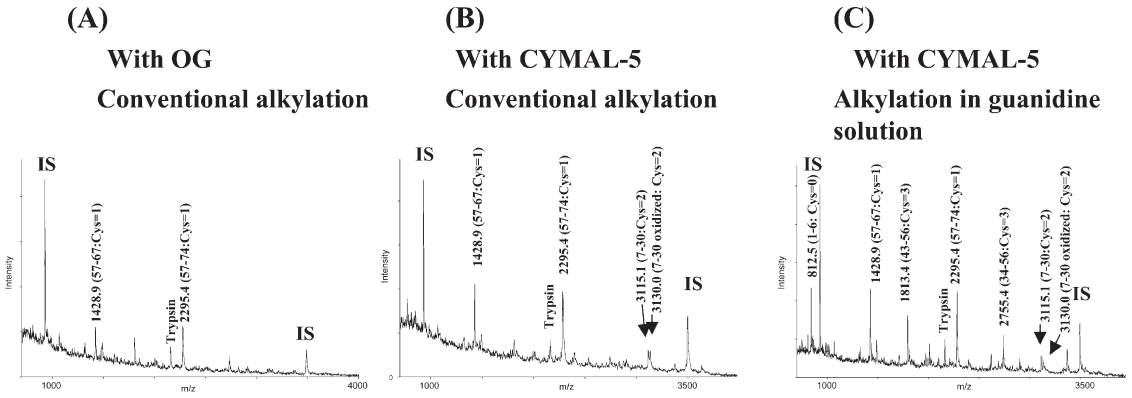

Figure 2. MALDI-TOFMS spectra of in-gel digestion products of phospholipase A2. Conditions: Zn-imidazole staining with 0.8 mm gel; phospholipase A2 1 pmol. (A) With OG and conventional alkylation, (B) with CYMAL-5 and conventional alkylation, and (C) with CYMAL-5 and alkylation in the presence of 6 M guanidine hydrochloride. Other conditions are described in the text.

the peak intensity, but this was still not enough to allow protein identification by MASCOT searching (Fig. 2(B)). However, use of 6 M guanidine hydrochloride as the in-gel reducing and alkylating buffer gave good performance; 8 Cys residues out of a total of 14 Cys residues were detected, and the sequence coverage was 62% (gi/129410) (Fig. 2(C)). Using 6 M guanidine hydrochloride for in-gel alkylation did not affect trypsin autolysis nor keratin contamination. Use of another reducing reagent, tributylphosphine, or another alkylating reagent, iodoacetamide, instead of DTT or acrylamide, did not yield any significant improvement, and use of 8 M urea gave a similar result to that obtained with 6 M guanidine hydrochloride (data not shown). Although many researchers do not use a denaturing buffer such as 8 M urea or 6 M guanidine hydrochloride for in-gel alkylation of Cys residues, we consider that the use of a denaturing buffer during in-gel alkylation is to be strongly recommended.

In-gel digestion of HCT116 samples

For real-world proteome analysis, we selected the LC/MS approach to identify large numbers of proteins in order to test the various in-gel digestion protocols listed in Table 1. The results are displayed in Table 2. Shevchenko's method (procedure 1) was improved by adding OG in our first protocol (procedure 2),[2] and even more proteins were identified with our second-generation protocol (procedure 3),[5] which employs the combination of a thinner (0.8 mm) gel and negative staining. In the present study, the gel thickness was further decreased to 0.5 mm (procedure 4), and this afforded a further improvement. Although a thinner gel thickness is more efficient for in-gel digestion, 0.5 mm is probably close to the practical limit because of difficulties in handling the gel sheets.

Next, we found that use of CYMAL-5 (procedure 5) as a detergent gave a better result than OG (procedure 4), supporting the result obtained in the case of bacteriorhodopsin. The effect of reducing and alkylating Cys residues under denaturing conditions was also studied by comparing the results obtained with procedures 4 and 6, or procedures 5 and 7. An apparent improvement was obtained by using 6 M guanidine hydrochloride as the in-gel alkylation buffer. Procedure 7, involving the combination of both CYMAL-5 and 6 M guanidine hydrochloride, identified the largest number of proteins and is clearly superior to Shevchenko's protocol.

Tryptic peptides containing 0.1% w/v (3.4 mM) OG or 0.1% w/v (2.0 mM) CYMAL-5 were analyzed by LC/MS/MS. Although most peptides seemed to be separated from the OG (retention time 84–85 min) and CYMAL-5 (retention time 88–89 min) peaks, some peptides overlapped with the OG peak. Also, separation of the OG peak seemed to be insufficient, because strongly retained peaks were still observed after the OG peak in procedures 3, 4 and 6, suggesting that some peptide signals were suppressed by OG. On the other hand, CYMAL-5 showed stronger retention than any peptide observed in this study. This means CYMAL-5 is superior to OG for identifying hydrophobic peptides. As shown in Fig. 3, we compared the retention times of identified peptides obtained with various in-gel digestion protocols. The use of CYMAL-5 (procedures 5 and 7) gave the largest number of peptides throughout the whole retention time range. In particular, strongly retained peptides (Fig. 3, inset) were more clearly observed in the presence of CYMAL-5, while relatively few strongly retained peptides were seen in the absence of detergent.

A representative MS/MS spectrum obtained with procedure 7 (CYMAL-5 addition) is shown in Fig. 4. The retention time of this peak was 86 min and the hydrophobic sequence FLFLLLGPAGK was identified; this peptide was only clearly observed when CYMAL-5 was added. CYMAL-5 is thus able to extract hydrophobic peptides, and significant carryover of CYMAL-5 or variation in retention times was not observed during 120 runs.

The amount of detergent added in the digestion step is important, because addition of 1% w/v (34 mM) OG severely suppressed the electrospray ionization of peptides in the region of OG elution from the LC column (data not shown), though significant interference was not observed in MALDI-MS spectra.[16] CYMAL-5 has a low CMC value of approximately 2 mM. Therefore, 2 mM CYMAL-5 is expected to show the same dissolving power as 20 mM OG.

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Table 2. Results of LC/MS/MS analysis after in-gel tryptic digestion of HCT116

		Gel (mm)	Stain	Alkylation condition	Detergent	Unique peptides	Unique proteins
Procedure 1[a]	Soluble frac.	1.0	Silver	Normal	—[d]	2344	786
	Membrane frac.	1.0	Silver	Normal	—[d]	2270	744
	(Membrane protein)[e]					352	138
Procedure 2[b]	Soluble frac.	1.0	Silver	Normal	OG	2684	836
	Membrane frac.	1.0	Silver	Normal	OG	2633	815
	(Membrane protein)[e]					390	142
Procedure 3[c]	Soluble frac.	0.8	Zn-imidazole	Normal	OG	2829	867
	Membrane frac.	0.8	Zn-imidazole	Normal	OG	2780	845
	(Membrane protein)[e]					420	151
Procedure 4	Soluble frac.	0.5	Zn-imidazole	Normal	OG	3157	914
	Membrane frac.	0.5	Zn-imidazole	Normal	OG	3102	895
	(Membrane protein)[e]					455	156
Procedure 5	Soluble frac.	0.5	Zn-imidazole	Normal	CYMAL-5	3421	941
	Membrane frac.	0.5	Zn-imidazole	Normal	CYMAL-5	3222	904
	(Membrane protein)[e]					505	167
Procedure 6	Soluble frac.	0.5	Zn-imidazole	Guanidine	OG	3485	965
	Membrane frac.	0.5	Zn-imidazole	Guanidine	OG	3396	921
	(Membrane protein)[e]					483	162
Procedure 7	Soluble frac.	0.5	Zn-imidazole	Guanidine	CYMAL-5	3650	987
	Membrane frac.	0.5	Zn-imidazole	Guanidine	CYMAL-5	3431	962
	(Membrane protein)[e]					544	175

[a] Based on Shevchenko's method.
[b] Based on our method (trypsin (OG)).[2]
[c] Based on our method (negative staining+thinner gel separation+trypsin (OG)).[5]
[d] Detergent not added.
[e] Annotated by Gene Ontology[17] from membrane fraction samples.

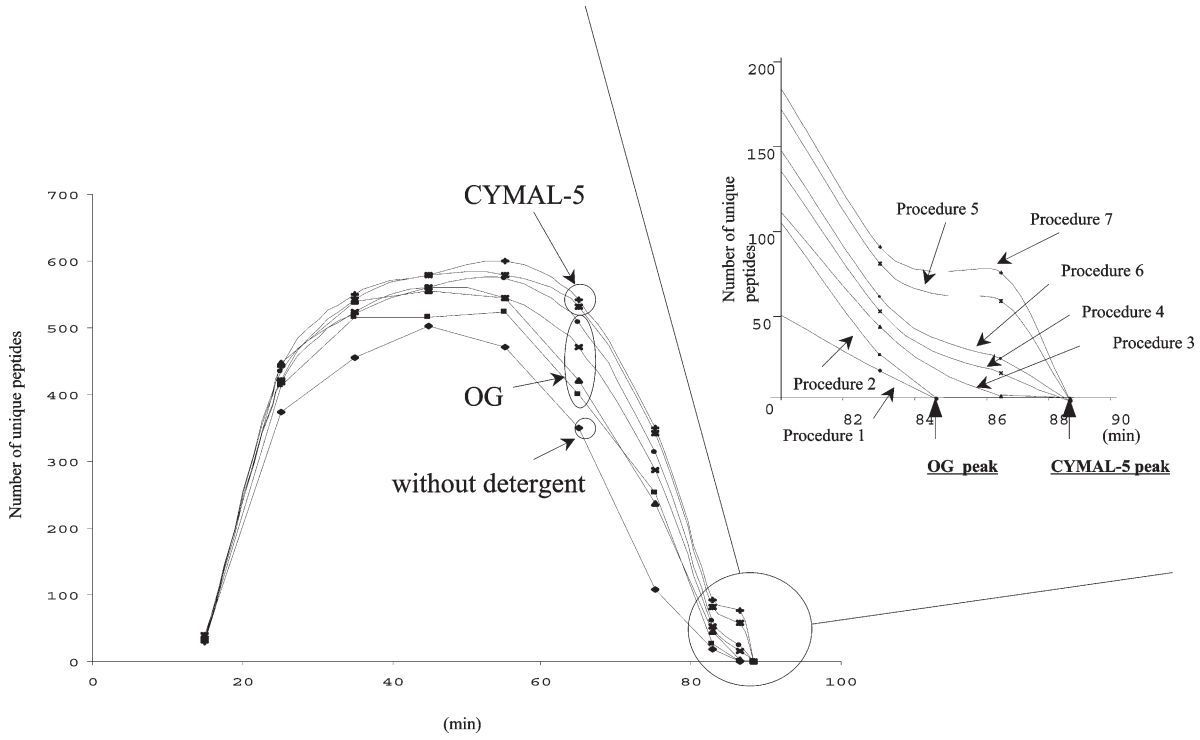

Figure 3. Number of peptides identified from membrane fraction using various in-gel protocols. Peptides were identified by LC/MS/MS, and each point expresses the total number of peptides found every 10 min up to 80 min retention time, and every 2 min after 80 min retention time. For other conditions, see Table 1 and the text.

The results of evaluation of in-gel alkylation efficiency of Cys residues are shown in Fig. 5. The number of Cys residues (counted from the Cys-containing peptides) obtained under denaturing conditions was greater than that obtained using the procedure with conventional buffer (without denaturing reagents) in both soluble and membrane fractions. This result suggests that the in-gel alkylation was improved by using 6 M guanidine hydrochloride. Further, proteins were not lost from the gel pieces because the number of identified peptides was increased by using 6 M guanidine hydrochloride (Table 2). The results suggest that the increase in the number of identified peptides was due to the denaturing effect during

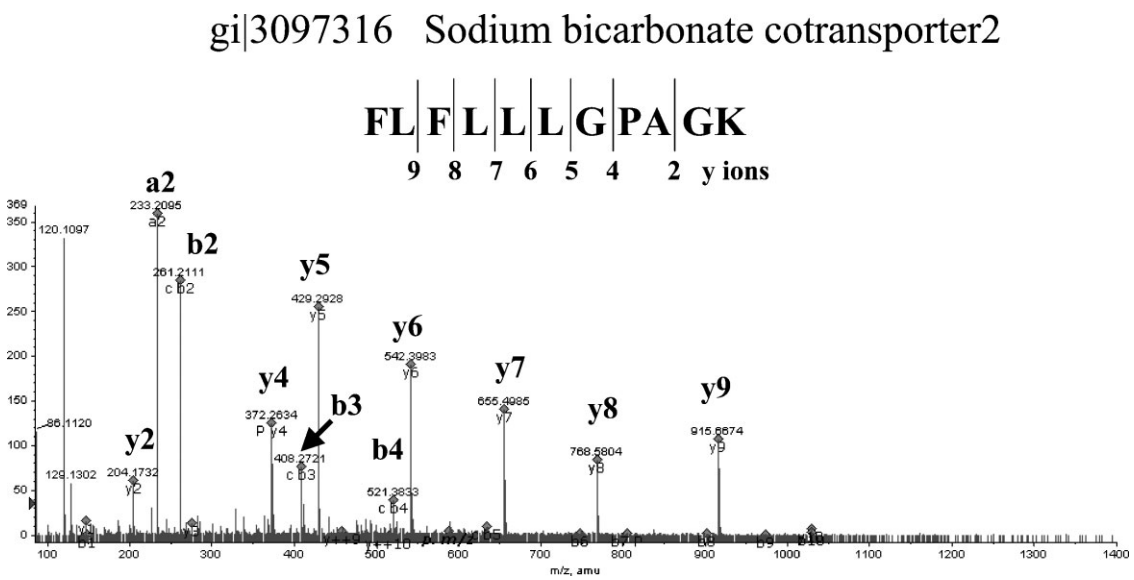

Figure 4. Representative MS/MS spectrum of hydrophobic peptides obtained by trypsin digestion in the presence of CYMAL-5. Conditions are described in the text.

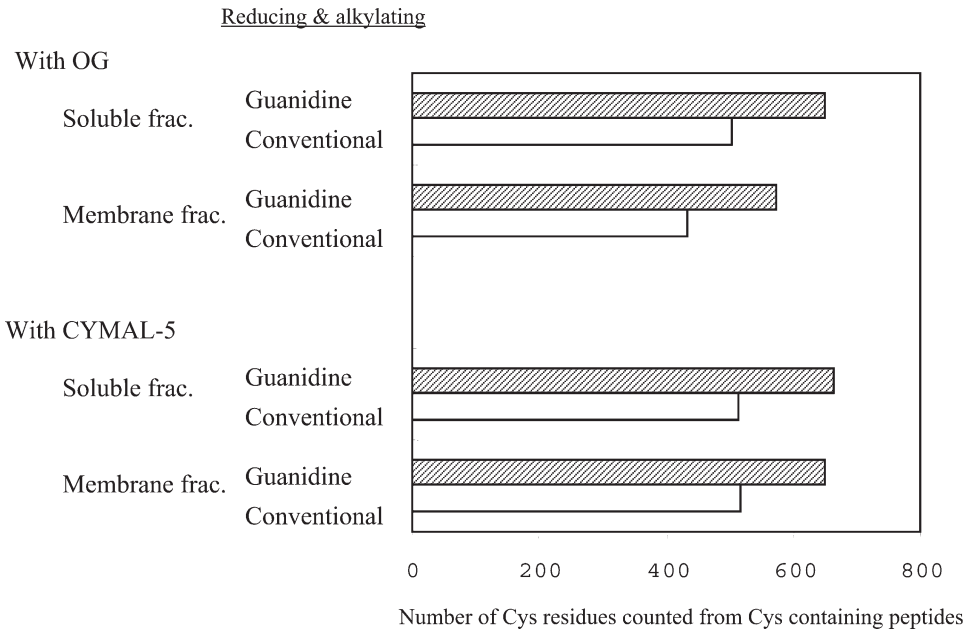

Figure 5. Number of identified Cys residues counted from the Cys-containing peptides. Conditions are described in the text.

the in-gel alkylation step, and not only Cys-containing peptides, but also other peptides, were recovered more efficiently. Here, we note that, although in-solution alkylation of proteins before SDS-PAGE would give better results, in practice proteins are often separated on SDS-PAGE without alkylation. Therefore, a convenient in-gel alkylation procedure under denaturing conditions is a significant advance.

Identified proteins from the membrane fraction of HCT116 cells were annotated based on Gene Ontology data.[17] Although information about many cellular protein components is incomplete, numbers of membrane proteins were identified with the procedures tested here (Table 2). Procedure 1 without detergent in the in-gel digestion step gave the lowest number, and adding CYMAL-5 in the in-gel digestion step (procedures 5 and 7) increased the number of membrane proteins identified. The combination of thinner gel (0.5 mm), negative staining, a 6 M guanidine hydrochloride in-gel alkylation step, and CYMAL-5 in the in-gel digestion step, was clearly superior in allowing identification of more membrane proteins.

CONCLUSIONS

Use of the non-ionic detergent CYMAL-5 as an additive in our high-throughput in-gel digestion system using 96-well plates significantly improved performance. The combination of a thinner gel, negative staining, a 6 M guanidine hydrochloride

in-gel alkylation step, and CYMAL-5 in the in-gel digestion step, gave the best performance when HCT116 proteins were analyzed by LC/MS after SDS-PAGE separation. The protocol shown here is simple and convenient, and should be widely applicable to various samples, including membrane proteins. It is also considered to be suitable for use in a fully automated robotic system.

Acknowledgements

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