

Figure 4. MALDI MS spectrum of the N-terminal peptides obtained from lysozyme, cytochrome c and BSA. Each protein (5 pmol) was separated by SDS-PAGE. After these bands were excised from the gel, they were mixed in a micro tube and were prepared as described.

Blue staining and then destained overnight in 25% isopropanol/10% acetic acid.

Mass spectrometry

The sample dissolved in 0.1% TFA was desalted with a ZipTip C18 (Millipore Corp.) and eluted with 2.5 μ L 50% acetonitrile/0.05% TFA solution before analysis by MALDI-MS, and α -cyano-4-hydroxycinnamic acid (CHCA) saturated in 50% acetonitrile/0.05% TFA solution was used as an appropriate matrix to perform MALDI-PSD-MS analysis. A portion (0.5 μ L) of each sample solution was

mixed with 0.5 μ L of the matrix solution on the MALDI target and analyzed after drying. The m/z values of the spectra were externally calibrated with a bradykinin fragment (monoisotopic mass: 757.40) and an ACTH fragment. Mass spectra were measured on an AXIMA CFR-plus V2.3.2 (Shimadzu/Kratos, Manchester, UK) mass spectrometer. A nitrogen laser (337 nm) was used to irradiate the sample for ionization. The acceleration voltage of the instrument was set to 20 kV using a gridless-type electrode. The reflectron mode was set to detect positive ions. The PSD spectrum was obtained by focusing all the product ions in a single spectrum by curved

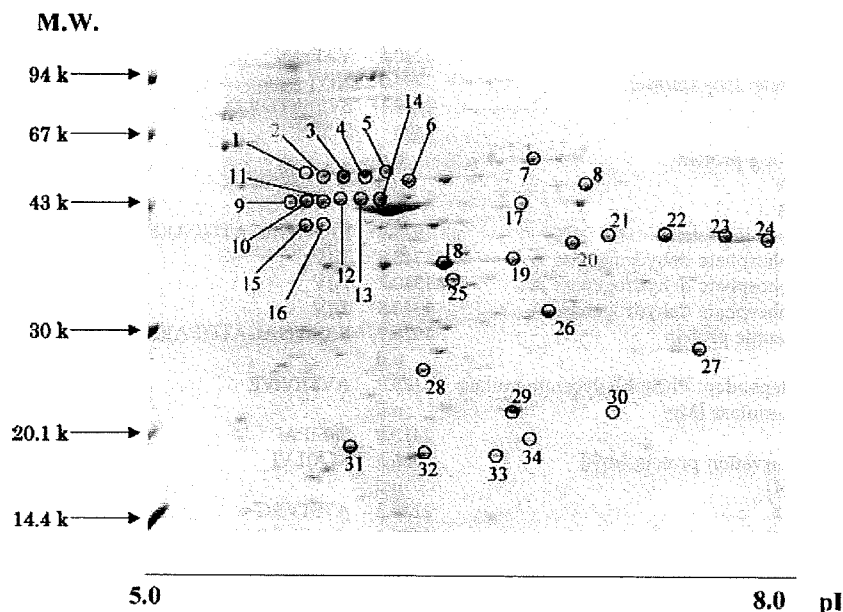


Figure 5. Two-dimensional profile of *Escherichia coli* extracts. Spots shown with a number were prepared as described.

field reflectron.¹⁶ Selected product ions generated from angiotensin II were used for external PSD calibration.

RESULTS AND DISCUSSION

Application for proteins analyzed 'in-gel'

In the previous preliminary work¹⁴ we described a *de novo* sequencing method for N-terminal peptides isolated from proteins blotted on PVDF membranes. We have since developed methods to analyze proteins separated by 1D or 2D SDS-PAGE that do not require blotting on PVDF membranes, leading to higher recovery of N-terminal peptides. Chemical modifications including carboxyamido-methylation, guanidination and N-terminal derivatization of proteins were performed according to the methods described above. At each step of the chemical modification processes, washing the reagents thoroughly from the gel is key to obtain effective mass spectra necessary for N-terminal sequencing by this method.

The effectiveness of this procedure was confirmed for four model proteins (lysozyme, GP3, cytochrome *c* and BSA). Figure 2(a) shows a MALDI mass spectrum obtained from cytochrome *c* after isolation of the N-terminal peptide from avidin resins as described in the Experimental section, where 1 pmol of the protein was loaded on the SDS-PAGE gel. The mass peak corresponding to the N-terminal peptide was mainly detected at *m/z* 1159.2. Furthermore, the five N-terminal amino acid residues were *de novo* sequenced by PSD analysis of the peak at *m/z* 1159.2. The MALDI-PSD mass spectra of N-terminal peptides isolated from two model proteins (G3P and BSA) using this method are summarized in Fig. 3, which shows that this method is sensitive enough to analyze sub-pmol levels of proteins.

To assess the ability of this method to identify each protein in a heterogeneous protein sample, we mixed the following three standard proteins: lysozyme, cytochrome *c* and BSA, and then separated them on a gel (Fig. 4). The signals corresponding to three N-terminal fragments derived from

Table 1. Sequencing results for proteins separated using 2D-PAGE of *Escherichia coli* extracts

Spot No	Candidate protein by PMF	N-terminal peptide [M+H]	Identified sequence	N-terminal sequence
1	Outer membrane protein tolC precursor	1547.8	ENImQVm	ENLMQVYQQAR
2	Glutamate decarboxylase beta	773.3	mDJJ	MDKK
	Glutamate decarboxylase alpha	1477	mDQJmm(TD)	MDQKLLTEDFR
3	Glutamate decarboxylase beta	773.3	mDJJ	MDKK
	Glutamate decarboxylase alpha	1476.9	mDQJmm(TD)	MDQKLLTEDFR
4	Glutamate decarboxylase beta	773.3	mDJJ	MDKK
	Glutamate decarboxylase alpha	1476.9	mDQJmm(TD)	MDQKLLTEDFR
5	Glutamate decarboxylase beta	773.3	mDJJ	MDKK
6	Alkaline phosphatase, chain A	1354	TPEmP	TPEMPVLENR
7	H ⁺ transporting two-sector ATPase alpha chain	2000.2	mQiNS	MQLNSTEISELIK
8	Serine hydroxymethyltransferase	757.3	mi(JR)	MLKR
9	Isocitrate dehydrogenase	1524.9	mESJVVV(AE)GJ	MESKVVVAEGK
10	Isocitrate dehydrogenase	1524.9	mESJVVV	MESKVVVAEGK
11	Isocitrate dehydrogenase	1524.9	mESJVVV(AE)GJ	MESKVVVAEGK
12	Enolase ([2-phosphoglycerate dehydratase)	794.5	SJiVJ	SKIVK
13	Enolase ([2-phosphoglycerate dehydratase)	794.5	SJiVJ	SKIVK
	Putative aminotransferase	1240.8	VAEmN	VAEMNKIR
14	Enolase ([2-phosphoglycerate dehydratase)	794.5	SJiVJ	SKIVK
15	Phosphoglycerate kinase	1893.1	SVijm(TD)LD	SVIKMTDLDLAGKR
16	Phosphoglycerate kinase	1893	SVijm	SVIKM
17	Glycerol-3-phosphate-binding protein	n.d		
18	Outer membrane proteinA	n.d		
19	Outer membrane proteinA	n.d		
20	Phosphate-binding periplasmic protein	1928.9	EASiTGAGATF(PA)(PV)mAJ	EASLTGAGATFPAPVYAK
21	gapA, Glyceraldehyde-3-phosphate dehydrogenase A	1340.8	TiJV	TIKVINGFGR
22	gapA, Glyceraldehyde-3-phosphate dehydrogenase A	1340.7	TiJV	TIKVINGFGR
23	gapA, Glyceraldehyde-3-phosphate dehydrogenase A	1340.8	TiJV	TIKVINGFGR
24	Phosphate-binding periplasmic protein	1928.9	EASiTGAGATF(PA)(PV)mAJ	EASLTGAGATFPAPVYAK
25	Chaperone protein hchA	n.d		
26	2,3-Bisphosphoglycerate-dependent Phosphoglycerate mutase	1178.9	AVTJiViVR	AVTKLVLVR
27	Poo448, and superoxide dismutase [Mn]	n.d		
28	Superoxide dismutase	1413.8	SFEiPAi	SFELPALPYAK
29	DNA protection during starvation protein [dps]	966.6	STAJLVJ	STAKLVK
30	yfbU,UPF0304 protein yfbU	n.d		
31	Outer membrane protein X	2225.3	ATSTVRG	ATSTVRGGYQAQSDAQGMNK
32	DNA-binding protein H-NS	1450.4	SEAiKiNniR	SEALKILNNIR
33	Gluconate-6-phosphate dehydrogenase	n.d		
34	Outer membrane protein X	n.d		

N-Terminal peptides were purified from the tryptic digests of each spot and analyzed by MALDI-PSD. The candidate proteins obtained by PMF analyses using Mascot from a database (MSDB), identified by *de novo* sequencing using MALDI-PSD analyses and matching to N-terminal sequences are listed.

each protein could be detected on one mass spectrum in contrast to other methods that involve analysis of complicated internal tryptic digests, and as a result the sequence of each N-terminal fragment could be determined *de novo* by PSD-MALDI analysis. It should also be noted that Met and Trp are oxidized to Met(O₂) and Trp(O)/Trp(O₂)/Trp(O₃), respectively, in this protocol (data not shown). As a result, Met and Tyr cannot be distinguished, as they are too close in molecular weight (MW).

Application of the method to *Escherichia coli* extracts

To prove that this method is practical, we attempted N-terminal sequencing of proteins present in *Escherichia coli* extracts. The protein extracts from lyophilized cells were first separated by 2D PAGE in the range of pH 5–8. After staining the gel with Coomassie Brilliant Blue, 34 protein spots with MWs ranging from 15 to 70 kDa were randomly excised from the gel (Fig. 5) and subjected to N-terminal sequencing analysis by the method developed here. The results were completely compatible with results of PMF analysis that utilized one-tenth of their tryptic digests (Table 1). The mass lists for PMF analysis were converted manually by AXIMA software and database searches were performed using Mascot. When two or more peaks were observed in the MALDI-TOF spectrum, analyses in negative mode were carried out to confirm the identity of the N-terminal peptides, as the intensity in negative mode is generally increased by

introduction of a sulfate group.^{14,17} The precursor ions chosen by the procedure described above were analyzed by MALDI-PSD. Moreover, detection of the neutral loss peak of the derivatives (m/z -136) or the derivatives and -NH₃ molecule (m/z -153) in the case where this fragment contains homoarginine in the internal sequence was used to determine whether these ions were N-terminal fragments. Two peaks (m/z 773 and 1476) appeared mainly in the MALDI-TOF spectrum after isolation of the N-terminal peptide of spot No. 2, and each peak ion was analyzed as a precursor ion by MALDI-PSD (Fig. 6). The sequences were identified from two precursor ions (m/z 773 and 1476). The sequence (MVKK) obtained by MALDI-PSD analysis of the precursor ion at m/z 773 was in agreement with the N-terminal sequence of glutamate decarboxylase beta, and another sequence (MDQKLLTDFR) obtained from an ion at m/z 1476 was in agreement with that of glutamate decarboxylase alpha. Therefore, it was confirmed that there were two proteins in the same spot, which had an extremely high homology except for their N-terminal regions, even though the top-scoring protein in the PMF analysis was glutamate decarboxylase alpha. This result shows the effectiveness of carrying out N-terminal sequencing of proteins that cannot be separated, even by 2D-PAGE. As described above, the N-terminal sequences from 26 spots were identified (Table 1). Determination of the N-terminal sequences of targeted proteins by this method relies on the presence of an arginine or homoarginine residue in the

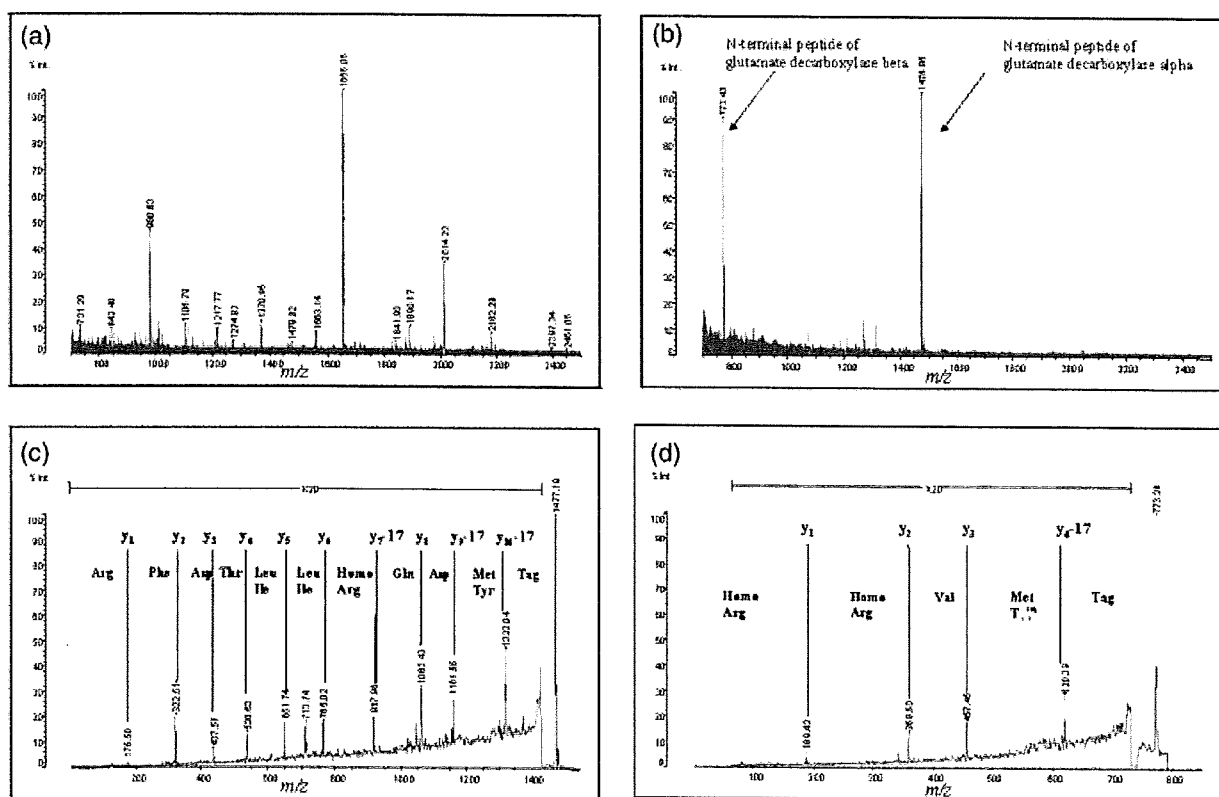


Figure 6. MALDI spectra obtained at each step during N-terminal sequencing of spot No. 2 from *Escherichia coli* extracts separated by 2D-PAGE: (a) MALDI-TOF MS spectrum after trypsin digestion; (b) MALDI-TOF MS spectrum of the N-terminal peptides specifically isolated from the trypsin digests; (c) MALDI-PSD spectrum of the precursor ion at m/z 773; and (d) MALDI-PSD spectrum of the precursor ion at m/z 1476.

C-terminal region of the peptide. It may be difficult to use MALDI-MS analysis to detect some proteins that are cleaved by trypsin quite close to their N-termini. Conversely, other proteins that produce too long an N-terminal fragment after trypsin digestion may be difficult to identify by MS analysis. We observed significantly different sensitivities in detecting different N-terminal fragments by MALDI-MS analysis. In general, N-terminal sulfonation of peptides resulted in increased sensitivity in the negative mode of MALDI MS analysis, compared with the positive mode. However, some peptides containing more than one arginine or homoarginine residue due to miscleavage by trypsin digestion gave less sensitivity in the negative mode. Therefore, MS analysis in both positive and negative ion modes is recommended to identify the targeted N-terminal fragments.

CONCLUSIONS

In this report, we describe a new method to determine N-terminal amino acid sequences of proteins by MALDI-TOF MS. After reduction and alkylation of cysteine residues, and blocking of ϵ -amino groups of lysine residues by guanidination with *O*-methylisourea hemisulfate, α -amino groups of N-termini of proteins are derivatized by sulfo-NHS-SS-biotin. By employing sulfo-NHS-SS-biotin instead of BCA, which was used previously,¹⁴ specific isolation of N-terminal peptides from trypsin digests of proteins followed by introduction of sulfonyl groups into their N-termini proceeds sequentially at rather better yields. Amino acid sequences of these N-terminal peptides are easily determined *de novo* by exclusive observation of γ -series fragment ions in the MALDI-PSD spectrum. The sensitivity of this method is sufficient to analyze low pmol levels of proteins, and this level is almost equivalent to that of the Edman method using a gas-phase sequencer. However, our method provides several advantages. The first is that it can support high-throughput N-terminal analysis of many proteins in parallel, and the second is its applicability to heterogeneous protein samples. Even if the protein sample in a gel contains more than one protein, each N-terminal sequence analysis can be carried out independently, without

requiring time-consuming purification of each protein. A third advantage is that this method could prove useful as a tool to identify each protein comprising a multiprotein complex in living cells. This method developed here is not effective for N-terminally blocked proteins, but a revised method, which is effective regardless of such N-terminal modifications, has been developing based on the principle described in this paper.

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ARTICLES

Identification on Membrane and Characterization of Phosphoproteins Using an Alkoxide-Bridged Dinuclear Metal Complex as a Phosphate-Binding Tag Molecule

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We have developed a method for on-membrane direct identification of phosphoproteins, which are detected by a phosphate-binding tag (Phos-tag) that has an affinity to phosphate groups with a chelated Zn²⁺ ion. This rapid profiling approach for phosphoproteins combines chemical inkjet technology for microdispensing of reagents onto a tiny region of target proteins with mass spectrometry for on-membrane digested peptides. Using this method, we analyzed human epidermoid carcinoma cell lysates of A-431 cells stimulated with epidermal growth factor, and identified six proteins with intense signals upon affinity staining with the phosphate-binding tag. It was already known that these proteins are phosphorylated, and our new approach proved to be effective at rapid profiling of phosphoproteins. Furthermore, we tried to determine their phosphorylation sites by MS/MS analysis after in-gel digestion of the corresponding spots on the 2DE gel to the rapid on-membrane identifications. As one example of use of information gained from the rapid-profiling approach, we successfully characterized a phosphorylation site at Ser-113 on prostaglandin E synthase 3.

KEY WORDS: on-membrane digestion, phosphate-binding tag, chemical inkjet technology, phosphoproteomics, mass spectrometry.

Phosphorylation is a major post-translational modification of proteins, and a large number of proteins in living cells are regulated by phosphorylation by kinases, or dephosphorylation by phosphatases.¹ These modifications are essential for cellular events including signal transduction, DNA transcription, protein synthesis, cell-cycle progression, and cell metabolism.²⁻⁴ In order to elucidate relationships between regulation mechanisms by phosphorylation/dephosphorylation of proteins and such cellular events, it is necessary to investigate the phosphorylation status of proteins, identifying the specific sites at which they have been modified. This is still a challenging

task, even with recent advances in the proteomics field, which includes powerful methods to analyze proteins by mass spectrometry. Several approaches using different strategies have been developed to investigate protein phosphorylation in cells and tissues. Immunostaining or immunoprecipitation approaches using specific phosphoserine, phosphothreonine, and phosphotyrosine antibodies have been utilized for detection or enrichment of phosphoproteins.⁵ Traditional metabolic labeling methods involving incorporation of the radioactive isotope ³²P have also frequently been used in the phosphoproteomics field.⁶ Furthermore, analyses of phosphorylations by kinases and dephosphorylations by phosphatases *in vitro* have been effectively used to estimate dynamic movements of target proteins in phosphorylation/dephosphorylation systems.⁷ However, characterization of phosphorylation sites on target proteins using these approaches is technically challenging. Analysis by mass spectrometry, especially tandem mass spectrometry, has been relied on most often for char-

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acterization of phosphoproteins and their phosphorylation sites.⁸ Database searches are used to analyze MS/MS spectra from tryptic digested phosphoproteins, and these can indicate the phosphorylation sites of proteins. In general, phosphoproteins comprise less than 10% of cellular proteins in typical mammalian cells. Therefore, a method for enrichment of phosphoproteins from cells and tissues is required prior to MS analysis.⁹ Selective isolation and enrichment of phosphoproteins and -peptides can be carried out using antibodies against phosphorylated amino acids, or by β -elimination chemistry performed on phospho-Ser/Thr residues.¹⁰ However, it has been known that these methods have several drawbacks. For example, false-positive enrichment can happen when using antibodies against phospho-Ser/Thr because these antibodies have a low specificity in comparison with a phospho-Tyr antibody. In the β -elimination method, unwanted side reactions can occur, and reproducibility may be problematic. On the other hand, the enrichment of phosphoproteins and -peptides by immobilized metal ion affinity chromatography (IMAC), where chelated metal ions having an affinity for phosphate groups are used (such as Fe^{3+} , Ga^{3+}), is a fast, easy to use, and economical procedure.¹¹ Ficarro et al. analyzed proteins extracted from yeast lysates that were digested with trypsin, and the resulting phosphopeptides were then identified by LC-MS/MS analysis after enrichment of phosphopeptides by IMAC.¹² Furthermore, they have shown that methyl esterification of carboxylic groups decreased nonspecific binding of these peptides to the IMAC column.¹² A phosphate-binding tag molecule, which is a novel type of IMAC utilizing the chelated Zn^{2+} ion and has a strong affinity to phosphate groups, has been recently developed.^{13,14} In the present study, we describe an approach consisting of both microscale identification of phosphoproteins on-membrane by using a Phos-tag molecule and characterization of their phosphorylation sites by MS/MS after enrichment of in-gel digests. The Phos-tag molecule is an alkoxide-bridged dinuclear metal complex (i.e., 1,3-bis[bis(pyridine-2-ylmethyl)amino]propan-2-olato dizinc(II) complex). This method also utilizes on-membrane peptide mass fingerprinting (PMF) analysis of a microscale region using inkjet technology, followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).¹⁵ To achieve the process described above, a chemical inkjet printer instrument is used to capture the sample image, and to micro-dispense reagents onto the visually designated positions. Trypsin is microdispensed by chemical inkjet printer onto the membrane used for affinity staining, and then the matrix solution is dispensed directly onto the same position prior to MALDI-TOF MS analysis, which is carried out directly from the membrane surface. This approach

has the advantage that PMF analysis of the visualized proteins is performed on the same membrane, without the necessity to extract proteolytic peptides after digestion. Furthermore, this microdispensing function of reagents using piezoelectric inkjet technology could be used to improve on-membrane PMF analysis in the microscale region of the protein spots without cross-contamination between proximate proteins. As a result, reagents at sub-nanoliter volume levels can be microdispensed, allowing digestion of only a tiny region within a protein spot. By using phospho-enriched whole-cell lysates of A-431 cells that had been stimulated with epidermal growth factor (EGF), we could successfully identify six proteins with intense signals in affinity staining with biotin-pendant Phos-tag on a 2DE blot.¹⁶ However, we could not identify the phosphorylation sites on these peptides by on-membrane MS analysis because of ion suppression by negative charges in a number of the nonphosphorylated peptides that were produced by tryptic digestion. Therefore, we further performed enrichment of phosphopeptides using Phos-tag agarose from in-gel digested peptides.

Firstly, we characterized the phosphopeptides from ovalbumin digests to confirm the detection limit for the MS approach after enrichment by Phos-tag agarose. Phosphopeptides were enriched using Phos-tag agarose, and a few hundred femtomoles were analyzed by SDS-PAGE/MALDI-MS as described above. Subsequently, we carried out enrichment of phosphopeptides followed by MALDI-MS analysis for 2DE-separated proteins of phospho-enriched whole-cell lysates of A431 cells stimulated with EGF. A phosphorylation site in a phosphopeptide of prostaglandin E synthase 3, one of the proteins identified by on-membrane PMF analysis, was characterized by MS/MS analysis to be at Ser 113. In this experiment a phosphorylation site of prostaglandin E synthase 3 was directly characterized, although phosphorylation sites of this protein were predicted to be at Ser 113 and Ser 118 by mutation analysis, including measurements of enzyme activity.¹⁷ Here we describe an effective approach for analysis of phosphoproteins consisting of both rapid on-membrane identification using chemical inkjet technology by detection with biotin-pendant Phos-tag and selective enrichment using Phos-tag agarose followed by MS/MS analysis to determine the specific phosphorylation sites.

MATERIALS AND METHODS

Materials

Phospho-enriched whole-cell lysates of A-431+EGF/PE were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ovalbumin, tributylphosphine, iodoacetamide, ProteoPrep Total Extraction Sample Kit,

polyvinylpyrrolidone (PVP-40), Direct Blue 71, α -cyano-4-hydroxy-cinnamic acid (CHCA), and 2,5-dihydroxy benzoic acid (2,5-DHB) were obtained from Sigma-Aldrich (St. Louis, MO). Trypsin was obtained from Promega (Madison, WI) and the Immobilon-FL PVDF membrane was purchased from Millipore (Bedford, MA). Streptavidin, Alexa Fluor 633 conjugate, was obtained from Invitrogen (Carlsbad, CA). Pharmalyte (pH 3–10) and Immobiline DryStrip (pH 3–10 NL, 13 cm) were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). 1-O-n-Octyl- β -D-glucopyranoside was obtained from Nacalai Tesque (Kyoto, Japan). Phos-Tag Agarose was purchased from MANAC, Inc. (Hiroshima, Japan), and biotin-pendant Phos-tag ligands were obtained from the Phos-tag consortium (<http://www.phos-tag.com/english/index.html>).

Instruments

Direct analysis on the PVDF membrane was performed using a MALDI-TOF MS instrument, AXIMA-CFR plus (Shimadzu Corporation, Kyoto, Japan, and Kratos Analytical, Manchester, UK), which was operated in positive-ion mode by using an internal calibration method with trypsin autodigest and ACTH (18–39) (m/z 842.51, 2465.20). For MS/MS of enriched phosphopeptides, a MALDI-QIT TOF MS instrument, AXIMA-QIT (Shimadzu Corporation, Kyoto, Japan, and Kratos Analytical, Manchester, UK), was used, with external calibration using angiotensin II and ACTH (18–39) (m/z = 1046.54 and 2465.20, respectively). For on-membrane digestion on a microscale region of protein spots, the chemical inkjet printer (Shimadzu Corporation, Kyoto, Japan) was used for microdispensing the reagents onto blotted protein spots, as previously reported.¹⁵

Sample Preparation of In-Gel Digested Ovalbumin for Enrichment of Phosphopeptides

Ovalbumin (5, 2, 1, 0.5, 0.2 pmol) was separated with SDS-PAGE (10–20%) and visualized with Coomassie brilliant blue (CBB) staining. The gel pieces were excised and washed in 50 mM NH_4HCO_3 /50% (v/v) acetonitrile for 10 min. After reducing with 10 mM dithiothreitol at 56°C for 60 min, the protein in the gel was alkylated with 55 mM iodoacetamide for 60 min at room temperature. The following in-gel digestion was performed according to the protocol in the Experimental section, In-Gel Trypsin Digestion, as described below. The digested peptides were utilized for both PMF analysis and enrichment of phosphopeptides for MS analysis.

Sample Preparation for 2DE

The proteins from phospho-enriched whole-cell lysates of A-431+EGF/PE (200 μg), were recovered by TCA pre-

cipitation and then dissolved in 200 μL Protein Extraction Reagent Type 3 solution (from ProteoPrep Sample Extraction Kit). Protein solutions from the cell lysates of A-431+EGF were prepared according to the manufacturer's protocol for isoelectric focusing. Solubilized proteins were reduced with 5 mM tributylphosphine for 60 min at room temperature and then alkylated with 15 mM iodoacetamide for 60 min at room temperature. Pharmalyte (pH 3–10) was added to a final concentration of 0.2% (w/v) and a trace of bromophenol blue (BPB) was also added. The protein solution was centrifuged at 15,000 g for 20 min at 20°C and the supernatant was used for rehydration of IPG strips. Amersham IPG strips (pH 3–10, 13 cm) were rehydrated for 8 h with the prepared sample solution (200 μL) and focused on a Protean IEF Cell apparatus (Bio-Rad, Hercules, CA) for 100 kV/h at a maximum of 8 kV. The focused IPG strips were equilibrated for 10 min with equilibration buffer including 20 mg/mL dithiothreitol, and the second dimensional SDS-PAGE (10–20%) was then performed for these strips. Separated gels were supplied to the following electroblotting for detection of phosphoproteins using a phosphate-binding tag, and they were also utilized for the in-gel digestion technique to enrich phosphopeptides using affinity-tag agarose.

Detection of Phosphoproteins Using Phos-Tag Biotin and Streptavidin Conjugate Labeled with Alexa Fluor 633

The proteins separated by 2DE were blotted onto the Immobilon-FL membrane using the semi-dry electroblotting method described previously.¹⁸ The blotted membrane was rinsed with water and air dried. Dried PVDF membranes were rewetted by dipping into 100% methanol for 10 sec and then were air dried on a filter paper for 15 min. Subsequently, the blot was dried in a vacuum chamber for 30 min. The blot was incubated at 37°C for 30 min and then was air dried for 2 h. According to the method reported by Kinoshita et al. with some modifications, phosphoprotein spots were detected with Phos-tag biotin and streptavidin labeled with Alexa Fluor 633.¹⁶ In order to form the zinc(II)-bound Phos-tag molecule, 10 μL of 0.1 M Phos-tag biotin in MeOH was incubated with 20 μL of 10 mM $\text{Zn}(\text{NO}_3)_2$, 2 μL of streptavidin conjugate, and 468 μL 10 mM Tris-HCl, 100 mM NaCl containing 0.25% (w/v) PVP-360, pH 7.4 (TBS-PVP), for 30 min at room temperature. After desalting on a filter unit (molecular weight cut off = 10 kDa), this active Phos-tag solution was diluted into 50 mL TBS-PVP buffer. The blot membrane was completely dried according to a slightly modified rapid immunodetection approach, as reported previously.¹⁹ The blot was immersed into the active Phos-tag solution and incubated for 30 min. The

blot was then washed with TBS buffer for 5 min twice and then rinsed with water. The fluorescent image was detected with an excitation wavelength of 635 nm using the FLA-5000 analyzer (Fujifilm, Tokyo, Japan). After image acquisition, the Phos-tag molecule was removed by incubation in 1 N aqueous NH_3 for 15 min three times. This was followed with Direct Blue 71 staining of the blot membrane.²⁰ A direct on-membrane peptide mass fingerprinting (PMF) approach was followed for the dark spots corresponding to spots detected on fluorescent images.

Direct On-Membrane Peptide Mass Fingerprinting

The blot membrane was cut and adhered to the stainless steel plate using 3M electrically conductive tape 9713 (St. Paul, MN). A visualized image of the adhered blot was acquired with a scanner in the chemical inkjet printer, and the target protein spots were selected on the basis of the scanned images. Subsequently, the reagents for on-membrane digestion were printed onto microscale regions of the protein spots.¹⁵ Ten nL of 0.1% (w/v) PVP solution in 60% (v/v) MeOH was printed to pre-wet the membrane, and then 50 nL of trypsin at 40 $\mu\text{g}/\text{mL}$ in 10 mM NH_4HCO_3 containing 10% (v/v) 2-propanol was microdispensed to each target position. On-membrane digestion was performed for 16 h at 30°C in a humidified chamber. After digestion, 100 nL of 5 mg/mL α -cyano-4-hydroxycinnamic acid in 0.1% (v/v) trifluoroacetic acid (TFA) containing 50% (v/v) acetonitrile was printed onto each position on the membrane. The blot was then subjected to on-membrane MS analysis using the AXIMA-CFR plus instrument. Positional information for the printed region was transferred to the mass spectrometer as an output file from the chemical inkjet printer, and MS analysis was performed for the digested region on the basis of this information. A database search from the obtained MS spectrum was conducted using the Sprot database with the aid of Mascot software (Matrix Science, MA), with fixed parameters including a tolerance of 0.3 Da for the MS analysis, and one missed cleavage site.

In-Gel Trypsin Digestion

Relatively dark protein spots on a CBB-stained 2DE gel corresponding to the affinity-stained spots were excised and washed in 50 mM NH_4HCO_3 containing 50% (v/v) acetonitrile for 10 min. The gel pieces were washed with 100 mM NH_4HCO_3 for 10 min and then washed twice in 50 mM NH_4HCO_3 /acetonitrile 50% (v/v) for 10 min. After removing supernatant, the gel pieces were dried *in vacuo*. An aliquot of 10 $\mu\text{g}/\text{mL}$ trypsin in 100 mM NH_4HCO_3 containing 0.1% (w/v) 1-O-n-octyl- β -D-glucopyranoside was added and the pieces were incubated on ice for 15 min. Next, 6 μL of 100 mM NH_4HCO_3 was added and

pieces were incubated at 37°C overnight. Digested peptides were then extracted with 0.1% (v/v) TFA/66% (v/v) acetonitrile. Half of the extract was used for purification of phosphopeptides using Phos-tag agarose, and the other half was used for general peptide mass fingerprinting.

Enrichment of Phosphopeptides Using Phos-tag Agarose

Phos-tag agarose (30 μL of swelled gel) was added to a sample reservoir (a SUPREC-01 centrifugal filter unit). The storage buffer was removed by centrifugation (2000 g , 20 sec, 20°C) and then binding/washing buffer (0.1 M Tris- CH_3COOH , 1.0 M CH_3COONa , pH 7.5) was added to the sample reservoir. After centrifugation at 2000 g for 20 sec, the balancing buffer (0.1 M Tris- CH_3COOH , 1.0 M CH_3COONa , 10 μM $\text{Zn}(\text{CH}_3\text{COO})_2$, pH 7.5) was added to form the active zinc (II)-bound Phos-tag agarose. The solution was incubated for 20 min at room temperature and the filtrate was removed by centrifugation (2000 g , 20 sec, 20°C). This washing by centrifugation was repeated with binding/washing buffer (100 μm) three times. Subsequently, the digested peptides was solubilized in 50 μL binding/washing buffer (0.1 M Tris- CH_3COOH , 1.0 M CH_3COONa , pH 7.5) and placed in the sample reservoir. The filter unit was centrifuged at 2000 g for 20 sec after incubation for 20 min at room temperature. The filter unit was washed with binding/washing buffer three times and the filtrate was discarded. Elution buffer (1 N aqueous NH_3) was placed in the sample reservoir and incubated for 5 min at room temperature. Elutant was recovered by centrifugation (2000 g , 20 sec, 20°C). This elution step was repeated three times and the elutant was used for MS analysis after a desalting operation.

MALDI-TOF Analysis and Database Search

The elutant was dried *in vacuo* and then resolved in 0.1% (v/v) trifluoroacetic acid. A desalting operation was then performed with ZipTip according to the manufacturer's protocol. Aliquots of 0.5 μL were dispensed onto a stainless steel plate with a micropipette and then an equal volume of 5 mg/mL CHCA in 50% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid (TFA) was dispensed for PMF analysis and MS analysis for enriched phosphopeptides. After air drying, the MS operation was performed in positive mode with a MALDI-TOF MS instrument. PMF analysis was then carried out in reflectron mode, and MS operation in linear mode was used for MS analysis of enriched phosphopeptides. MS/MS analysis for phosphopeptides was performed with an AXIMA-QIT instrument with 5 mg/mL 2,5-DHB in 50% (v/v) acetonitrile/0.1% (v/v) TFA. A database search for identification of proteins from their MS spectrum was conducted as described

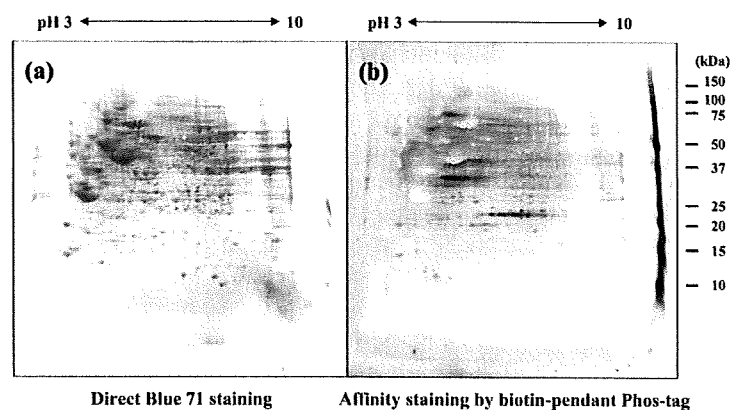


FIGURE 1

Specific detection of phosphoproteins by biotin-pendant Phos-tag and fluorescence-conjugated streptavidin. (a) The blot membrane for phospho-enriched whole-cell lysates of A-431+EGF separated with 2DE was stained with Direct Blue 71. (b) Phosphoproteins on the blot membrane of phospho-enriched whole-cell lysates of A-431+EGF separated with 2DE were detected with a biotin-pendant Phos-tag followed by fluorescence-conjugated streptavidin.

in the section on-membrane PMF analysis. MS/MS ion search of enriched phosphopeptides was performed using the Sprot database with the aid of Mascot software, with fixed parameters including a tolerance of 0.3 Da for the MS analysis, and one missed cleavage site allowed.

RESULTS AND DISCUSSION

Detection of Phosphoproteins on Blot Membranes by a Biotin-Pendant Phos-tag

Proteins extracted from phospho-enriched whole-cell lysates of A-431 stimulated with EGF were separated by 2DE and electroblotted onto a PVDF membrane. Detection of phosphoproteins using Phos-tag molecules was performed according to a modified rapid immunodetection procedure, as reported previously.^{16,21} This method permits Western blotting without a blocking procedure, providing the advantage that direct on-membrane MS analysis is unaffected by blocking proteins such as bovine serum albumin, casein, or skim milk. In this study we used a complex of the biotin-pendant Phos-tag and fluorescence-conjugated streptavidin for visualization of phosphoproteins. The method might facilitate the removal of the complexes from the membrane just by incubation in 1 N aqueous NH_3 without using a special stripping buffer containing detergents or reducing agents, as described previously.¹⁶ This improvement would induce the decrease of the loss of the target proteins on the membrane, conferring a great benefit to subsequent MS analysis. Phosphoproteins on the blot membrane were specifically detected using the complexes. Figure 1b shows a fluorescent image of detected phosphoproteins. After detection of phosphoproteins, Phos-tag molecules bound to phosphoproteins were removed by washing three times with 1 N aqueous NH_3 . The blot membrane was stained with Direct Blue 71 (Figure 1a). It has been previously reported that the limit for detection of pro-

teins with Direct Blue 71 staining is approximately 10 ng of protein.²⁰ Most of the proteins detected by affinity staining with the biotin-pendant Phos-tag did not overlap spots on the Direct Blue 71-stained image, and most protein spots with intense signals in Figure 1b were estimated to be present at less than 10 ng. Comparing a Direct Blue 71-stained image and an affinity-stained image in Figure 1, we see that intense signals by the biotin-pendant Phos-tag were particularly observed in the molecular-weight range of approximately 24–30 kDa, as described previously (region 1 and region 2 in Figure 2).²² These proteins were not also observed in the Direct Blue 71-stained image of Figure 1a and are suggested to be present at a very small amount. This result indicates that Phos-tag molecules are highly sensitive in detecting phosphoproteins on membrane.

On-Membrane Direct PMF Analysis for Phosphoproteins Using Chemical Inkjet Technology

We carried out microscale on-membrane PMF analysis for six proteins that were detected upon Direct Blue 71 staining and that also gave intense signals from detection with Phos-tag molecules (Figure 2). As described previously, trypsin and matrix solution were microdispensed onto a tiny region of the target protein spots using chemical inkjet technology, and the resulting digested peptides were directly analyzed on the membrane with a MALDI-TOF MS instrument.²¹ This piezoelectric inkjet technology enables to microdispense reagents at sub-nanoliter volume levels onto microscale region of proximate protein spots without cross-contamination. Furthermore, this microscale on-membrane PMF analysis allows detection and identification of phosphoproteins on a single blot membrane. It is a rapid, easy-to-use method that does not require a desalting procedure (e.g., by using a ZipTip). Therefore, this technology is very attractive for rapid on-membrane identification

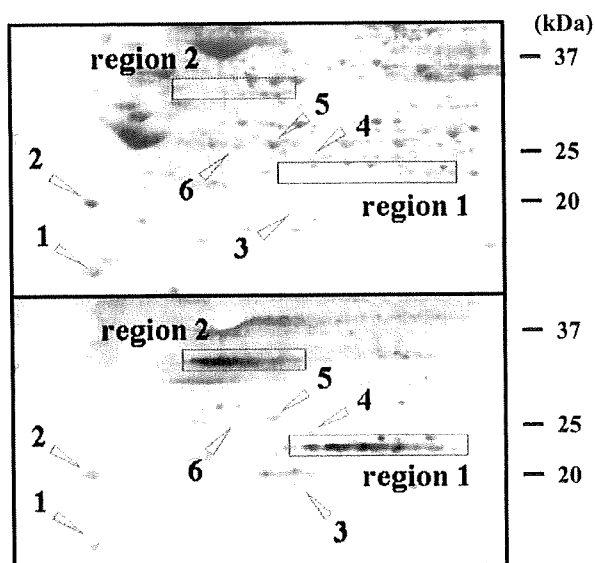


FIGURE 2

Protein spots used for on-membrane PMF analysis for phosphoproteins from phospho-enriched whole-cell lysates of A-431+EGF separated by 2DE. The blot membrane was stained with Direct Blue 71 after removing bound Phos-tag (*upper image*). The blot membrane was affinity stained with a biotin-pendant Phos-tag followed by fluorescence-conjugated streptavidin (*lower image*).

of proteins detected by affinity staining such as antibody, biotin-pendant affinity tag. The digested proteins were identified by a database search on the basis of the obtained MS spectrum. Table 1 shows the results of a database search (protein of spot 1 was also identified by PMF analysis of in-gel digests). All identified proteins were previously reported to be phosphorylated. By combining the Phos-tag molecules detection with on-membrane direct protein identification using inkjet technology, this rapid on-membrane profiling approach can be highly effective for phosphoprotein analysis.

Enrichment by Phos-Tag Agarose of Phosphopeptides from In-Gel Digested Ovalbumin

An MS signal derived from phosphopeptides could not be observed upon on-membrane PMF analysis. It appeared that a negative charge on a phosphate group suppressed MS signals of phosphopeptides in a number of non-phosphopeptides. Therefore, we tried to enrich phosphopeptides from in-gel digested peptides using Phos-tag agarose to characterize phosphorylation sites of identified proteins. We first demonstrated phosphopeptide isolation for ovalbumin to confirm the detection limit in MALDI-MS analysis after enrichment of phosphopeptides using Phos-tag agarose. Ovalbumin (5, 2, 1, 0.5, 0.2 pmol) was separated by SDS-PAGE (10–20%) and gel pieces corresponding to each protein band were excised. In-gel digestion of the gel pieces was performed, and phosphopeptides were enriched from the resulting tryptic digested peptides by Phos-tag agarose to give MS analysis in a linear mode. Figure 3a shows the result of MS analysis for in-gel digested ovalbumin without enrichment by Phos-tag agarose. A number of non-phosphopeptide signals and a single signal from phosphopeptide (340–359, $[M+H]^+ = 2088.91$) were observed in typical MS spectra of in-gel digested ovalbumin. An MS spectrum of phosphopeptides enriched from tryptic digests of ovalbumin is shown in Figure 3b. Four signals from phosphopeptides are seen, except at the lowest concentration, 0.2 pmol ovalbumin. Furthermore, a number of non-phosphopeptides observed in Figure 3a were hardly detected after enrichment. This result highlights the advantages of detecting phosphopeptides after enrichment by Phos-tag agarose. The observed MS signals correspond to the regions 340–359 (m/z 2088.91), 62–84 (m/z 2525.15), and 59–84 (m/z 2915.34) including a single phosphate group (80 Da), and MS signals after β -elimination (approximately –98 Da) were also confirmed in a spectrum taken in reflection mode (data not shown). Two phosphopeptides at m/z 2525.15 and m/z 2915.34 were likely modified with

TABLE 1

Phosphoproteins Identified by Microscale On-Membrane PMF Analysis Using a Chemical Inkjet Printer and MALDI-TOF MS.

	Accession No.	Protein Name	M.W.	Mascot Score
Spot 1	P05387	60S acidic ribosomal protein P2	11658	34
Spot 2	Q15185	Prostaglandin E synthase 3	18971	86
Spot 3	P33316	Deoxyuridine 5'-triphosphate nucleotidohydrolase	26975	58
Spot 4	P04792	Heat-shock protein beta-1	22826	62
Spot 5	P04792	Heat-shock protein beta-1	22826	114
Spot 6	P04792	Heat-shock protein beta-1	22826	83

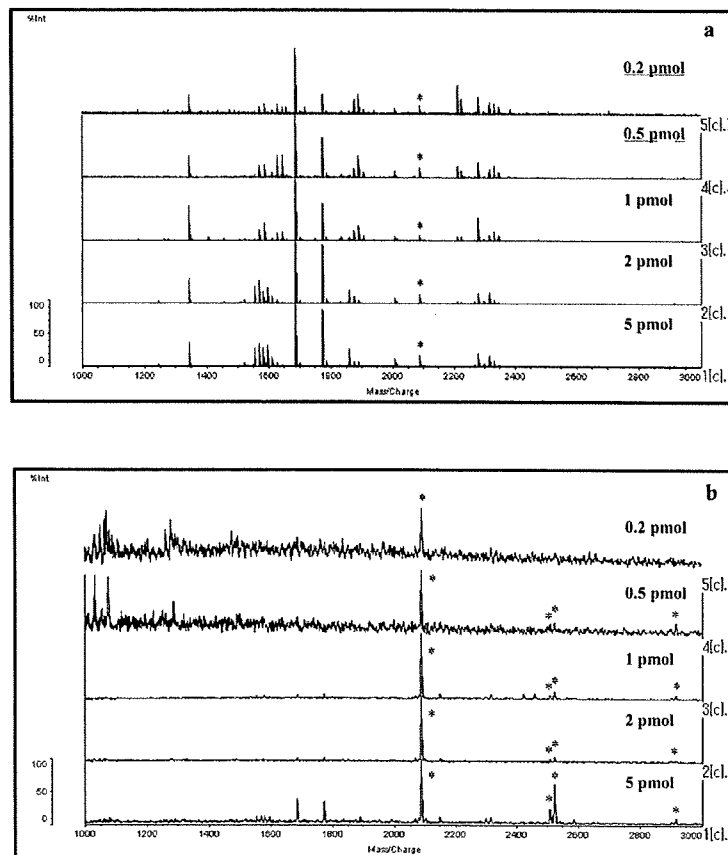


FIGURE 3

MALDI-MS spectra of in-gel digested peptides of ovalbumin (5, 2, 1, 0.5, 0.2 pmol) separated by SDS-PAGE (10–20%). (a) without enrichment of phosphopeptides using Phos-Tag Agarose (b) with enrichment of phosphopeptides using Phos-Tag Agarose. The phosphorylated peptides detected here are marked with asterisks (*).

acrylamide on a cysteine residue. A signal corresponding to phosphopeptides alkylated with iodoacetamide was also observed at m/z 2511.14 (the region 62–84). In this study we successfully detected phosphopeptides processed by in-gel digestion of ovalbumin at concentrations above 0.2 pmol. Our results show that MS signals from non-phosphorylated ovalbumin peptides were suppressed after enrichment, whereas Phos-tag agarose enriched phosphorylated peptides with high specificity.

Characterization of Phosphorylation Sites in Phosphoproteins Identified by On-Membrane Direct PMF Analysis

Subsequently, we tried to characterize phosphorylation sites on phosphoproteins identified by on-membrane microscale PMF analysis. Intense phosphopeptide signals were obtained upon MS analysis of prostaglandin E synthase 3 when enrichment of phosphopeptides using in-gel digests was carried out for six proteins. Figure 4 shows MS signals of phosphopeptides specifically selected by Phos-tag agarose. The MS signals observed in reflectron mode consisted of the phosphopeptide at m/z

1955.59 (DWEDDSDEDMSNFDR), its oxidized form (+16 Da), and the peptides that estimated to be cleaved with β -elimination. MS spectra of the other proteins after enriching for phosphopeptides did not detect any phosphopeptides (data not shown). There were faint spots for some of the other proteins on a Direct Blue 71-stained image, indicating they were not abundant to detect in MS mode due to loss of the samples during the enrichment procedure. In relation with heat-shock protein beta-1, which is relatively abundant (especially spot 5), some non-phosphorylated peptides observed on PMF analysis were retained after the enrichment procedure, and the phosphopeptides could not be completely enriched (data not shown). It seemed that nonspecifically bound nonphosphorylated peptides could not be removed, and that they inhibited ionization of phosphopeptides on MS analysis after enrichment. However, future refinements of this method should lead to improvements in characterization of phosphorylation sites. Heat-shock protein beta-1 is also known to be phosphorylated on multiple Ser/Thr residues, and identification of highly phosphorylated peptides is more difficult because of ion suppression with multiple

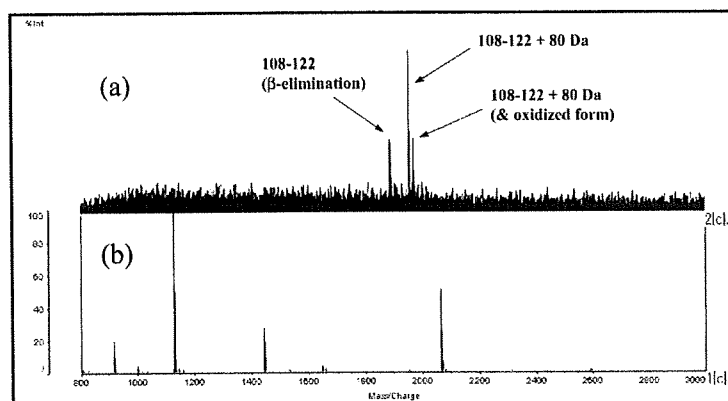


FIGURE 4

MS spectrum of prostaglandin E synthase 3 with/without enrichment of phosphopeptides. (a) MS analysis after enrichment of phosphopeptides using in-gel digests. (b) MS analysis of in-gel digests without enrichment of phosphopeptides.

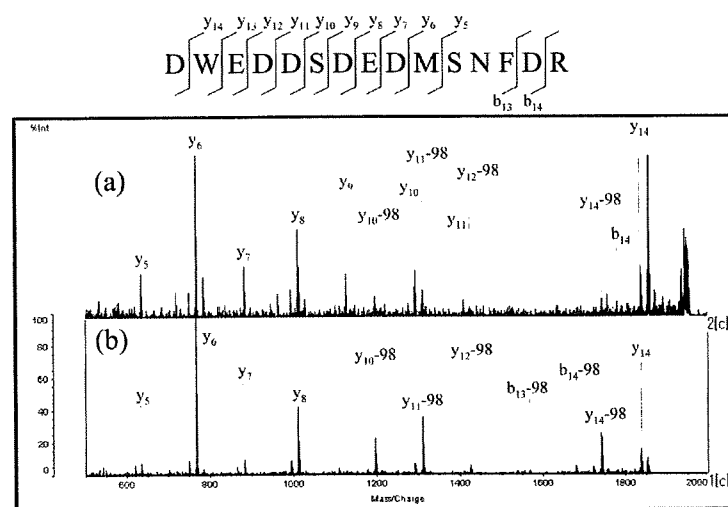


FIGURE 5

MS/MS spectrum of enriched phosphopeptide of prostaglandin E synthase 3. (a) MS/MS analysis of enriched phosphopeptide ($[M+H^+]=1955.59$). (b) MS/MS analysis of β -eliminated phosphopeptide ($[M+H^+]=1857.60$).

phosphates in comparison with singly phosphorylated peptides.²³

We performed MS/MS analysis for isolated phosphopeptides to characterize phosphorylation sites in prostaglandin E synthase 3 (Figure 5a). Although the enriched phosphopeptide was confirmed to be the region 108–122, corresponding the sequence DWEDDSDEDM SNFDR by MS/MS ion search, the phosphorylation sites could not be identified from the results shown in Figure 5a. Because this peptide has Ser residues at positions 113 and 118, no fragment ions containing a phosphate group (+80 Da) could be observed in the MS/MS spectra. Therefore, we could not determine which serine residue has a phosphate group by MS/MS ion search of the phosphopeptide. Subsequently, we tried to characterize the phosphorylation site by comparing the MS/MS spectrum for the phosphopeptide ($[M+H^+]=1955.59$) described above with that of the β -eliminated phosphopeptide ($[M+H^+]=1857.60$) (Figure 5b). Some fragment ions with β -elimination (–98 Da) were seen in a series

of y ions (y_{10} , y_{11} , y_{12} , y_{13} , and y_{14}) that were observed in both MS/MS spectra. If Ser 118 is phosphorylated, y_5 , y_6 , y_7 , y_8 and y_9 ions should be signals of fragment ions with β -elimination (–98 Da) in Figure 5 (b). However, the observed fragment ions with β -elimination (–98 Da) were y_{10} , y_{11} , y_{12} , y_{13} , and y_{14} ions, and the phosphorylation site was determined to be at Ser 113 by comparing information about the y_{10} to y_{14} ions. These results indicate that it is highly efficient to compare MS/MS for two peptides with/without β -elimination to determine the actual phosphorylation sites of peptides containing multiple predicted phosphorylation sites (Ser/Thr). Phosphorylation sites of prostaglandin E synthase 3 have been previously predicted to be at Ser 113 and at Ser 118 on the basis of mutation analysis.¹⁷ Our results confirm a previous report showing phosphorylation of Ser 113, even though no fragment ions derived from phosphorylation at Ser 118 were detected in MS/MS mode.

In conclusion, we report an effective rapid profiling approach consisting of detection with the biotin-pendant

Phos-tag and microscale on-membrane protein identification, which utilizes a combination of inkjet printing technology and MALDI-TOF MS. The characterization of phosphorylation sites by enrichment of phosphopeptides using Phos-tag agarose followed by MS/MS analysis could become a powerful approach in the field of phosphoproteomics.

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RESEARCH ARTICLE

Expression of proteins containing disulfide bonds in an insect cell-free system and confirmation of their arrangements by MALDI-TOF MS

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Escherichia coli alkaline phosphatase (AP) and human lysozyme (h-LYZ), which contain two and four disulfide bonds, respectively, were expressed in a cell-free protein synthesis system constructed from *Spodoptera frugiperda* 21 (Sf21) cells. AP was expressed in a soluble and active form using the insect cell-free system under non-reducing conditions, and h-LYZ was expressed in a soluble and active form under non-reducing conditions after addition of reduced glutathione (GSH), oxidized glutathione (GSSG), and protein disulfide isomerase (PDI). The *in vitro* synthesized proteins were purified by means of a *Strep*-tag attached to their C termini. Approximately 41 µg AP and 30 µg h-LYZ were obtained from 1 mL each of the reaction mixture. The efficiency of protein synthesis approached that measured under reducing conditions. Analysis of the disulfide bond arrangements by MALDI-TOF MS showed that disulfide linkages identical to those observed in the wild-type proteins were formed.

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Cell-free protein synthesis system / Disulfide bond / Insect cell / MALDI-TOF MS

1 Introduction

We have established a cell-free protein synthesis system (Transdirect insect cell) derived from *Spodoptera frugiperda* 21 (Sf21) insect cells [1]. The productivity of the cell-free system is approximately 10–15-fold higher than that of the rabbit

reticulocyte cell-free system. It is therefore expected to be sufficient to perform gene expression analyses including not only the measurement of enzymatic activity and western blotting, but also investigation of PTMs by MS [2, 3]. The insect cell-free system thus may find application as a useful tool for post-genomic studies, as an effective protein production method for targeted proteins.

One significant PTM is the formation of disulfide bonds. This plays a very important role in both the biological activity and stabilization of native protein structures. However, it has been reported that formation of disulfide bonds in cell-free synthesis systems can differ from the arrangement found in the corresponding native proteins, because reducing agents such as DTT, which are required to preserve the protein synthesis activity of the extract during extraction, storage, and translational reactions in conventional cell-free systems,

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Abbreviations: AP, *Escherichia coli* alkaline phosphatase; GSH, reduced glutathione; GSSG, oxidized glutathione; h-LYZ, human lysozyme; PDI, protein disulfide isomerase; Sf21, *Spodoptera frugiperda* 21

are usually added to the buffer to prepare the cell extract [4–8]. Recent reports of syntheses of several proteins containing disulfide bonds describe *Escherichia coli* and wheat germ cell-free systems [9–14] that were constructed for expression of these proteins in soluble and active form. This was accomplished by the addition of reduced glutathione (GSH), oxidized glutathione (GSSG), protein disulfide isomerase (PDI), and molecular chaperones such as GroEL, GroES. However, even in these cases, the exact confirmation of the disulfide bond arrangements has not been carried out.

Here, we describe a simple method to prepare an insect cell extract, the conditions required for optimal translation of proteins containing disulfide bonds, and analyses of protein disulfide bonds by MALDI-TOF MS. We describe the production and analysis of two model proteins, *E. coli* alkaline phosphatase (AP) and human lysozyme (h-LYZ), which contain two disulfide bonds (AP; Cys¹⁷⁰-Cys¹⁸⁰, Cys²⁸⁸-Cys³³⁸ in the wild-type form) [15, 16] and four disulfide bonds (h-LYZ; Cys⁷-Cys¹²⁹, Cys³¹-Cys¹¹⁷, Cys⁶⁶-Cys⁸², Cys⁷⁸-Cys⁹⁶ in their wild-type forms) [17, 18], respectively. Using the system developed here, both proteins were obtained in soluble and active form, and were shown to be completely identical to the wild-type proteins by analyses of their disulfide bond arrangements using MALDI-TOF MS. This is the first detailed confirmation of disulfide bond arrangements for proteins synthesized in a cell-free system.

2 Materials and methods

2.1 Materials

Restriction endonucleases and DNA modifying enzymes were purchased from Toyobo (Osaka, Japan) and New England Biolabs, Inc. (Ipswich, MA, USA). PDI from bovine liver was obtained from Takara Bio (Shiga, Japan). Desthiobiotin, TFA and CHCA were from Sigma (St. Louis, MO, USA). *Strep*-Tactin superflow was from Qiagen (Düsseldorf, Germany). Plasmid pTD1 (the nucleotide sequence of this vector has been submitted to the GenBank databases under accession no. AB194742) [19], containing a 5'-untranslated region of the polyhedrin gene as a translational enhancer sequence, was obtained from Shimadzu (Kyoto, Japan).

2.2 Construction of plasmid

For AP synthesis, the expression plasmid (pTD1-strep-AP) was constructed as follows. A vector named pTD1-strep, which includes an eight-glycine spacer and a *Strep*-tag sequence at the C terminus of the target protein, was constructed according to the following procedure. The oligonucleotides G8-strep-F (5'-GGGAATTCGGTACCGGATCCGGTGGAGGTGGAGGTGGAGGTGGATGGAGCCATCCGCAGTTTAAAAGTAATCTAGAGC-3') and its complementary sequence were annealed. After digestion with *EcoRI* and *XbaI*, the DNA fragment was subcloned into the *EcoRI*-*XbaI* sites

of the pTD1 vector. Next, the AP gene was amplified by PCR using the AP-N primer (5'-ATGCGGACACCAGAAATGCC-3') as the sense primer, the AP-C primer (5'-GGGGTACCTTTCAGCCCCAGAGCGG-3') as the antisense primer, and genomic DNA prepared from *E. coli* BL21 (DE3) pLysS as the template. The amplified DNA fragment was then treated with T4 polynucleotide kinase. After digestion with *KpnI*, the amplified fragment was subcloned into the *EcoRV*-*KpnI* sites of a pTD1-strep vector, and the resulting vector, pTD1-strep-AP, containing AP having a *Strep*-tag at its C terminus, was constructed.

For h-LYZ synthesis, the expression plasmid (pTD1-strep-h-LYZ) was constructed as follows. The h-LYZ gene was amplified by PCR using the LYZ-N primer (5'-ATGAAGGTTTTTCGAGAGATGCG-3') as the sense primer, the LYZ-C primer (5'-GGGGTACCAACACCACAACCTTGAACG-3') as the antisense primer, and pER18602 [17] as the template. The amplified DNA fragment was then treated with T4 polynucleotide kinase. After digestion with *KpnI*, the amplified fragment was subcloned into the *EcoRV*-*KpnI* sites of a pTD1-strep vector, and the resulting vector, pTD1-strep-h-LYZ, containing h-LYZ having a *Strep*-tag at its C terminus, was constructed. The DNA sequences of these recombinants were confirmed by the dideoxynucleotide chain termination method.

2.3 Preparation of mRNAs for cell-free protein synthesis

The expression plasmids prepared as described above were linearized by *HindIII* digestion, then purified by ethanol precipitation following gel filtration. The mRNAs were synthesized as described previously [1].

2.4 Expression and purification of AP and h-LYZ

The Sf21 extract and cell-free protein synthesis system was prepared as reported previously [1] except for the use of DTT and other reducing agents. For cell-free protein synthesis of AP (1 mL), the reaction mixture consisted of 40 mM HEPES-KOH (pH 7.9), 100 mM KOAc, 1.5 mM Mg(OAc)₂, 0.25 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, 200 µg/mL creatine kinase, 80 µM of each of the 20 amino acids, 0.1 mM EGTA, 0.25 mM PMSF, 200 µg/mL tRNA, 320 µg/mL mRNA, and 50% Sf21 extract. For h-LYZ synthesis (10 mL), GSH, GSSG and PDI were also added to the reaction mixture at a concentration of 0.5 mM, 0.5 mM and 2 µM, respectively. The reactions were carried out at 25°C for 5 h.

Next, the reaction mixture for AP (1 mL) was centrifuged at 15 000 rpm for 15 min (4°C), and the supernatant was applied to the *Strep*-Tactin superflow column (0.5 mL) equilibrated with 50 mM Tris-HCl, 300 mM NaCl, pH 8.0 (buffer A). The column was washed with the same buffer (2.5 mL), and then the protein was eluted with buffer A containing 2 mM desthiobiotin (1.5 mL). The eluate was concentrated to 50 µL by ultrafiltration (molecular weight cutoff = 10 kDa).

In the case of h-LYZ, its preparation was carried out at ten times scale, and the eluate was concentrated to 500 μ L by ultrafiltration (molecular weight cutoff = 3 kDa). The proteins thus obtained were stored at -20°C until use.

2.5 Measurement of enzymatic activities

AP activity was determined by three independent experiments using an alkaline phosphatase assay kit (Wako, Osaka, Japan). One unit of phosphatase activity was defined as the amount of enzyme that released 1 nmol *p*-nitrophenol/min at 37°C in Tris-HCl buffer (100 mM, pH 9.8).

Lysozyme activity was assayed by turbidity decrements at 450 nm of 240 $\mu\text{g}/\text{mL}$ *Micrococcus luteus* (Wako) in Tris-HCl buffer (50 mM pH 8.0), and the activity was determined by three independent experiments. One unit was defined as the amount of enzyme that decreased 0.001 OD units/min at 37°C in Tris-HCl buffer (50 mM, pH 8.0).

2.6 Protein assay

The protein concentration was quantified by three independent experiments using the QuantiProTM BCA Assay Kit (Sigma). BSA was used as the standard protein.

2.7 Confirmation of disulfide bonds in AP by MALDI-TOF MS

The purified AP (1 μg) was electrophoresed on a 12.5% polyacrylamide gel containing SDS under non-reducing conditions and then stained with CBB. The protein bands on the gel were treated under three following conditions: (a) reduced by DTT and *S*-alkylated by iodoacetamide, (b) *S*-alkylated by iodoacetamide, (c) no treatment; subsequently, they were digested with trypsin (100 ng, Promega, Madison, WI, USA) overnight. The tryptic digests were extracted from gels using 0.1% TFA followed sequentially by 50% ACN containing 0.1% TFA. These extracts were mixed and concentrated to approximately 10 μL . Sample (2 μL) was mixed with 0.8 μL of CHCA solution (5 mg/mL in 50% ACN containing 0.1% TFA) on the MALDI target plate and analyzed after drying. The mass spectra of the resulting tryptic digests were acquired in both reflectron positive ion mode and linear positive ion mode with an AXIMA-CFRTM-plus MALDI-TOF MS instrument (Shimadzu/Kratos, Manchester, UK) according to the standard method [20].

2.8 Confirmation of disulfide bonds in h-LYZ by MALDI-TOF MS

The purified h-LYZ (10 μg) was denatured in 10 μL 8 M urea solution and then treated under three conditions as described in Section 2.7. Each reaction mixture was diluted by adding 50 mM ammonium bicarbonate into 100 μL and digested with trypsin (350 ng) overnight. The tryptic digests were desalted and concentrated to approximately 8 μL by

ZipTip μ -C18 (Millipore, Billerica, MA, USA). The mass spectra for a 0.5- μL portion of the tryptic digests were acquired in a similar manner to that described above by reflectron positive ion mode.

Next, to confirm the disulfide bond assignments that were not identified using the procedures as described above, the purified h-LYZ (100 μg) was denatured in 100 μL 8 M urea, then diluted by adding 50 mM ammonium bicarbonate into 1 mL and digested with trypsin (3.5 μg) overnight. The tryptic digests were separated by reverse-phase HPLC using an ODS column (4.6 \times 150 mm, Shimadzu) from which they were eluted with a linear gradient against ACN containing 0.1% TFA from 5 to 50% for 50 min at a flow rate of 1 mL/min. Each eluted fraction (2 μL) was mixed with 1 μL CHCA solution, and the peptides included in the fractions were identified by MALDI-TOF MS. Peptides linked by disulfide bonds among Cys⁶⁶, Cys⁷⁸, Cys⁸², and Cys⁹⁶ were further digested with thermolysin (200 ng, Calbiochem, Darmstadt, Germany) in 100 μL Tris-HCl (100 mM, pH 8.0) at 37°C for 1 h, and the digests were desalted, concentrated and their mass spectra were acquired.

3 Results

3.1 Expression, purification and characterization of AP and h-LYZ

The cell-free system normally contains a reducing agent such as DTT to keep the reducing conditions similar to those inside a living cell. However, the above conditions seem to be inappropriate for synthesis of functional AP and h-LYZ. In fact, although bands corresponding to both authentic AP and h-LYZ were observed on SDS-PAGE at similar level by a fluorescence labeling method [1] for proteins expressed under such conditions, the activities of these proteins were not detected at all (data not shown). This means that the correct formation of their disulfide bonds is essential for both activity and stability. Therefore, translation products of mRNAs prepared from plasmid pTD1-strep-AP and pTD1-strep-h-LYZ were examined in an insect cell-free system with or without a reducing agent as described in Materials and methods.

Phosphatase activity was detected strongly in the case of AP synthesized under non-reducing conditions, whereas it was not active under reducing conditions (Fig. 1). On the other hand, activity of h-LYZ was not detected under either condition, but it was detected strongly after the addition of GSH, GSSG, and PDI to the reaction mixture (Fig. 2).

Using the respective conditions where activities of AP and h-LYZ were expressed at the highest levels, both proteins were synthesized, and then purified by affinity column chromatography, and their purities were judged on SDS-PAGE by staining with CBB (Fig. 3). The AP and h-LYZ ran as almost single bands having molecular masses of about 49 and 17 kDa, respectively. These observed molecular masses

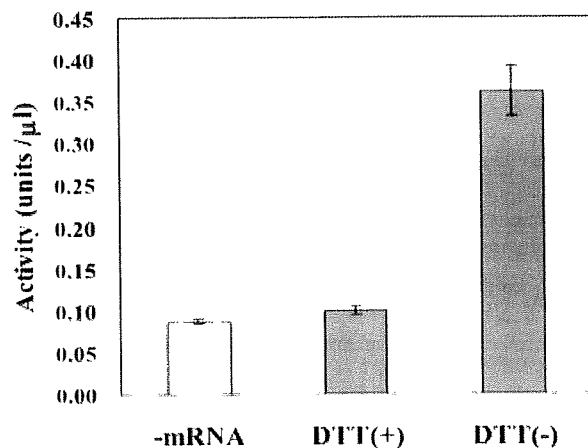


Figure 1. Measurement of phosphatase activities for AP synthesized in the insect cell-free system. The cell-free syntheses were carried out under reducing and non-reducing conditions at 25°C for 5 h. The symbols, -mRNA, DTT(+), and DTT(-) indicate the reactions were performed without mRNA (negative control), and under reducing and non-reducing conditions, respectively. The means and SDs of three replicate experiments are indicated.

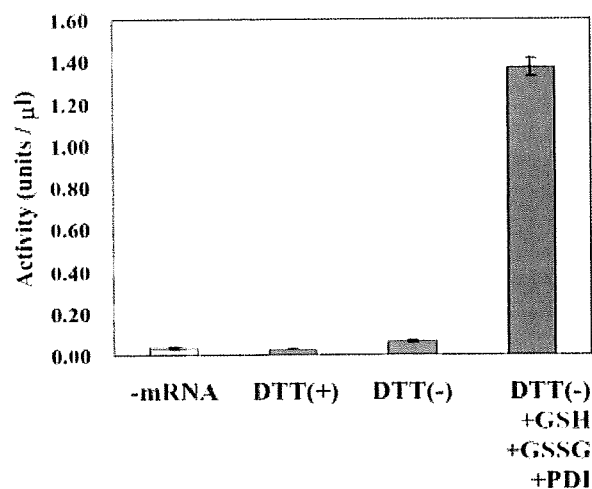


Figure 2. Measurement of lysozyme activities of h-LYZ synthesized in the insect cell-free system. The reactions were carried out under three conditions at 25°C for 5 h: (i) reducing, (ii) non-reducing, (iii) non-reducing in the presence of GSH, GSSG, and PDI. The symbols, -mRNA, DTT(+), DTT(-), and DTT(-) + GSH + GSSG + PDI indicate the reactions were performed without mRNA (negative control), and under reducing, non-reducing, and non-reducing in the presence of GSH, GSSG, and PDI, respectively. The means and SDs of three replicate experiments are indicated.

corresponded to the theoretical values calculated from the amino acid sequences of the proteins' mature forms. The final yields of AP and h-LYZ were approximately 41 μg from 1 mL reaction mixture and 300 μg from 10 mL reaction mixture, respectively.

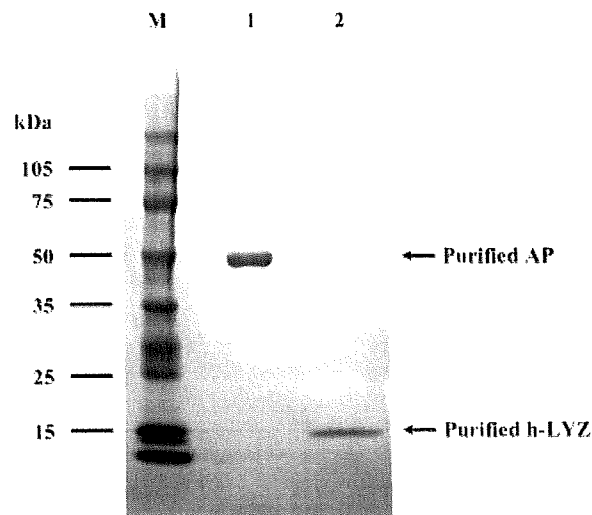


Figure 3. SDS-PAGE analysis of purified AP and h-LYZ synthesized in the insect cell-free system. The purified proteins (1 μg) were electrophoresed on 5–20% gradient SDS-PAGE. Lanes M, 1, and 2: marker, AP, and h-LYZ, respectively.

By analyzing the specific activities of APs synthesized both *in vitro* and *in vivo* (Table 1), it was shown that the specific activity of the AP synthesized *in vitro* was slightly less than that expressed *in vivo* in *E. coli* K12 SW 1033/pk 1-5 (Toyobo). This slight difference in the specific activities might be caused by the attachment of the affinity tag ($^{452}\text{GTGSGGGGGGGWSHPQFEK}^{471}$) to the AP produced *in vitro*. On the other hand, the specific activity of the h-LYZ synthesized *in vitro* was similar to that of the wild type h-LYZ (Sigma) prepared from human milk (Table 1).

3.2 Confirmation of disulfide bond arrangements in proteins synthesized using the insect cell-free system

To confirm the disulfide bond arrangements of proteins synthesized in the insect cell-free system developed here, the disulfide pairings of two proteins, AP and h-LYZ, were determined by MALDI-TOF MS. Figure 4 shows the location of disulfide bonds for the two wild-type proteins along with their complete amino acid sequences, including the affinity tag used here. Each protein was treated under the three conditions (reduced and S-alkylated, S-alkylated, or no treat-

Table 1. Specific activities of AP and h-LYZ synthesized *in vitro* or *in vivo*

Protein	Specific activity (U/ μg)	
	<i>In vitro</i>	<i>In vivo</i>
Alkaline phosphatase	20.05 \pm 0.40	22.37 \pm 0.45
Human lysozyme	26.33 \pm 0.71	25.67 \pm 0.77

A

¹MRTPEMPVLE NRAAQGDITA PGGARRLTGD QTAALRDSLS DKPAKNIILL¹¹⁰
⁵¹IGDGMGDSEI TAARNYAEGA GGFFKGIDAL PLTGQYTHYA LNKKTKGPDY¹⁰⁰
¹⁶¹VTDSAASATA WSTGVKTYNG ALGVDIHEKD HPTILEMAKA AGLATGNVST¹⁵⁰
¹⁵¹AELQDATPAA LVAHVTSRKC YGPSATSEKC PGNALKEGGR GSITFQLLNA²⁰⁰
²⁰¹RADVTLGGGA KTF AETATAG EWQGKTLRFQ AQARGYQLVS DAASLNAVTE²⁵⁰
²⁵¹ANQKPLLGL FADGNMPVRW QGPKATYHGN IDKPAVTCTP NPQRNDSVPT³⁰⁰
³⁰¹LAQMTDKAIE LLSKNEKGFF LQVEGASIDK QDHAANPCGQ IGETVDLDEA³⁵⁰
³⁵¹VQRALFEFAK DGNTLVIVTA DHAHASQIVA PDTKAPGLTQ ALNTKDGAVM⁴⁰⁰
⁴⁰¹VMSYGNSEED SQEHTGSQLR LAAYGPHAAAN VVGLTDQTF FYTMKAALGL⁴⁵⁰
⁴⁵¹KGTGSGGGGG GGGWSHPQFE K
 Spacer sequence Strep-tag sequence

B

¹MKVFERCELA RTLKRLGMDG YRGISLANWM CLAKWESGYN TRATNYNAGD⁵⁰
⁵¹RSTDYGHQI NSRYWCNDGK TPGAVNACHL SCSALLQDNI ADAVACAKRV¹⁰⁰
¹⁰¹VRDPQGRRAW VAWRNRCQNR DVRQYVQCGG VGTGSGGGGG GGGWSHPQFE¹⁵⁰
¹⁵¹K
 Spacer sequence Strep-tag sequence

Figure 4. Amino acid sequences and the locations of disulfide bonds in both wild-type AP and h-LYZ. Disulfide linkages of wild-type AP (A) and h-LYZ (B) are indicated by lines. Spacer and Strep-tag sequences are underlined.

ment, see Section 2.7) followed by overnight trypsin digestion. Tryptic peptides derived from each protein were analyzed by MALDI-TOF MS. The PMF for each protein sample treated under these three conditions was almost identical, with the exception of regions corresponding to cysteine-containing peptides, regardless of conditions for sample preparation, and the values obtained were in good agreement with the theoretical values calculated assuming the presence of wild-type disulfide bonds (Tables 2 and 3).

As shown in Fig. 5, in the case of the reduced and S-alkylated AP treatments, three peaks, which apparently corresponded to tryptic fragments containing carbamidomethyl-cysteine(s) of this modified protein, were specifically observed at m/z values of 2097.05 (Fig. 5A), 2240.16 (Fig. 5B) and 2523.15 (Fig. 5C). These values were in good agreement with the theoretical values (m/z 2096.97, 2240.09, 2523.15) calculated from the amino acid sequence of the wild-type protein (Table 2).

On the other hand, peaks with the m/z values described above were not observed for the untreated AP or for the AP treated with S-alkylation without reduction. Instead, peaks were observed at m/z values of 1980.97 (Fig. 5A) and 4649.03 (Fig. 5D) for these proteins. Each value was in good agreement with the theoretical values (m/z 1980.93, 4649.06) calculated from the tryptic peptide fragments containing wild-type disulfide linkages (Table 4). However, peaks corresponding to incorrect disulfide linkages were not detected. Therefore, the AP synthesized in the insect cell-free system developed here formed wild-type disulfide linkages.

In the case of h-LYZ, two stages of protease digestion were required to confirm the disulfide bond arrangements,

because peptide fragment having two disulfide bonds was obtained by the first tryptic digestion (Fig. 4B). As shown in Table 3, the calculated mass values of the peptides containing carbamidomethyl-cysteine(s) obtained by tryptic digestion of h-LYZ were 577.25, 648.31, 942.38, 1363.69, 2708.19, and 2927.38. In the case of the reduced and S-alkylated h-LYZ, six peaks were clearly observed that were in good agreement with the theoretical values. The observed m/z values were 577.24, 648.19, 942.32, 1363.68, 2708.17, and 2927.29, and each was assigned to a peptide containing carbamidomethyl-cysteine(s).

On the other hand, these peaks were not detected for S-alkylated h-LYZ without reduction or for untreated h-LYZ. The calculated mass values of the peptides containing disulfide linkages, obtained by tryptic digestion of these h-LYZs, are 1823.89, 3239.46, and 3636.67. Three peaks were clearly observed that were in good agreement with the theoretical values. The m/z values of 1823.97 (Fig. 6A), 3239.22, and 3636.36 (Fig. 6B) were assigned to peptides containing disulfide linkages (Table 5). No peaks corresponding to incorrect disulfide linkages were detected. From these results, we concluded that the h-LYZ synthesized in the cell-free system formed four disulfide bonds, and two of these (Cys⁷-Cys¹²⁹ and Cys³¹-Cys¹¹⁷) were the same as those observed in the wild-type protein. However, the locations of the other two disulfide bonds between Cys⁶⁶, Cys⁷⁸, Cys⁸², and Cys⁹⁶ were still undetermined.

To determine the remaining disulfide linkages, the tryptic fragment observed at the m/z value of 3636.36 was isolated using reverse-phase HPLC. Of eight peaks observed in the reverse-phase HPLC elution profile, peak 8 was identified

Table 2. Theoretical and observed monoisotopic mass values for tryptic digests of AP

Mass value	Position	MC ^{a)}	Modification(s)	Modified mass value	Observed mass value ^{b)}			Peptide sequence
					(a)	(b)	(c)	
3687.88	235–269	0	MSO ^{c)} : 266	3703.87	3703.65			GYQLVSDAASLNAVTEANQQKPLLGLFADGNMPVR
2792.45	140–168	0			2792.39			AAGLATGNVSTAEIQDPAALVAHVTSR
2730.32	421–445	0	MSO: 444	2746.32	2730.31			IAAYGPHAAANVVLDTQDFFYTMK
2727.16	396–420	0	MSO: 400, 402	2759.15	2727.16			DGAVMVMVSYGNSEEDSQEHTGSQLR
2473.27	361–384	0			2473.36			DGNTLVIVTADHAHASQIVAPDTK
2466.13	331–353	0	Cys_CAM ^{d)} : 338	2523.15		2523.15	N. D. ^{e)}	QDHAANPCGGIGETVLDLDEAVQR
2213.07	95–116	0			2213.16			TGKPDYVTDASAATAWSTGVK
2183.07	275–294	0	Cys_CAM: 288	2240.09		2240.16	N. D.	ATYHGNDKPAVTCTPNPQR
1982.93	169–187	2	Cys_CAM: 170, 180	2096.97		2097.05	N. D.	KCYGPSATSEKCPGNALEK
1975.03	76–93	0			1975.05			GIDALPLTGQYTHYALNK
1959.02	46–64	0	MSO: 55	1975.02	1975.05			NIILLIGDGMGSEITAAR
1816.80	452–471	0			1816.92			GTGSGGGGGGGGWSHPQFEK
1496.70	212–225	0			1496.79			TFAETATAGEWQGK
1419.68	295–307	0	MSO: 304	1435.67	1435.71			NDSVPTLAQMTDK
1416.71	117–129	0			1416.79			TYNGALGVDIHEK
1410.73	318–330	0			1410.82			GFFLQVEGASIDK
1201.65	191–201	0			1201.71			GSITEQLLNAR
1185.59	3–12	0	MSO: 6	1201.59	1201.71			TPEMPVLENR
1184.60	13–25	0			1184.67			AAQGDITAPGGAR
1170.55	169–179	1	Cys_CAM: 170	1227.57	N. D.			KCYGPSATSEK
1160.54	65–75	0			1160.58			NYAEGAGGFFK
1154.59	130–139	0	MSO: 137	1170.58	1170.60			DHPTILEMAK
1113.63	385–395	0			1113.65			APGLTQALNTK
1045.56	27–36	0			1045.61			LTGDQTAALR
1042.45	170–179	0	Cys_CAM: 170	1099.47	N. D.			CYGPSATSEK
960.50	37–45	0			960.49			DSLSDKPAK
888.48	202–211	0			888.47			ADVTLGGGAK
831.40	180–187	0	Cys_CAM: 180	888.42	N. D.			CPGNALEK

The mass values more than 800 are shown.

a) MC, number of missed cleavages.

b) (a) (b) (c) stand for the sample conditions described in Section 2.7. In these samples, the peptides containing unmodified cysteine(s) were not observed.

c) MSO, methionine sulfoxide (artificial modifications). MSO was observed in several tryptic digests of AP, because these samples were concentrated using SpeedVac after extraction from gels.

d) Cys_CAM, carbamidomethyl-cysteine.

e) Not detected.

as the peptide of interest by MALDI-TOF MS (Fig. 7 and Table 6). Therefore, the peptide (Fig. 8A) was subjected to thermolytic digestion as described in Materials and methods. Numerous peaks were seen on the mass spectra of thermolytic digests, but most were judged to be derived from the enzyme preparation, because they were also observed in the mass spectrum of the control sample. The control sample was prepared by thermolytic digestion under the same conditions, but without the peak 8 peptide (data not shown). Two peaks were thus clearly observed at *m/z* values of 1362.58 (Fig. 8B) and 2112.33 (Fig. 8C), which were not observed in the control sample. These values were in good agreement with the theoretical values (*m/z* 1362.57, 2112.01) calculated for the peptide fragments containing native disulfide linkages. From these

results, it was demonstrated that the h-LYZ synthesized using the insect cell-free system developed here formed wild-type disulfide linkages.

4 Discussion

In this report, we have established an effective method for synthesis of proteins in soluble and active form using an insect cell-free system. The proteins *E. coli* AP and h-LYZ were produced as examples of the system's performance. We have demonstrated, using MALDI-TOF MS, that the disulfide linkages of these proteins were identical to those of the wild-type proteins. This is the first detailed analysis of disulfide bond formation in proteins produced using a cell-free system.

Table 3. Theoretical and observed monoisotopic mass values for tryptic digests of h-LYZ

Mass value	Position	Modification(s)	Modified mass value	Observed mass value ^{a)}		Peptide sequence	
				(a) (b) (c)	(a) (b) (c)		
2756.32	71–98	Cys_CAM ^{b)} : 78, 82, 96	2927.38		2927.29	N. D. ^{d)}	TPGAVNACHLSCSALLQDNI ADAVACAK
2651.17	124–151	Cys_CAM: 129	2708.19		2708.17	N. D.	QYVQCGVGTGSGGGGGGGG WSHPOFEK
1400.68	52–63			1400.68			STDYGIFQINSR
1306.66	23–34	Cys_CAM: 31	1363.69		1363.68	N. D.	GISLANWMCLAK
		MSO ^{c)} : 30	1322.66				
1012.45	35–42			1012.44			WESGYNTR
981.44	43–51			981.41			ATNYNAGDR
885.36	64–70	Cys_CAM: 66	942.38		942.32	N. D.	YWCNDGK
811.38	16–22	MSO: 18	827.37		811.32		LGMDGYR
788.42	109–114			788.36			AWVAWR
685.36	103–108			685.27			DPQGISR
591.29	7–11	Cys_CAM: 7	648.31		648.19	N. D.	CELAR
550.30	3–6			550.15			VFER
520.23	117–120	Cys_CAM: 117	577.25		577.24	N. D.	CQNR

The mass values more than 500 are shown.

a) (a) (b) (c) stand for the sample conditions described in Section 2.7. In these samples, the peptides containing unmodified cysteine(s) were not observed.

b) Cys_CAM, carbamidomethyl-cysteine.

c) MSO, methionine sulfoxide (artificial modifications).

d) Not detected.

AP was synthesized in a soluble and active form using an insect cell-free system without any reducing agents, because the folding of this particular protein allowed proper formation of disulfide bonds, which occurred spontaneously under these conditions [21]. Thus, the non-reducing environment was very important for correct disulfide bond formation in this case. The specific activity of the AP synthesized in the presence of GSH, GSSG, and PDI was almost the same as that of AP synthesized in the absence of these components (data not shown). Thus, for the synthesis of active AP, the addition of GSH, GSSG, and PDI seems to be unnecessary in this cell-free system. However, the situation was different in the case of synthesis of active h-LYZ. The optimum concentrations of GSH, GSSG, and PDI were determined for the synthesis of active h-LYZ, and they were found to be 0.5 mM, 0.5 mM, and 2 μ M, respectively. PDI catalyzes both the oxidation of disulfides and the isomerization of incorrect disulfides in newly synthesized polypeptides during folding in the oxidizing environment of the ER. Furthermore, PDI is a multifunctional protein involved in the folding, assembly, and PTM of many proteins, and its concentration in the lumen of the ER may approach millimolar levels [22]. In this study, the optimum concentration of PDI (2 μ M = 200 μ g/mL) was comparatively high, and therefore it is thought that the concentration of endogenous PDI in the insect cell extract is not sufficient to synthesize active h-LYZ; alternatively, the insect cell extract may lack some of the enzymes existing in the lumen of the ER, or their properties may differ somewhat.

On the other hand, neither the addition of the chaperone molecules such as GroEL and GroES, nor complicated preparation methods of cell extracts, as reported in other cell-free systems [9–14], were required for the insect cell-free system developed here. Even a single improvement, i.e., not adding DTT or any other reducing agents, will be effective in producing active proteins in some cases. For example, h-LYZ was expressed in a soluble and active form, and approximately 300 μ g-purified protein was obtained from 10 mL of the reaction mixture. The efficiency of protein synthesis approached that attained under reducing conditions [1–3]. This result means that the protein synthesis activity in the insect cell extract can be preserved without reducing agents. We therefore conclude that our method of preparing the insect cell extract by freeze-thawing [1] probably prevents the essential components for protein synthesis from inactivation by oxidation, which may occur for example when a Dounce homogenizer is used.

The results of this study show that the insect cell-free protein synthesis system can produce proteins containing disulfide bonds identical to those in the wild-type protein by adding the correct amounts of DTT, GSH, GSSG, and PDI. Therefore, the insect cell-free protein synthesis system can be an effective tool for synthesizing active proteins containing disulfide bonds.

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