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A method for simple identification of signature peptides derived from polyUb-K48 and K63 by MALDI-TOF MS and chemically assisted MS/MS fragmentation

Short Communication

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Summary. A simple method is described to identify signature peptides derived from polyubiquitin (polyUb) chains. The method is based on MALDI-TOF MS/MS analysis after chemically assisted fragmentation, and works on peptides isolated from polyacrylamide gels. PolyUb chains branched at K48 and K63 were chosen as models for Ub-protein conjugates. They were resolved by SDS-PAGE, and their tryptic peptides (in-gel-trypsinolysis) derivatized with 3-sulfopropinic acid NHSester to obtain chemically assisted fragmentation during the MS/MS analysis. PolyUb-K63 produced a single peptide identified as ^{55}TLSDYNIQK^{63} (GG)ESTLHLVLR^{72}. PolyUb-K48 produced two branched signature peptides identified as ^{43}LIFAGK^{48}(GG)QLEDGR^{54} and ^{43}LIFAGK^{48} (LRGG)QLEDGR^{54}. The recovery of signature peptide with LRGG as branched chain underscores the need to take limited proteolysis into account in the search for detection of ubiquitinated peptides in proteomics studies. In conclusion, a simple method has been described allowing the identification of signature peptides, which are diagnostic markers of the majority of polyUb-conjugated proteins. In principle, the method should be applicable also for other more rare signature peptides.

Keywords: MALDI-TOF MS/MS – Chemically assisted fragmentation – Polyubiquitin chains – Isopeptides – Signature peptides

Abbreviations: Ub, ubiquitin; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; CAF, chemical assisted fragmentation; PSD, post-source decay

Introduction

Ubiquitin (Ub) is a 8 kDa (76 residues) peptide. Ub is enzymatically conjugated via isopeptide bond to lysine residues of certain proteins as well as to Ub itself. This leads to the formation of mono/poly/multi-ubiquitinated proteins. Ubiquitination is a frequent posttranslational modification of proteins, involved in a number of biological processes. Polyubiquitination via ubiquitin K48 is often coupled to degradation of proteins by the 26S proteasome system, whereas polyubiquitination via ubiquitin K63 acts as non-proteolytic signals in several intracellular pathways (for review, see Pickart and Fushman, 2004). The digestion of polyUb chains by trypsin is sterically blocked at the branching points K48 and K63. The presence of these specific signature peptide(s) in tryptic digest of proteins obtained by one-dimensional or two-dimensional gel electrophoresis are diagnostic of free polyUb conjugates and polyubiquitinated proteins. Due to the fact that Ub is conserved from yeast to mammals (Schlesinger and Goldstein, 1975), it is expected that the signature peptides are identical among species.

The high current interest in polyubiquitinatioin has led to considerable efforts to define the "ubiquitome" of cells. In an important study in yeast Peng et al. (2003) used the highly work-intensive and technically demanding method of LC/LC-MS/MS to identify ubiquitinated proteins.

The purpose of the present study was to identify signature peptide(s) for proteins poly-ubiquitinated via either K48 or K63 by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS/MS. The K48 and K63 polyUb-chains were separated by SDS electrophoresis and in-gel digested. To facilitate the MS/MS detection chemically assisted fragmentation was used after peptide derivatization with 3-sulfopropinic acid NHS-ester (Keough et al., 2002). This procedure was sensitive, simple and produced

ions leading to a simple and unambiguous identification of both fully digested and partially digested signature peptides.

Material and methods

Material

K48-linked Ub chains were obtained from Biomol/Affiniti and K63-linked Ub chains (Ub_2–Ub_7) from BostonBiochem. Proteomics grade trypsin was purchased from Sigma or Stratagen. The sulfonic acid derivatization reagents used for chemical assisted fragmentation (CAF) was from Amersham Biosciences.

Sample preparation

Free polyUb chains, branched at K48 and K63, were separated into individual species (Ub_2–Ub_7) by SDS-PAGE and stained with Coomassie Brillant Blue (see inset in Figs. 1 and 2). The resolved components, including mono-Ub used as control, were excised from gel (12.5% w/v acrylamide; Figs. 1 and 2 insert), de-stained, and vacuum-dried before being subjected to in-gel trypsinolysis. For this, the dried gel pieces were covered by 25 μl of fresh trypsin solution (0.01 μg/μl in 100 mM bicarbonate buffer) and digested at 37°C for 18 h. The peptides were recovered by 3 successive extractions, each consisting of 20 min incubation of the gel pieces with 40 μl extraction solution at 20°C with continuous shaking and removal of supernatant after centrifugation. The extraction solutions were 1% trifluoroacetic acid (TFA), followed by 0.1% TFA in 50% acetonitrile (ACN), and finally 100% ACN. The pooled peptide extracts were concentrated in a vacuum centrifuge to approximately 25 μl. From this a 0.5 μl sample (corresponding to about 500 femtomol of protein) was mixed with 0.5 μl of matrix (saturated solution of α-cyano-4-hydroxycinnamic acid from LaserBio Labs, Sophia-Antipolis Cedex, France), deposited on a polished steel target plate, and air-dried.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS)

Samples of tryptic peptides were analyzed by MALDI-TOF MS (Ultraflex from Bruker Daltonik GmbH, Bremen, Germany), being irradiated with a nitrogen laser. The spectra (Figs. 1 and 2), acquired in reflector positive mode at an accelerating voltage of 25 kV, were externally calibrated. Annotation of the tryptic peptides was by XMASS and BioTools 2.1 software (Bruker Daltonics), and the sets of peptide masses used to search the NCBI or Swiss-prot database. The search for peptides of unique molecular mass derived from branching at K48 and K63 as well as the confirmation of the ubiquitin site was accomplished by Profound software (Zhang and Chait, 2000) (http://prowl.rockefeller.edu) by scanning for the addition of the C-terminal tryptic branched signature peptides of Ub, i.e. GG (114.05 Da) or LRGG (383.23 Da).

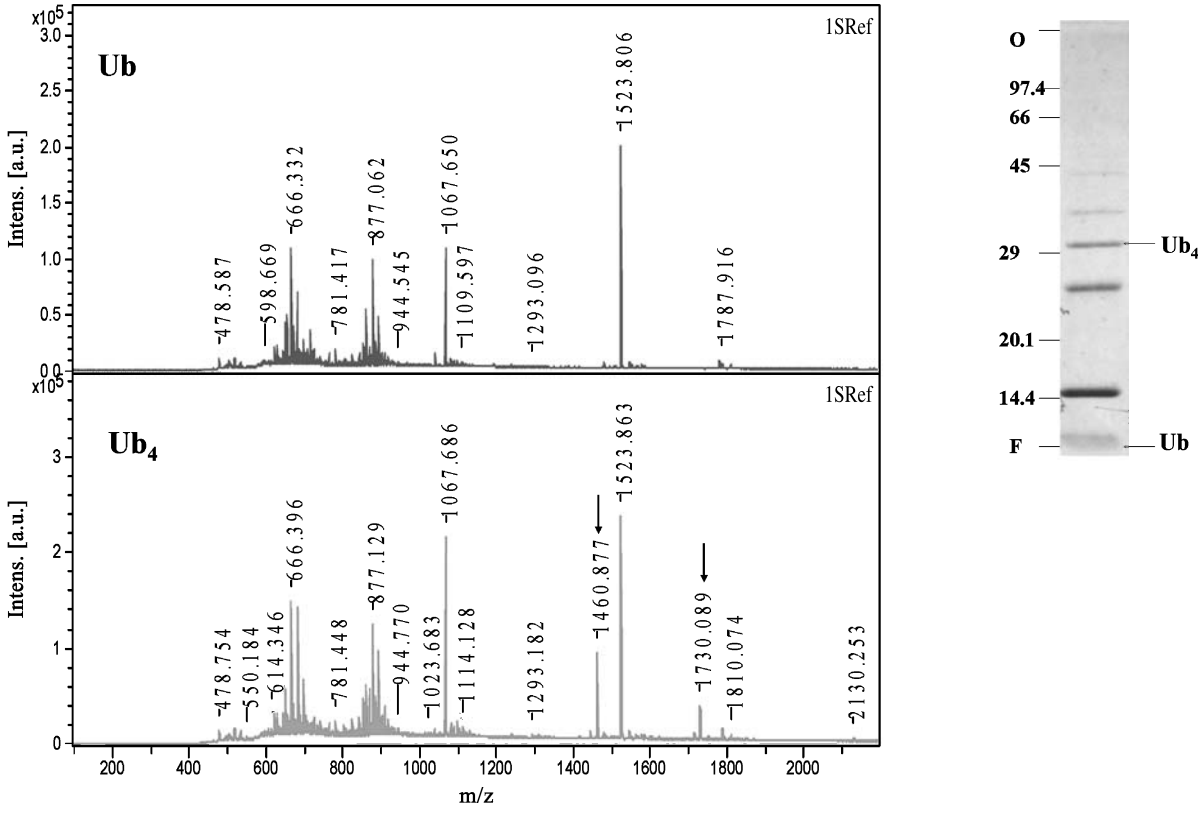

Fig. 1. Identification of the branched signature peptides ^{43}LIFAGK^{48}(LRGG^{76})QLEDGR^{54} and ^{43}LIFAGK^{48}(LRGG^{76})QLEDGR^{54} in polyUb-K48. A mixture of polyUb-K48 of various chain length, containing also Ub, was resolved by SDS polyacrylamide gel electrophoresis (PAGE) (inset). Ub4 and Ub were trypsinized in-gel and analyzed by MS. Upper panel: MALDI-TOF mass spectrum of the tryptic digest of Ub. Lower panel: MALDI-TOF mass spectrum of the tryptic digest of Ub_4-K48. Note the appearance in the lower panel of two additional ions corresponding to signature peptides with m/z 1460.88 and 1730.09. For further details, see the Material and methods section

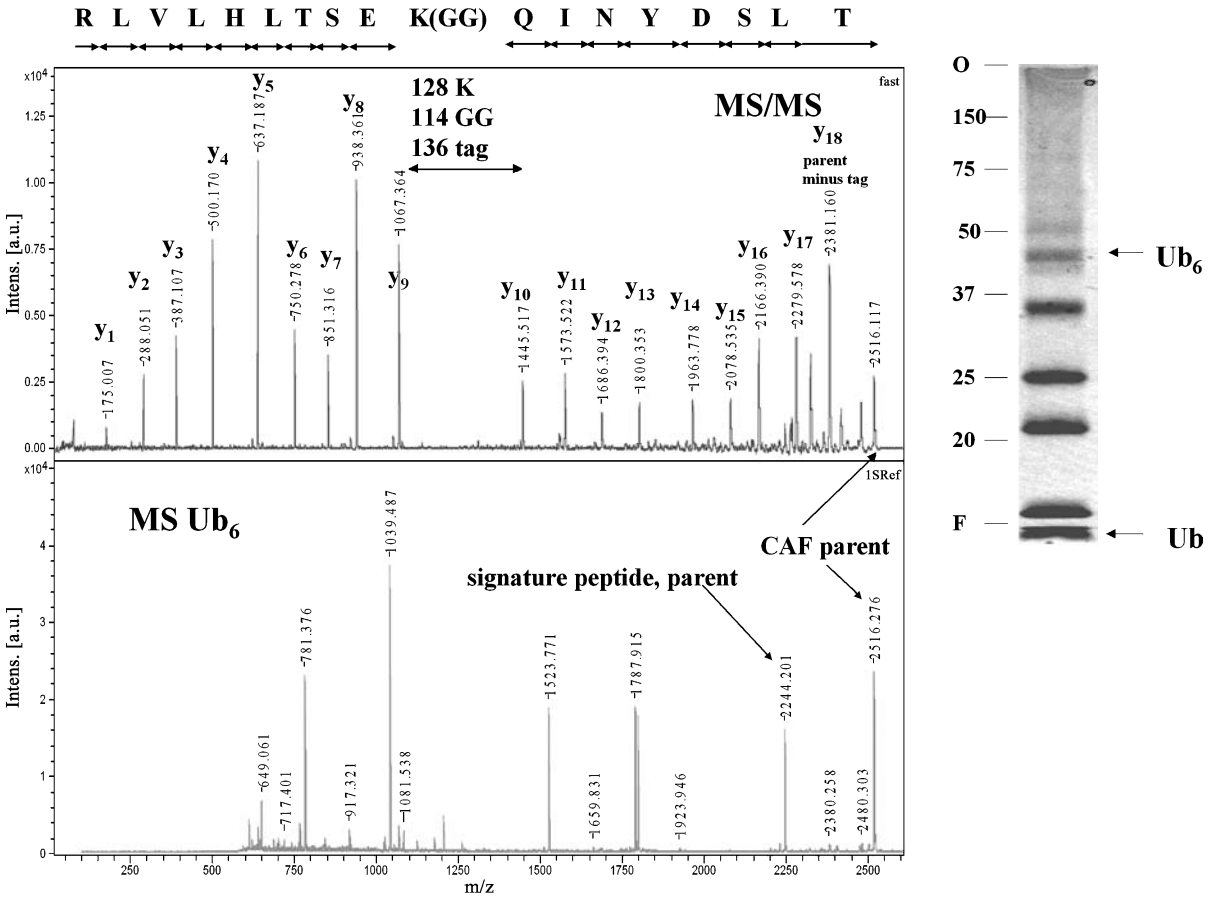

Fig. 2. Identification of the branched signature isopeptide ^{55}TLSDYNIQK^{63}(GG^{76})ESTLHLVLR^{72} in polyUb-K63. Following resolution and identification on SDS-PAGE (inset), Ub_6-K63 was trypsinized in-gel and analyzed by MALDI MS. The lower panel shows the MS spectrum of the tryptic peptides of Ub_6-K63 after derivatization of N-termini with sulfonic acid. Note the signature peptide with m/z 2244.20 which is also present as a robust peak in its CAF-derivatized form with a m/z 2516.27 due to the addition of 2 sulfonated groups of 136 atomic mass unit each, one as a tag on the N-terminal Threonine residue of the peptide chain and on the N-terminal glycine of the branched chain. Top panel: The MS/MS spectrum of the CAF-labelled branched signature peptide of m/z 2516.27 showing a clear y-ion series which illustrates that K63 is modified by GG. The 378.15 mass unit difference between y_{10} and y_9 corresponds to the sum of the monoisotopic masses of K (128.09) + two G (114.04) + the tag (136) at the N-terminus of the Gly-Gly branch. For further details, see the text and Table 2. Note that the relative strength of peptides derived from Ub itself differed from the experiment in Fig. 1. This is mainly due to the use of a different matrix and the use of ZipTip purification of the peptide mixture after derivatization. For further details see the Material and methods section

MALDI MS/MS analysis

Before MS/MS analysis the peptides derived from polyUb chains were subjected to sulfonic acid derivatization using 3-sulfopropinic acid NHS ester (Keough et al., 2002) on ZipTip as described by the manufacturer (Amersham Biosciences), except that 2,5-dihydroxybenzoic acid (20 mg/ml of 35% acetonitrile and 0.2% trifluoroacetic acid) replaced α-cyano-4-hydroxycinnamic acid as matrix. The MS/MS analysis was performed on Ultraflex (Bruker Daltonic, Germany).

Results

Comparison of the MS spectra of Ub and polyUb-K48 (Ub_{2-6}) revealed ions at m/z 1460 and 1730 (shown for Ub_4 in Fig. 1, lower panel), unique for polyUb-K48. They corresponded to the branched signature peptides ^{43}LIFAGK^{48}(GG^{76})QLEDGR^{54} and ^{43}LIFAGK^{48}(LRGG^{76})QLEDGR^{54}, respectively (Fig. 1; Table 1B). Both signature peptides had high amplitude and were well resolved, with isotopic distribution of 5 to 6 peaks. The sequence coverage was ~85% for Ub and similar for polyUb taking into account the contribution of the signature peptide(s).

Corresponding MS analysis of polyUb-K63 (Ub_{2-6}) revealed the branched signature peptide ^{55}TLSDYNIQK^{63}(GG^{76})ESTLHLVLR^{72} with m/z 2244.20 (Fig. 2, lower panel; Table 1). Low (subpicomolar) protein levels are often encountered in proteomics research (Chen et al., 2004). Peak signal-to-noise ratio ranging from 500 to 1000 was observed for the MS spectra for the major signature peptides upon the application of as little as 250–500 femtomoles of trypsinized polyubiquitin

protein on the target plate (Figs. 1, 2; data not shown). One reason why MALDI analysis works so well with the signature peptides may be that they were cleaved C-terminal to an arginine rather a lysine. We have found that peptides with C-terminal arginine tend to be more sensitively detected in time of flight instruments (not shown).

The MS/MS spectra confirmed the identity of the signature peptides (Fig. 2, upper panel). The use of the CAF method, in which only the y-ion fragments are detected because they retain a net positive charge, simplifies the interpretation of the MS spectra. It should be noted that upon CAF derivatization, the m/z of the parent peak in the MS/MS spectrum increased by 272 amu (atomic mass unit) due to double sulfonation, i.e. 136 amu for $O^-{}_3S$–CH_2–CH_2–CO– of the N-terminal residue of the peptide and another 136 amu for the terminus of the side chain (GG or LRGG) branched at K48/63 in polyUb-(K48/63).

Using the present derivatization protocol and the rather mild conditions for fragmentation the complete 18 residue mono-derivatized peptide (y_{18}) as well as every monoderivatized species from y_{10} to y_{18} was observed. The difference between y_{10} and y_9 was compatible with one lysine, a di-glycine and the sulfonic acid derivative (Fig. 2, upper panel). Also, the complete range of non-derivatized peptides (y_1–y_9) were observed (Fig. 2, upper panel). Similar clean results were obtained for the two signature peptides derived from polyUb-K48 (not shown).

Table 1. MALDI-TOF mass spectrometric identification of peptides derived from Ub alone (**A**) and the branched signature peptides derived from polyUb-K48 and polyUb- K63 (**B**) after tryptic digestion in-gel. In B, peptides branched to K through the carboxyterminal of G^{76} are indicated in parenthesis

A. Ub Sequence of tryptic peptides	m/z_{exp} (Da)	m/z_{calc} (Da)
^{43}LIFAGK^{48}	648.33	648.41
^{49}QLEDGR^{54}	717.30	717.35
^{55}TLSDYNIQK^{63}	1081.55	1081.55
^{64}ESTLHLVLR^{72}	1067.64	1067.62
B. PolyUb K48 and K63 linked-chains Sequence of signature isopeptides	m/z_{exp} (Da)	m/z_{calc} (Da)
^{43}LIFAGK^{48}(GG^{76}) QLEDGR^{54}	1460.78	1460.79
^{43}LIFAGK^{48}($LRGG^{76}$) QLEDGR^{54}	1729.97	1729.97
^{55}TLSDYNIQK^{63}(GG^{76}) ESTLHLVLR^{72}	2244.20	2244.20

Table 2. Comparison of predicted and observed mass values for y-ion series in the MS/MS analysis of the derivatized ^{55}TLSDYNIQK^{63} (GG^{76})ESTLHLVLR^{72} signature peptide for polyUbK63 with mass 2516.164 (MH+). The program GPMAW32 6.10 (see text for details) was used to predict the masses of y_1–y_{17}. The observed values of the same ions and for y_{18} are shown for comparison

Residue	y	m/z_{calc} (Da)	m/z_{exp} (Da)
Thr	18	–	2381.160
Leu	17	2279.134	2279.578
Ser	16	2166.050	2166.390
Asp	15	2079.018	2078.535
Tyr	14	1963.991	1963.778
Asn	13	1800.928	1800.353
Ile	12	1686.885	1686.394
Gln	11	1573.801	1573.522
Lys	10	1445.742	1445.517
Glu	9	1067.621	1067.364
Ser	8	938.579	938.36
Thr	7	851.547	815.316
Leu	6	750.499	750.278
His	5	637.415	637.187
Leu	4	500.356	500.170
Val	3	387.272	387.107
Leu	2	288.204	288.051
Arg	1	175.120	175.007

The amino acid sequence of the peptide was obtained from the experimentally determined y-ions series, by manual calculation of the difference between the consecutive y-ion masses. A considerably higher throughput and additional verification was obtained using the GPMAW32 6.10 software. By introducing the putative sequence suggested by the MS analysis, including the sulfonated GG (or LRGG), the theoretical y-values were automatically calculated, and found to fit very well with the experimentally determined values (Fig. 2; Table 2).

The analysis, illustrated for polyUb-K63 (Fig. 2, top panel; Table 2), revealed a spectrum of y-type fragment ions with clear background under MALDI-post-source decay (PSD) conditions and characterized by a simple profile for the derivatized signature peptide parent.

Discussion

The present method for detection of polyUb signature peptides is simple and was validated for in-gel trypsinolysis. This fact and the high sensitivity should make the method well suited to detect signature peptides in typical proteomics experiments where the amount of

ubiquitinated proteins typically is scarce because of the often high turn over of such proteins. The ease of detection of both the completely and incompletely digested signature peptides, ^{43}LIFAGK^{48}(GG^{76})QLEDGR^{54} and ^{43}LIFAGK^{48}(LRGG^{76})QLEDGR^{54}, derived from polyUb-K48 (Fig. 1) is an advantage for the analysis of in-gel digests of proteins separated by SDS electrophoresis, which often show incomplete digestion (Warner, 2005).

The chemically assisted fragmentation led to formation of a robust amount of biderivatized parent signature peptide (Fig. 2, lower panel), and a complete and clean range of y-ions. As shown for polyUb-K63 the whole range of y-ions from y_1 to y_{18} were detected with minimal noise (Fig. 2, upper panel), allowing simple sequencing using the GPMAW32 6.10 software (Table 2). The present study was limited to polyUb-K48 and polyUb-K63, but the method would be expected to be useful also for signature peptides derived from polyUb chains branching at the less frequent positions K6, 11, 27, 29 and 33 (Peng et al., 2003).

After the present study was completed the use of an alternative sulfonating reagent, 4-sulphonyl isothiocyanate, was reported for MALDI MS/MS analysis of synthetic peptides trypsinized in solution, including tetra-Ub-K48 (Wang and Cotter, 2005). Apparently, their method led to stronger fragmentation, including internal fragmentation of the tag, leading to a more complex profile in the signature portion of the MS/MS spectra.

In conclusion, the present method describes a straightforward way of identifying low concentrations of polyUb signature peptides after in gel digestion. Hopefully, this will facilitate the efforts in other laboratories and our own laboratory to find new targets for ubiquitination.

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