Summary

Human lysozyme (h-LYZ), which contains four disulfide bonds, was expressed in a cell-free protein synthesis system constructed from *Spodoptera frugiperda* 21 (Sf21) cells. h-LYZ was expressed in a soluble and active form under non-reducing conditions after addition of reduced glutathione (GSH), oxidized glutathione (GSSH), and protein disulfide isomerase (PDI). The *in vitro* synthesized proteins were purified by means of a *Strep*-tag attached to their C-termini. Approximately 30 μg of h-LYZ were obtained from 1 ml 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 protein were formed.

Results

Expression, purification and characterization of h-LYZ

mRNA prepared from plasmid a pTD1-strep-h-LYZ carrying h-LYZ gene was translated in the insect cell-free systems under following conditions: a) reducing condition by DTT, b) non-reducing condition, c) non-reducing condition in addition of GSH, GSSG, and PDI. Analysis of the synthesized proteins were carried out using FluoroTect™ (Promega Co.). The synthesized proteins were separated by centrifugation (15000rpm, 15min), and soluble and precipitated fractions were analyzed by 12.5 % SDS-PAGE and fluoroimager (Fig. 1A). The synthesized proteins were almost in the soluble fraction under such conditions. The protein synthesis efficiencies were similar to each other. However, lysozyme activity was detected strongly in the h-LYZ synthesized under non-reducing condition in addition of GSH, GSSG, and PDI (Fig. 1B). 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 400 μg/ml, respectively (Fig. 2).

Using the optimized condition where lysozyme activity was expressed at the highest levels, h-LYZ was synthesized, purified by affinity column chromatography, and the purity was judged on SDS-PAGE by staining with CBB (Fig. 3). The h-LYZ ran as almost single band having molecular mass of about 17 kDa. This observed molecular mass corresponded to the theoretical values calculated from the amino acid sequence of the proteins' mature form. The final yield of h-LYZ was approximately 30 μg from 1 ml reaction mixture.

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 (data not shown).

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

To confirm the disulfide bond arrangements of protein synthesized in the insect cell-free system developed here, the disulfide pairings of h-LYZ, were determined by MALDI-TOF MS. Fig. 4 shows the location of disulfide bonds for wild type protein along with the complete amino acid sequence, including the affinity tag used here. The protein was treated under the three following conditions: a) reduction followed by *S*-alkylation with iodoacetamide, b) *S*-alkylation with iodoacetamide, and c) non-treatment followed by overnight trypsin digestion. Tryptic peptides was analyzed by MALDI-TOF MS (Scheme 1). The PMF for h-LYZ sample treated under the three conditions described above was almost identical, with the exception of regions corresponding to cysteine-containing peptides, regardless of conditions for sample preparation (Fig. 5A), and the values obtained were in good agreement with the theoretical values calculated assuming the presence of wild-type disulfide bonds.

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. 4). 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 (data not shown). 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 3636.68 (Table 1). Three peaks were clearly observed that were in good agreement with the theoretical values. The m/z values of 1824.11 (Fig. 5B), 3239.53, and 3636.60 (Fig. 5C) were assigned to peptides containing disulfide linkages. 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 (Cys^{7}-Cys^{129} and Cys^{31}-Cys^{117}) were the same as those observed in the wild type protein. However, the locations of the other two disulfide bonds between Cys^{66}, Cys^{78}, Cys^{82}, and Cys^{96} were still undetermined.

To determine the remaining disulfide linkages, the tryptic fragment observed at the m/z value of 3636.60 was isolated using reverse-phase HPLC (Scheme 2). Of eight peaks observed in the reverse-phase HPLC elution profile, peak 8 was identified as the peptide of interest by MALDI-TOF MS (Fig. 6). Therefore, the peptide (Fig. 7A) was subjected to thermolytic digestion. Two peaks were thus clearly observed at m/z values of 1362.58 (Fig. 7B) and 2112.33 (Fig. 7C). 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.

Conclusion

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.

References

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Expression of proteins containing disulfide bonds in an insect cell-free system and confirmation of their arrangements by MALDI-TOF mass spectrometry. *PROTEOMICS*, 2007, *in press*.

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

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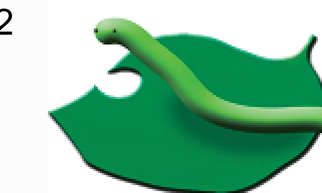
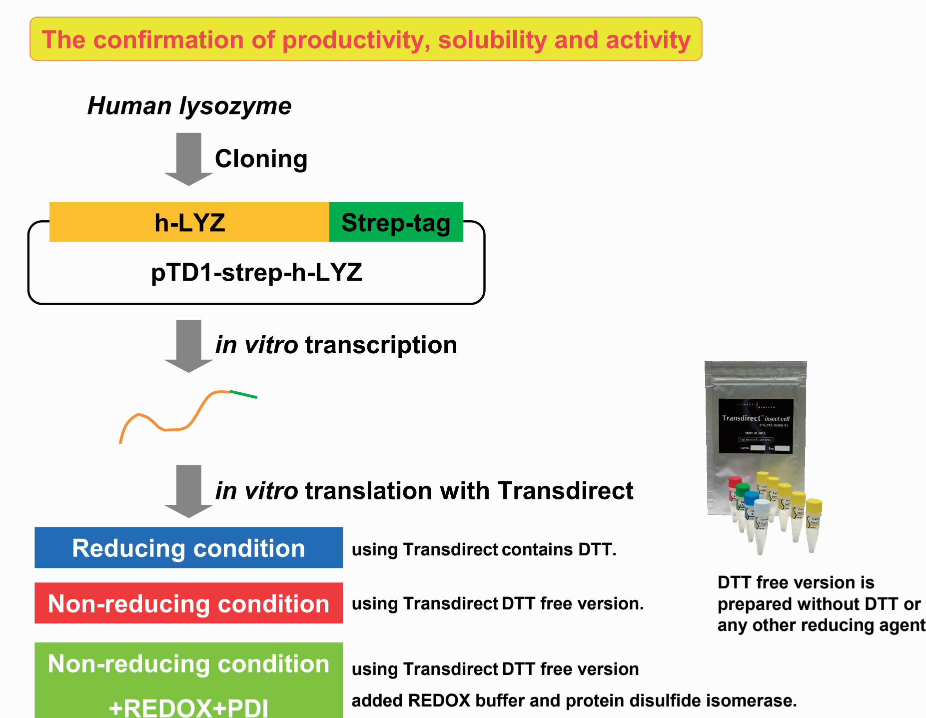
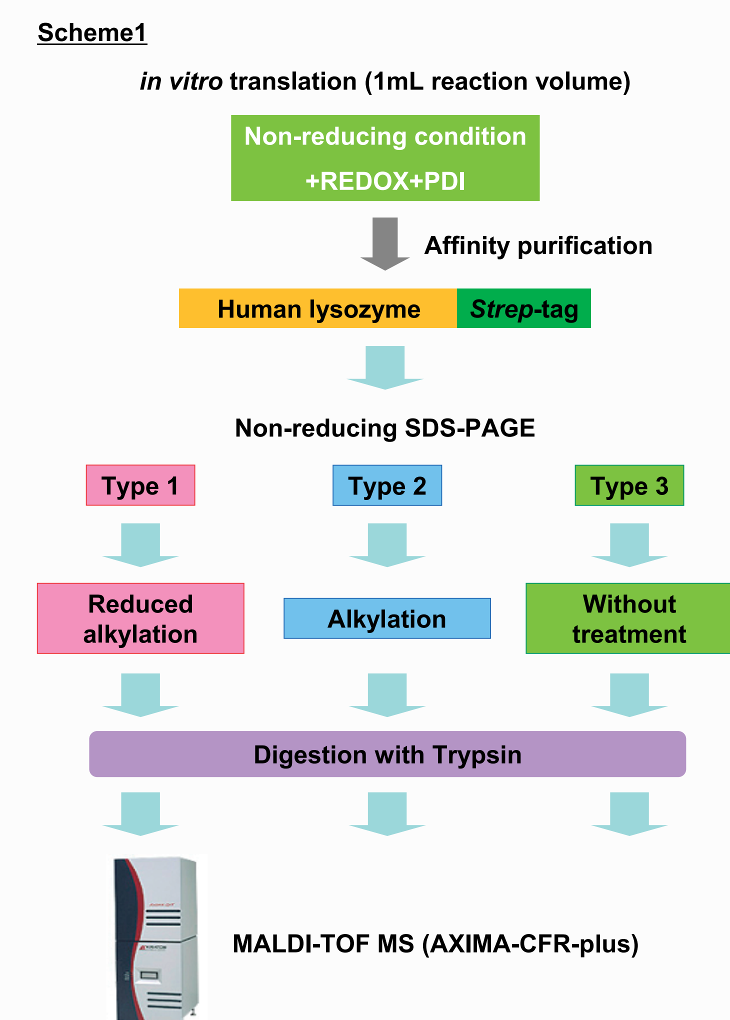
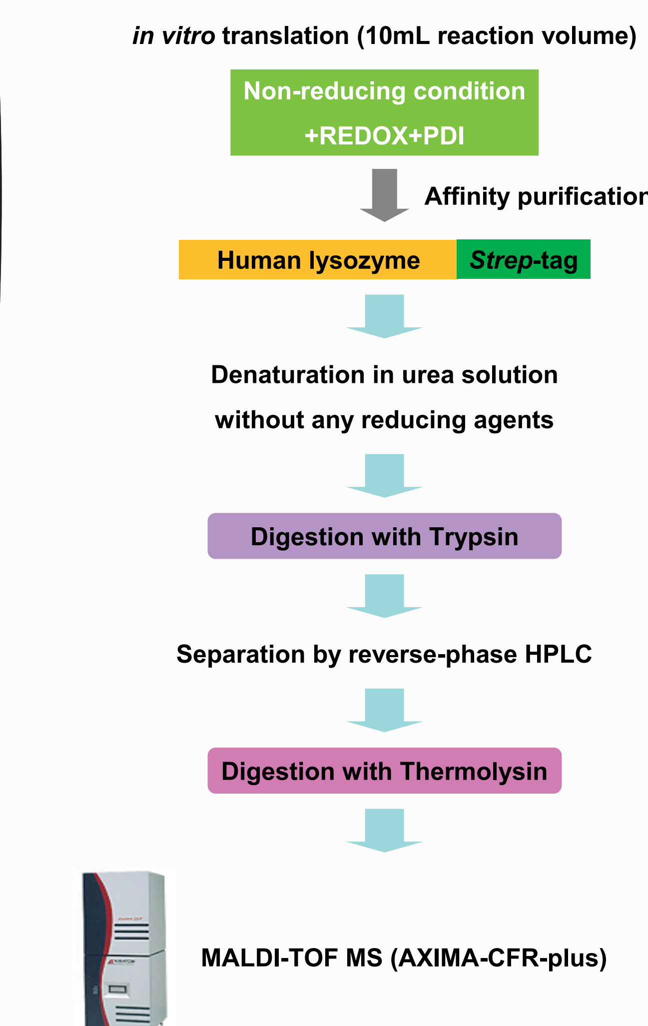


Experimental strategy



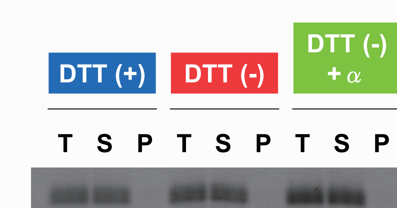
The confirmation of disulfide bond arrangements





Results

(A) Fluorescent detection



After reaction, samples (T) were centrifuged at 15000 rpm for 15 min to separate soluble (S) and precipitated (P) fractions. The pellet was resuspended in original volumeof distilled water. Lysozyme activity was assayed by turbidity decrements at 450 nm of 240 μg/ml Micrococcus luteus (Wako) in Tris-HCl buffer (50 mM pH 8.0).

(B) Enzymatic activity

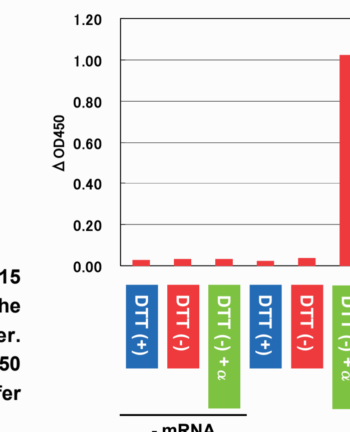


Fig. 1. Cell-free protein synthesis of h-LYZ under several conditions.

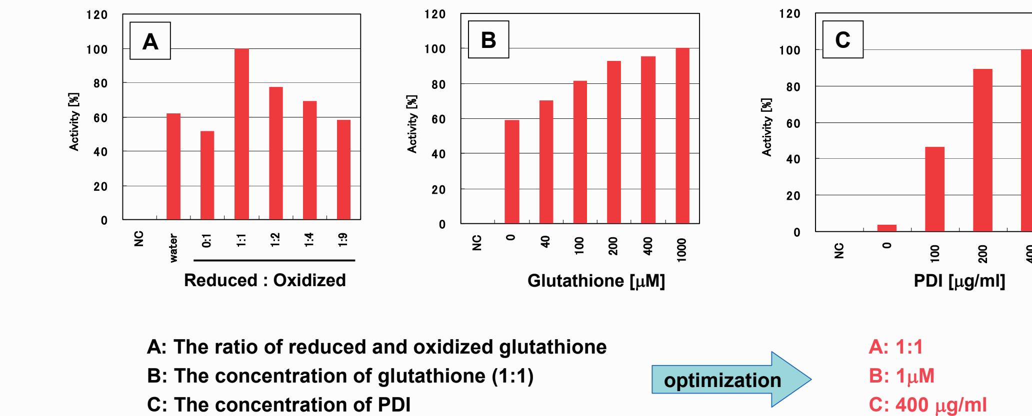


Fig. 2. Effect of REDOX buffer and PDI on the activity of synthesized h-LYZ.

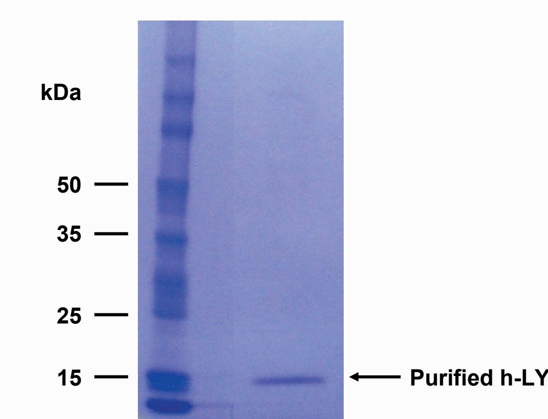


Fig. 3 SDS-PAGE analysis of purified h-LYZ.

[1]MKVFERCELA RTLKRLGMDG YRGISLANWM CLAKWESGYN[40]

[41]TRATNYNAGD RSTDYGIFQI NSRYWCNDGK TPGAVNACHL[80]

[81]SCSALLQDNI ADAVACAKRV VRDPQGIRAW VAWRNRCQNR[120]

[121]DVRQYVQGCG VGTGSGGGGG GGGWSHPQFE K

Spacer sequence — *Strep*-tag sequence

Disulfide linkages are indicated by lines.

Peptides containing cysteine(s) in tryptic digests indicated by colors.

Fig. 4. Amino acid sequence and the location of disulfide bonds in wild h-LYZ.

Table 1. Theoretical monoisotopic mass values for disulfide-linked peptides.

Position	Mass	Disulfide linkage (Cys^{7}-Cys^{129})
7-11 124-151	591.29 2651.17	3239.46

Position	Mass	Disulfide linkage (Cys^{31}-Cys^{117})
23-34 117-120	1306.66 520.23	1823.89

Position	Mass	Disulfide linkage (Cys^{66}-Cys^{82}, Cys^{78}-Cys^{96})
64-70 71-98	885.36 2756.32	3636.68

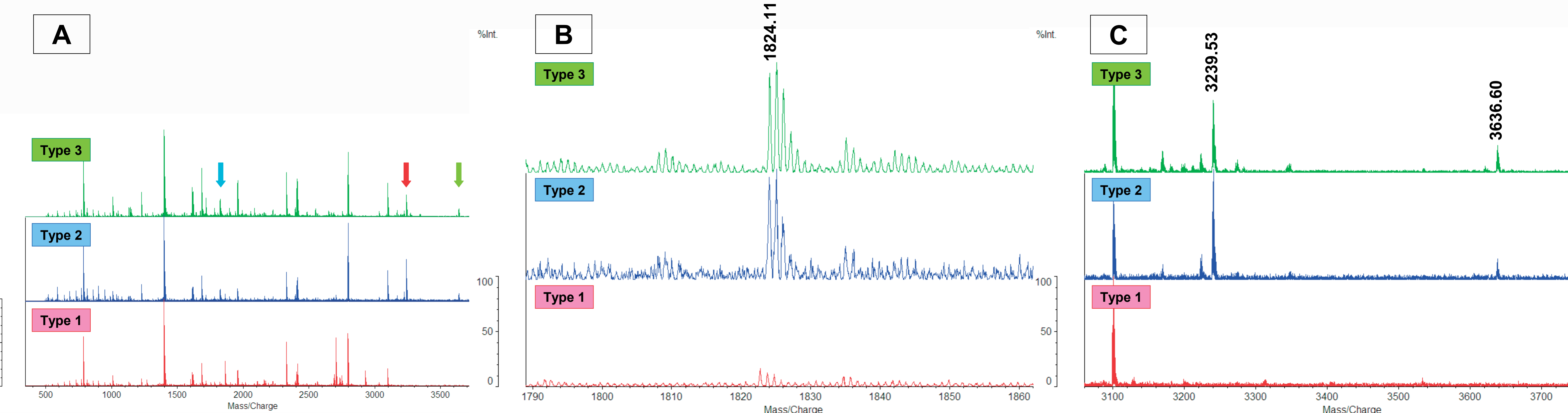
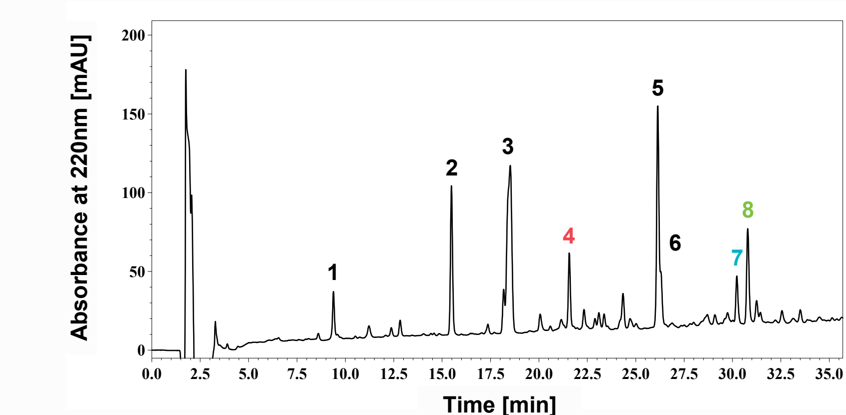


Fig. 5. MALDI-mass spectra of tryptic digests of h-LYZ.



The tryptic digests of h-LYZ were separated by reverse-phase HPLC with a 50 min linear gradient increasing from 5 to 50% (v/v) acetonitrilre containing 0.1% TFA.

Peak ID	Position	Modification(s)	Theoretical mass value	Observed mass value
1	43-51		981.43	981.49
2	35-42		1012.45	1012.40
3	16-22		811.38	811.45
4	7-11 124-131	Disulfide bond: 7-129	3239.46	3239.14
5	109-114		788.42	788.04
6	52-63		1400.68	1400.34
7	23-34 117-120	Disulfide bond: 31-117	1823.89	1823.99
8	64-70 71-98	Disulfide bond: 66-82, 78-96	3636.67	3636.39

Fig. 6. Theoretical and observed monoisotopic mass values for tryptic digests of synthesized h-LYZ separated using reverse-phase HPLC.

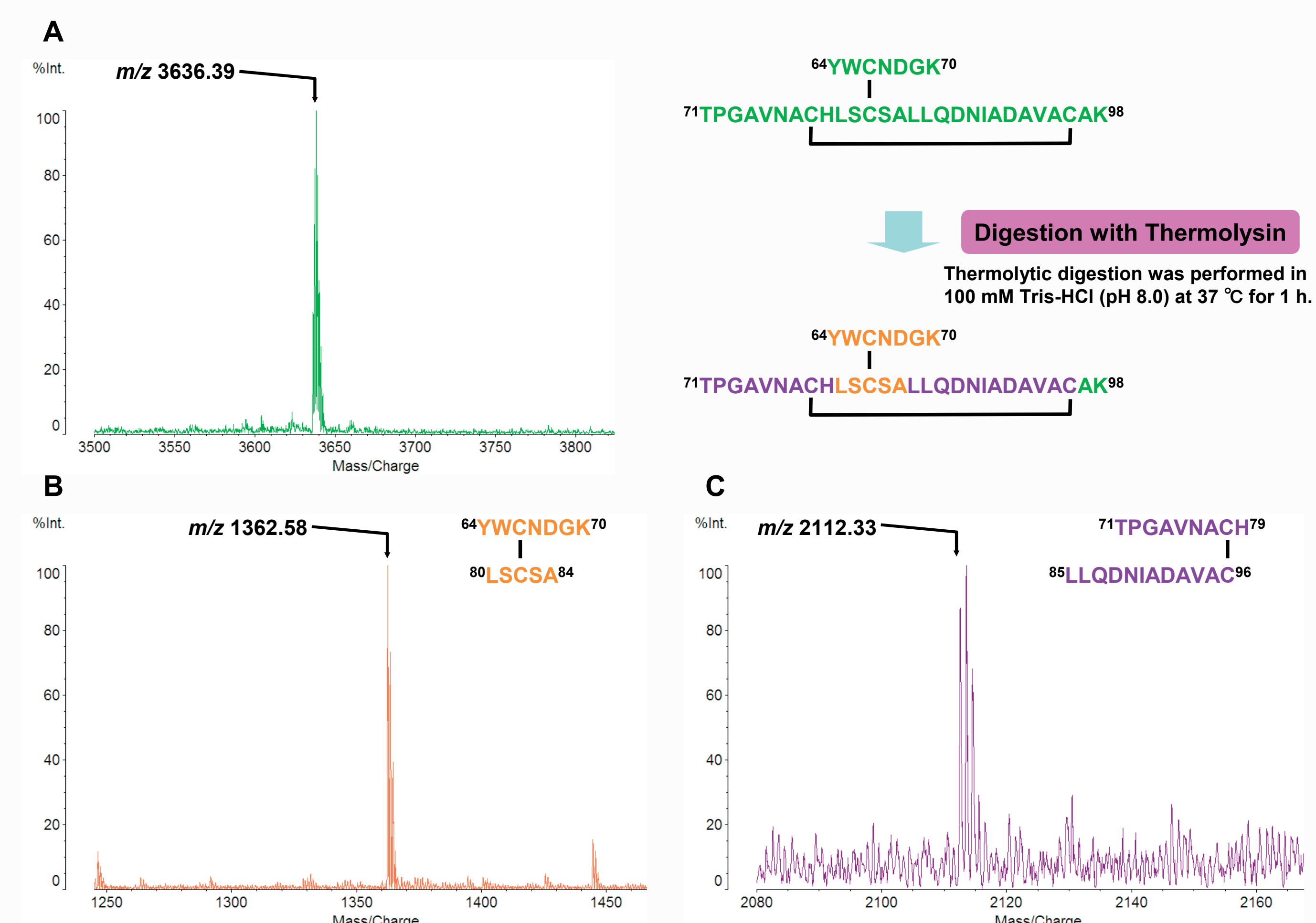


Fig. 7. MALDI-mass spectra of thermolytic digests of peptide 8.