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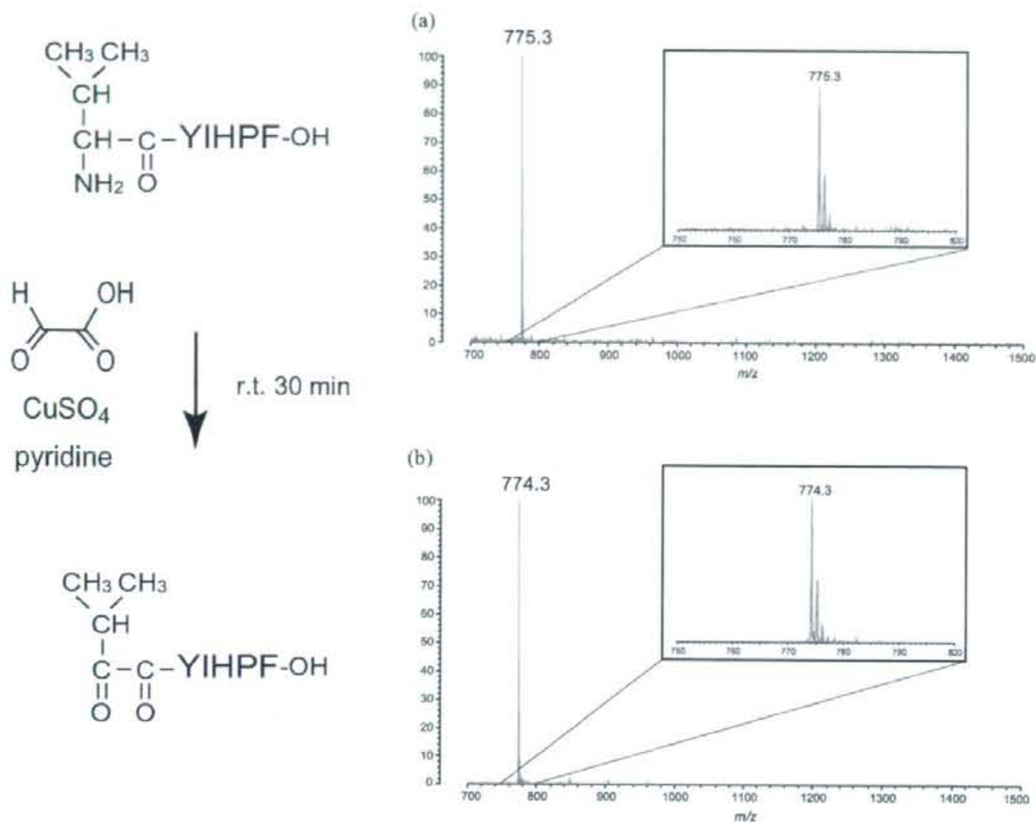

Figure 2. Transamination reaction of peptide VYIHPF (10 pmol, $[M+H]^+$: 775.4) and its MALDI-TOF mass spectra before (a) and after (b) the transamination reaction.

expected, only peptide **1** was recovered after treatment of the chemically modified mixture with DITC glass (Fig. 3(c)). Thus, the peptide having only an α-amino group, which was a model for the C-terminal peptide, was successfully isolated by the protocol. Hence, we next extended this protocol to proteins.

Application to proteins

Processing protein samples separated by 2D-PAGE, which is conventionally used for proteome analysis, would be a practical application for this protocol. Therefore, in this experiment, 1D-gel-separated proteins were used as starting samples.

Three model proteins (pig albumin, bovine cathepsin B, and bovine α-casein, all gel-separated, 30 pmol each) were chosen to test the protocol. These proteins were digested with LysC followed by transamination (Figs. 4(a), 4(c) and 4(e)) and treatment with DITC glass (Figs. 4(b), 4(d) and 4(f)). Figures 4(a) and 4(b) depict the results of pig albumin. The C-terminal peptide (F^tVIEIRGILA, $[M+H]^+$: 1130.7; the superscript 't' indicates transaminated residue) was detected after the transamination reaction (Fig. 4(a), arrow), but the signal of the peptide was weaker than other fragment signals. After treatment of the reaction mixture with DITC glass, the C-terminal peptide was selectively recovered (Fig. 4(b)). Figures 4(c) and 4(d) illustrate the results for bovine cathepsin B. The C-terminal peptide of bovine cathepsin B (I^tLRGQDHCGIESEIVAGMPCT, $[M+H]^+$: 2342.1) was isolated after treatment of the reaction mixture with DITC glass (Fig. 4(d)). Figures 4(e) and 4(f) depict the results for bovine α-casein which consists of two components, S1 and S2. The C-terminal peptides of bovine α-casein (T^tTMPLW, $[M+H]^+$:747.4 from S1, V^tIPYVRYL, $[M+H]^+$:1021.6 from S2) were isolated after treatment of the reaction mixture with DITC glass (Fig. 4(f)). In this case the C-terminal peptide from S2 was clearly detected using CHCA but not detected with DHBA as a matrix. In contrast, the isolated peptide from S1 was not detected using CHCA but with DHBA as a matrix (Fig. 4(f), inset).

Thus, it was demonstrated that the method can be applied to in-gel samples of proteins and that it can be complementarily used with the previously described method employing TMPP-Ac reagent.[7]

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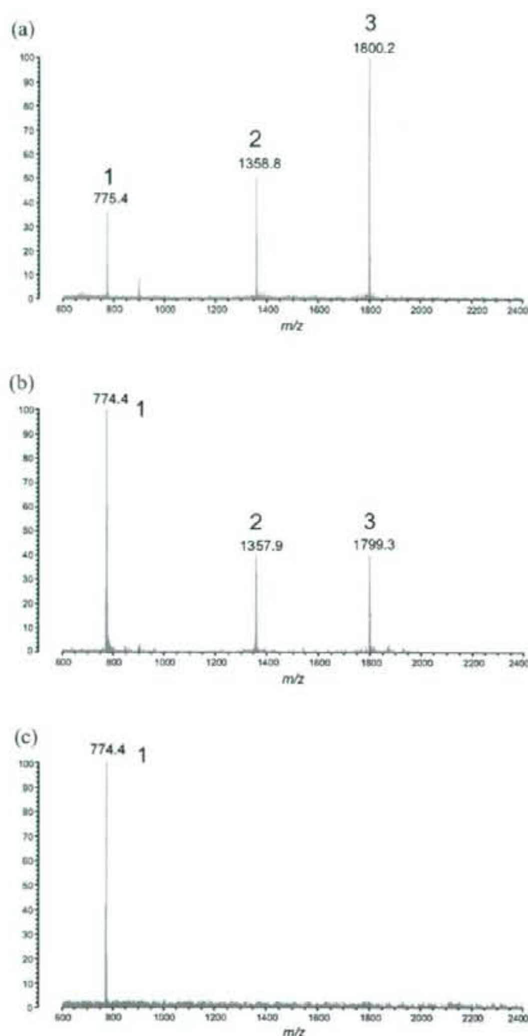

Figure 3. MALDI-TOF mass spectra of three-peptide mixture (a), after the transamination reaction (b) and after treatment with DITC glass (c). The three peptides are as follows: (1) VYIHPF ($[M+H]^+$: 775.4), (2) MHRQETVDCLK-NH_2 ($[M+H]^+$: 1358.7), and (3) AAKIQASFRGHMARKK ($[M+H]^+$: 1800.0). An aliquot (ca.10 pmol) of mixture was loaded on the target plate.

Modification of isolated C-terminal peptides

This method has the advantage that the isolated C-terminal peptide is open to introduction of virtually any type of functionality because it incorporates an α-ketocarbonyl group at its N-terminus that can accept nucleophilic attack.

As an example, we applied DNPH modification to the isolated C-terminal peptide of α_{S2}-casein (Fig. 5). The isolated C-terminal peptide was successfully modified with DNPH (Fig. 5(b)) and sequenced by MS (Fig. 5(c)).

DISCUSSION

It has been reported that some, but not all, N-terminal residues can be transaminated.[11,13,16] We examined the reactivity towards transamination of all 20 amino acid residues by using commercially available peptides. Sixteen of twenty residues (glycine, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, arginine, glutamic acid, glutamine, serine, threonine, asparagine, methionine, cysteine* (*: the SH group in the N-terminal cysteine residue was alkylated with iodoacetamide), and lysine) were transaminated, and peaks with a decrease of 1 m/z unit were detected (data not shown). For peptides having threonine, asparagine, cysteine*, or lysine residues at their N-termini, the signals of transaminated peptides were weak. Peptides incorporating histidine, tryptophan or proline residues at their N-termini afforded no transaminated product. However, such incompleteness of the reaction for peptides incorporating one of those seven residues (threonine, asparagine, cysteine*, lysine, histidine, tryptophan, or proline) at their N-termini does not affect the effectiveness of the method itself, because all peptides other than C-terminal peptides of proteins, which have lysine residues at their C-termini after LysC digestion, are depleted by DITC glass treatment whether or not their transamination reactions have proceeded to completion (data not shown). However, if a target C-terminal fragment of a protein has one of the three residues (threonine, asparagine or cysteine*: lysine residue is not placed at its N-terminus in this protocol) at its N-terminus, the signal in the mass spectrum can be detected but is weak after DITC depletion. For a C-terminal peptide having histidine, tryptophan or proline at its N-terminus, an orthogonal method should be employed.[5–7]

Optimized conditions or alternative routes to yield an α-ketocarbonyl group for peptides with these no-yield and low-yield residues should be exploited. For threonine and serine residues, there is an alternative technique that employs periodate oxidation.[22]

Dangers of side reactions have been mentioned in previous reports.[11,13,16,17] Peaks with a 56 and 74 Da increase were mainly detected among by-product peaks in the mass spectra. These by-products can generally form from almost all amino acid residues except for proline residues (data not shown). The mass increase of 56 Da may indicate the formation of a Schiff base with glyoxylate, and the increase of 74 Da can be explained by the addition of glyoxylate (before dehydration to the Schiff base). For the peptides containing aspartic acid at their N-terminus, the transamination reaction was incomplete although a 45-Da lighter species was detected as the principal species, which was attributed to the decarboxylated product.[13] This peak corresponds to that with an alanine residue at its N-terminus, so care should be taken of this special case.

The complexity of the mass spectrum that arose from the formation of these by-products was alleviated by DNPH modification. Figure 6 presents an example. A peptide incorporating an N-terminal serine residue (SFLLR) produced several by-products under this protocol (Fig. 6(a)). However, after DNPH modification, the modified peptide was selectively detected and the spectrum was simplified (Fig. 6(b)).

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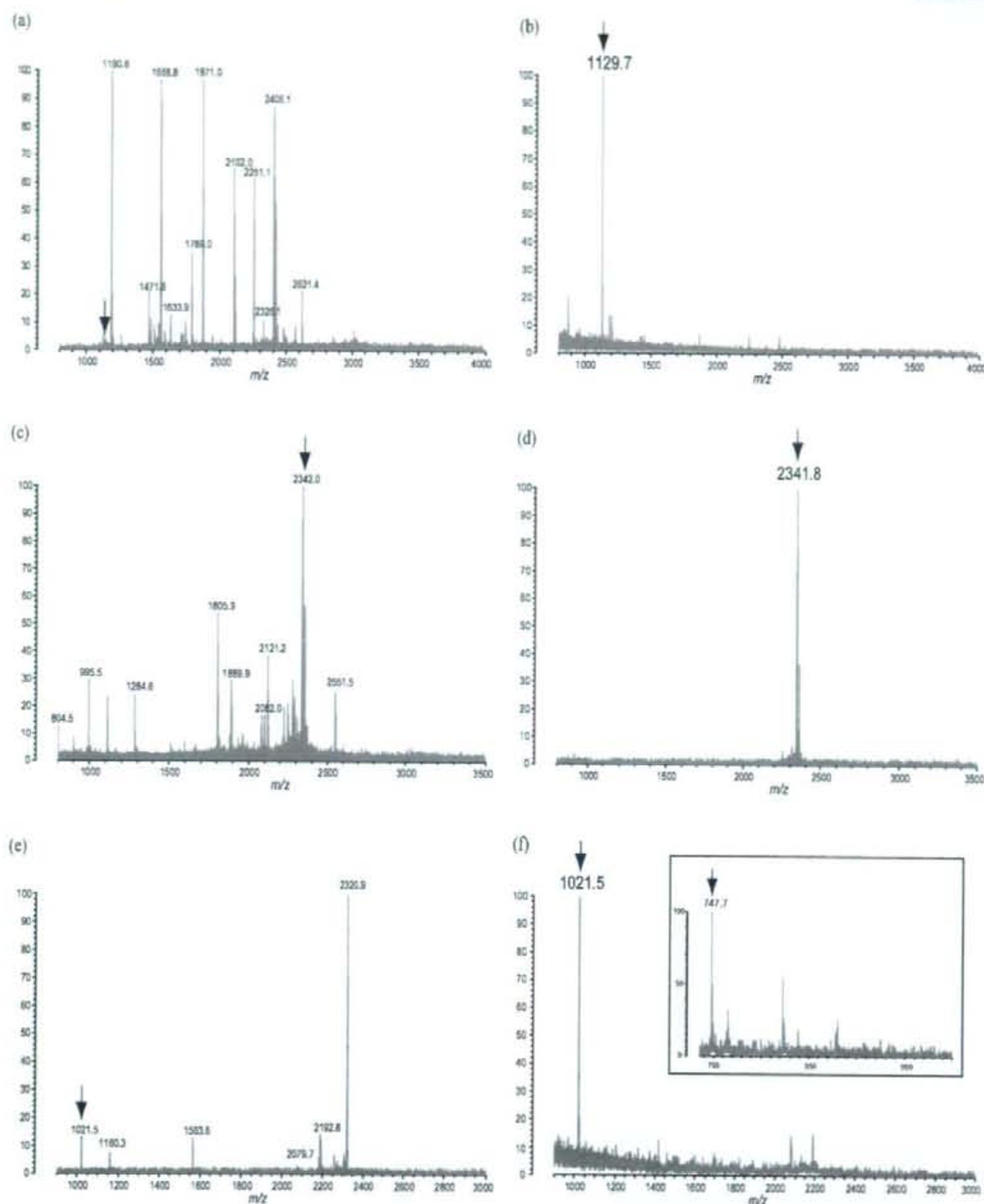

Figure 4. MALDI-TOF mass spectra of LysC digests after the transamination reaction (a, c, e) and after treatment with DITC glass (b, d, f). Three model proteins were used: pig albumin (a, b), bovine cathepsin B (c, d), and bovine α-casein (e, f). For bovine α-casein, two C-terminal peptides were isolated ($[M+H]^+$: 1021.06 from S2 and $[M+H]^+$: 747.4 from S1 (f: inset)). Arrows indicate the peaks of C-terminal peptides. An aliquot (ca. 3 pmol of digest) was loaded on the target plate.

It was reported that DNPH modification of peptides and use of DNPH as a matrix for MALDI-MS increase sensitivity.[20] With the experiment using the peptide (SFLLR, $[M+H]^+$: 635.4), the signal intensity of the modified peptide was enhanced. Successful application of the DNPH modification technique to transaminated peptides was thus demonstrated. This is partly explained by the higher affinity of DNPH-derivatized peptides for the DNPH matrix and structural similarity between derivatized peptides and the matrix.[20] It was reported that a nitrobenzene derivative functions as a matrix in MALDI-MS for selectively detecting 2-nitrobenzenesulfenyl-modified peptides and nitrotyrosine-containing peptides.[23]

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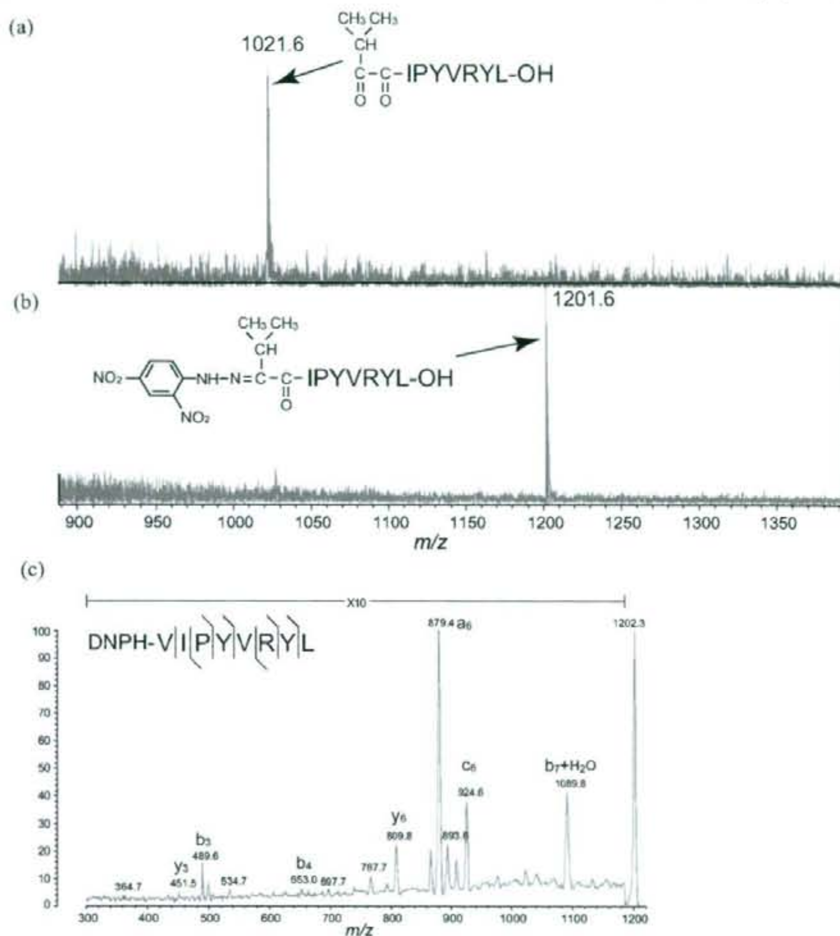

Figure 5. MALDI-TOF mass spectra of the isolated C-terminal peptide of α_{S2}-casein (a), after modification with DNPH reagent (b), and tandem mass spectrum (PSD mode) (c). An aliquot (ca. 3 pmol of digest) was loaded on the target plate. The b_7+H_2O ion arises from the loss of the C-terminal amino acid residue.[32]

The transamination reaction introduces a reactive carbonyl group into the peptide. It is beneficial for following chemical modifications because the resulting carbonyl group serves as a nucleophilic acceptor, whereas it increases the risk of undesired reactions, such as aldol reaction,[16,17] during subsequent steps. These undesired reactions can be minimized by the hydrazone or oxime formation just after the transamination reaction. This improved protocol is now under investigation.

In cases where proteins have a C-terminal lysine residue, the mass spectrum shows no peptide-derived signal because their C-terminal peptides, as well as internal peptides, are bound to DITC glass. This, in turn, suggests that the C-terminal residue can be lysine if no signal is observed in mass analysis. As mentioned already, the transamination reaction gives a couple of by-products which mainly derive from the addition reaction between the α-amino group of the peptide and the aldehyde group of glyoxylate. However, a C-terminal peptide whose N-terminus is proline gives no transaminated product and no adducts, which leaves the secondary amino group at the α-position intact (data not shown). Hence, in this case, the mass spectrum shows no peptide-derived signal as well. Therefore, if no signal is observed in the mass spectrum after the protocol, the C-terminal residue of a protein of interest can be lysine, or a C-terminal peptide whose N-terminus is proline.

For identification of proteins, N- or C-terminal analysis gives more reliable results than conventional methods such as peptide mass fingerprinting (PMF) or peptide fragment fingerprinting (PFF).[24,25] Hence, if the C-terminal analysis fails, N-terminal analysis can be complementarily used for this purpose.[8,26–28]

The N-terminus of a protein plays an important role as well.[4] As with C-terminal analysis, N-terminal structural determination serves for the analysis of post-translational modification and protein identification. We developed an N-terminal analysis method[8] by slightly modifying the method

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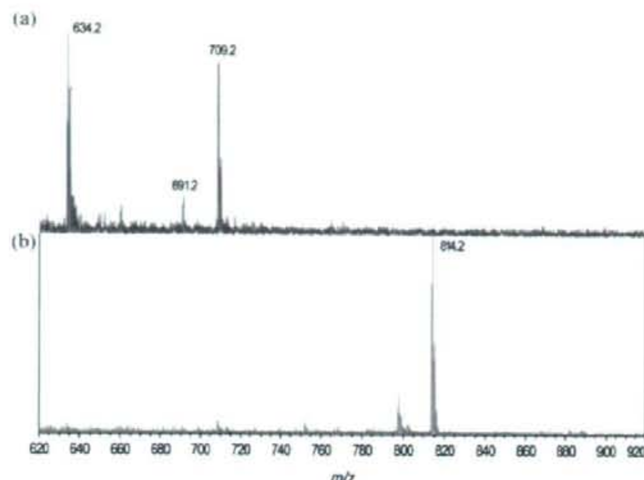

Figure 6. MALDI-TOF mass spectra of peptide SFLLR (10 pmol, $[M+H]^+$: 635.4) after the transamination reaction. The transaminated product (10 pmol, $[M+H]^+$: 634.4) was detected along with accompanying by-products (a), whereas the DNPH-modified product (10 pmol, $[M+H]^+$: 814.4) was mainly detected after the reaction (b).

for C-terminal analysis by just switching protease from LysC to *Grifola frondosa* metalloendopeptidase[29–31] (LysN). We are now studying N-terminal-specific isolation and sequencing by the transamination reaction (to be published elsewhere).

CONCLUSIONS

Isolation and sequencing of C-terminal peptides of proteins and their modification with DNPH were successfully performed. As far as we know, this is the first report of application of the transamination reaction to C-terminal analysis of protein by MS.

Owing to the α-diketone moiety, virtually any functionality can be introduced into the isolated C-terminal peptide. Therefore, C-terminal peptides isolated by this method can serve for a wide range of spectroscopic detection methodology.

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