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Location of Disulfide Bonds in Mature α-L-Fucosidase from Pea

ANNA CODINA[a], MARTA VILASECA[b], TERESA TARRAGÓ[c], IRENE FERNÁNDEZ[b], DOLORS LUDEVID[c] and ERNEST GIRALT[a,*]

[a] Department of Organic Chemistry, University of Barcelona, Barcelona, Spain
[b] Mass Spectrometry Service, III Division, University of Barcelona, Barcelona, Spain
[c] Department of Molecular Genetics, CID-CSIC, Barcelona, Spain

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Abstract: Fuc-9 is the mature form of a vacuolar α-L-fucosidase enzyme which seems to play an important role in plant growth regulation. Fuc-9 is a 202-residue protein containing five Cys residues located at positions 64, 109, 127, 162 and 169. In this study, the disulfide structure of Fuc-9 was determined by MALDI-TOF mass spectrometry (MS), with minimal clean-up of the samples and at a nanomolar scale. Two strategies, based on a specific chemical cleavage (with 2-nitro-5-thiocyanobenzoic acid and alkaline conditions) at the Cys residues and modification of Cys residues by acrylamide/deuterium labeled acrylamide alkylation, were used. Using these methods, the disulfide pairings Cys64-Cys109 and Cys162-Cys169 could be established. The advantages and limitations of our experimental approach are discussed.

Keywords: acrylamide; α-L-fucosidase; disulfide bonds; free cysteines; Fuc-9; MALDI-TOF mass spectrometry; NTCB; tryptic digestion

INTRODUCTION

α-1,2-L-Fucosidase is a 202-residue glycolytic enzyme which hydrolyses fucosyl-terminal groups of oligosaccharides [1]. The protein (Fuc) is synthesized in plant cells as a prepro-protein, reaching its mature form (Fuc-9) after the cleavage of a *C*-terminal propeptide of nine amino acids during its transit to the vacuoles. The *C*-terminal propeptide is necessary for Fuc sorting to the vacuole. Recently, several expressed sequence tags (EST) homologous to pea fucosidase have been identified in other leguminous plants as *Glycine max* (EST accession no. AI794968), *Cicer arietinum* (EMBL CAB76906) and *Medicago truncatula* (AW126318). The comparison of EST deduced amino acid sequences with Fuc protein shows a high degree of homology (80–56% identity). The five Cys residues (Cys64, 109, 127, 162 and 169) of Fuc protein [2], and most of the residues surrounding these Cys residues, are conserved in this family of fucosidases. It is unknown whether the Cys residues of the mature enzyme (Fuc-9) are involved in a catalytic dyad. Comparison between Fuc-9 and the pro-enzyme [3] suggests two disulfide bonds at Cys64-Cys109 and Cys162-Cys169. Cys127 would be a free Cys. In order to check this hypothesis, we decided to undertake an experimental study on Fuc-9 using MALDI-TOF mass spectrometry (MS) as the major analytical tool.

Locating disulfide bonds and free Cys residues in proteins is still a challenge, especially when dealing with compounds that have multiple disulfide bonds and when only nanomolar amounts of material are available. Although there is not a general method for disulfide bond location, the most commonly used strategy has been based on the enzymatic or chemical cleavage of the protein, followed by analysis of the resulting fragments containing the intact

* Correspondence to: Department of Organic Chemistry, University of Barcelona, Martí i Franquès 1, E-08028 Barcelona, Spain; e-mail: giralt@qo.ub.es

native disulfide bonds. In this kind of approach the selection of the proper enzymes and the optimal digestion conditions are critical issues.

Different studies have proved the efficiency of MS disulfide bonds elucidation [4]. Both electrospray ionization MS (ESI-MS) and MALDI-TOF MS provide the means to determine accurate masses of proteins and peptides in the low-to-sub-picomole range. On-line coupling of high-performance liquid chromatography (HPLC) with electrospray ionization MS (LC-MS) has traditionally been used in the disulfide structure determination of enzymatic digested proteins, as it provides a convenient means of obtaining mass information on peptide complex mixtures [5,6]. Nevertheless, more recently, examples of disulfide bonds located by MALDI-TOF MS have been reported [7,8]. This technique is clearly unaffected by many of the buffers and additives that are commonly associated with protein purification, so that it proves very useful in the analysis of complex mixtures without prior fractionation. In this study, we have assigned the disulfide structure of protein Fuc-9 using two different strategies which involve direct analysis by MALDI-TOF MS with minimal clean-up of the samples and at a nanomolar scale. The strategies exploited are based on a specific chemical cleavage at Cys residues and modification of Cys residues by alkylation, respectively.

MATERIALS AND METHODS

Chemicals

The following chemicals were used: tris(2-carboxyethyl)phosphine (TCEP) hydrochloride, Pierce (Rockford, IL); guanidine (Gu) hydrochloride, Boehringer-Mannheim Biochemicals (Indianapolis, IN); 2-nitro-5-thiocyanobenzoic acid (NTCB) and TPCK trypsin, Sigma (Steinheim); 4-vinylpyridine, Fluka (Neu-Ulm); unlabeled acrylamide, Bio-Rad (Hercules, CA); deuterium-labeled (2,3,3′-D_3-acrylamide, Cambridge Isotope Laboratories (Isomed S.L); dithiothreitol (DTT), Merck (Darmstadt). The water used in all procedures was purified using a Millipore (Bedford, MA) Milli-Q purification system. All of the organic solvents were HPLC grade.

MS

MALDI mass spectra were obtained on a Voyager DE-RP (PerSeptive Biosystems, Framinghan) time-of-flight (TOF) mass spectrometer equipped with a nitrogen laser (337 nm, 3 ns pulse). The accelerating voltage in the ion source was set to 20–25 kV. Data were acquired in the linear or reflector mode operation with delay times of 100–300 ns. Time-to-mass conversion was achieved by external calibration using different calibration mixtures (from CalBiochem) depending on the mass range of the analysed product: calibration mixture 1, m/z 905.05–1674.0 (des-Arg-bradykynin, angiotensin I, Glu1-fibrinopeptide B, neurotensin); calibration mixture 2, m/z 1297.5–5734.6 (angiotensin I, ACTH(1-17), ACTH(18-39), ACTH(7-38), insulin); cytochrome C, m/z 6181.0–12361; BSA, m/z 33216–66431. Experiments were performed using different matrices: α-cyano-4-hydroxycinnamic acid [CHCA, Aldrich], 3,5-dimethoxy-4-hydroxycinnamic acid [sinapinic acid, SA, Fluka (Neu-Ulm)] and 2,5-dihydroxy-benzoic acid [DHB, Aldrich]. Matrix solutions were prepared by dissolving 10 mg of compound in a 50% (v/v) solution of CH_3CN 0.1% aqueous trifluoroacetic acid (TFA). Equal volumes (1 μL + 1 μL) of matrix solution and diluted samples (see sample preparation) were mixed and spotted onto the stainless steel sample plate. The mixture was allowed to air dry before being introduced on the mass spectrometer.

Sample preparation for MALDI-TOF analysis. Crude mixtures (peptide concentration of 4 pmol/μL) were cleaned-up and concentrated over a micro-tip filled with chromatographic media (Zip-Tip C_{18} from Millipore). The sample (30–80 μL) was attached to the C_{18}-resin, washed with 0.1% aqueous TFA (10 μL × 3)[1] and step eluted in 2 μL fractions (× 6) of H_2O/CH_3CN (1:1, v/v) 0.1% TFA and CH_3CN 0.1% TFA. The eluted fractions were mixed with the matrix solutions and spotted onto the sample plate.

Chromatography

Analytical HPLC was carried out on a Shimadzu instrument (Kyoto, Japan) comprising two solvent delivery pumps (model LC-10AD) and an automatic injector (model SIL-10A XL). The detector was a diode array (model SPD-M10A VP). Instrument control and data acquisition and evaluation were performed using Class-LC-10 software run on a Compaq Deskpro Pentium processor, which was connected to the rest of the instrument by a communicator Bus module (model BM-10A).

Two hundred μL of peptide mixture (at a concentration of 4 pmol/μL) was injected and subjected to chromatography using a Nucleosil C18 column (10 μm particle size, 300 Å pore, 250 × 4 mm), a flow rate of 1 mL/min, eluant A [0.045% (v/v) aqueous

TFA], eluant B [0.036% (v/v) TFA in acetonitrile], and a linear 30-min gradient from 10 to 70% eluant B. Detection was achieved at 220 nm.

Strategy 1

Analysis of free sulfydryls (NTCB procedure and 4-vinylpyridine procedure). Protein sample (500 μL, 4 pmol/μL) dissolved in buffer X (Tris–HCl 20 mM pH 8, NaCl 0.5 M) was denatured with 6 M GuHCl (0.57g). Cyanylation of the free sulfydryl group was achieved at 37°C by adding a 10-fold molar excess of NTCB (over the protein). After 30 min, the pH was raised to 9.0 by adding Tris base (3.0 M). Cleavage of the cyanylated protein was accomplished after incubation at 37°C for 16 h.

In the case of alkylation with 4-vinylpyridine, a 60-fold molar excess of 4-vinylpyridine over free thiols was added and allowed to react for 12 h at room temperature, under nitrogen, and in the dark. The sample was then analysed by MALDI-TOF, after cleaning and concentrating the protein using a Zip-Tip.

Analysis of total sulfydryls (TCEP/NTCB procedure). Protein sample (500 μL, 4 pmol/μL) dissolved in buffer X was denatured with 6 M GuHCl (0.57 g). A 10-fold molar excess of TCEP was added, and was allowed to react at 37°C for 30 min to reduce the disulfide bonds in the protein. The denatured and reduced protein was then subjected to the above NTCB procedure to cyanylate the free sulfydryls groups and cleave the protein.

Strategy 2

Protein digestion. A 4 μM solution of recombinant Fuc-9 in buffer X (400 μL) was denatured at pH 1, 40°C for 1 h as described elsewhere [9]. The enzymatic digestion was performed at 37°C for 8 h by the addition of 50 μL (48 units/mg protein) of immobilized TPCK-trypsin. The enzyme was then eliminated by centrifugation (7000 × *g*, 3 min). The crude digest was analysed by MALDI-TOF MS after cleaning and concentrating the sample as explained.

DTT reduction/acrylamidation (10). Reduction of the tryptic peptides (60 μL at 4 pmol/μL in buffer X) was performed with DTT at 60-fold molar excess (6 μL of DTT 6 mM) over the disulfide bonds, in the dark, under nitrogen, for 20 h, at 40°C. Derivatization of reduced and non-reduced (60 μL at 4 pmol/μL, buffer X) digestion peptides was carried out with 10^4-fold molar excess of acrylamide (3.6 μL of acrylamide/2,3,3′-D_3-acrylamide 1:1) for 3 h at 40°C.

RESULTS AND DISCUSSION

We first applied a strategy to recognize the number and location of both cystine and Cys residues. The strategy was to use a specific chemical cleavage, which involves, in the first stage, the reaction of free sulfydryl groups with NTCB to selectively cyanylate Cys residues [11]. The *N*-terminal peptide bond of each *S*-cyanylated Cys residue can then be cleaved under alkaline conditions to form an *N*-terminal peptide and a 2-iminothiazolidine-4-carboxylyl (ITC) peptide (Figure 1). If the starting molecule contains several Cys residues, a series of ITC-peptides are formed. These peptides can be analysed by MALDI-TOF MS and mapped to the sequence.

In our case, a protein sample denatured with GuHCl 6 M was subjected to treatment with NTCB, followed by cleavage at pH 9 (Figure 2). The crude product was cleaned-up and concentrated over a micro-tip filled with chromatographic media (Zip-Tip C_{18}). The sample was attached to the C_{18}-resin, washed with 0.1% NH_4OH and step eluted with H_2O/CH_3CN/TFA mixtures [12,13]. The eluted fractions were mixed with matrix solutions and analysed separately by MALDI-TOF MS. The observation of the expected products by MS (Figure 3),

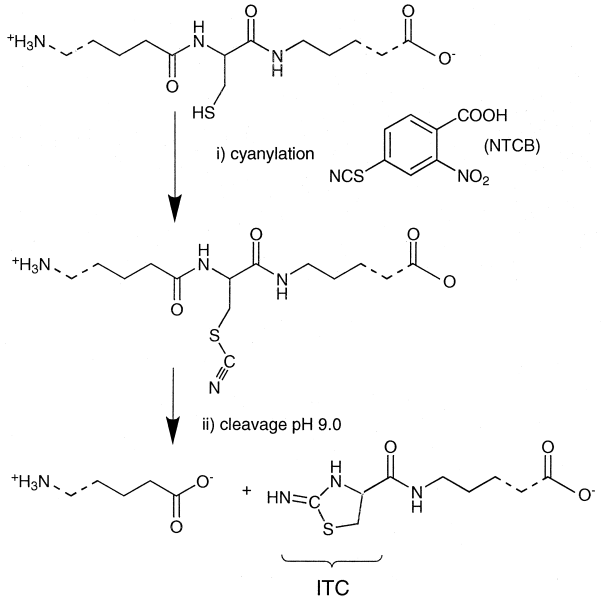

Figure 1 Cyanylation reaction of free thiols with NTCB followed by cleavage at pH 9.0 yielding the ITC peptide fragment.

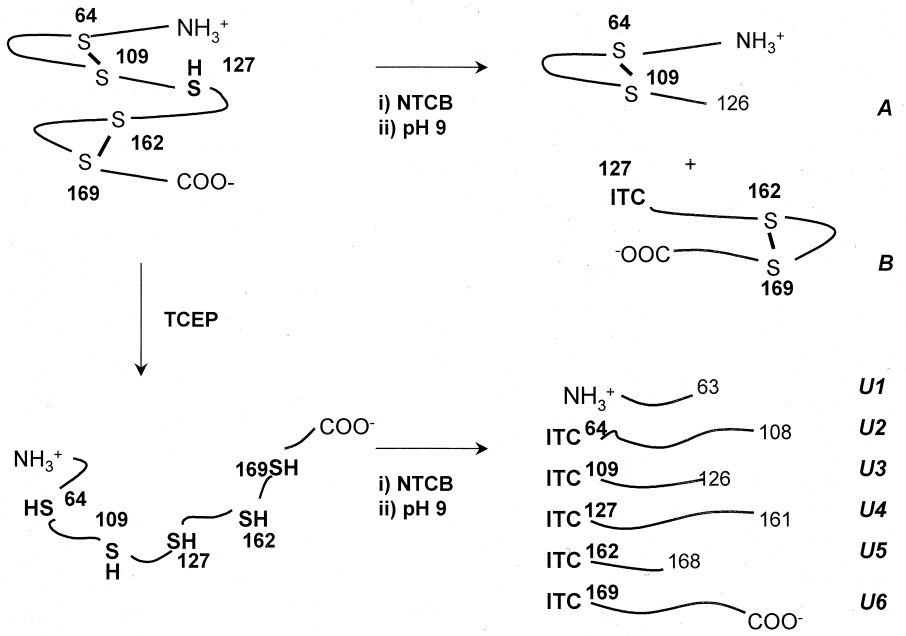

Figure 2 Schematic representation of reaction between NTCB and Fuc-9 with and without previous TCEP disulfide reduction. Direct cyanylation of Fuc-9 followed by reaction under basic conditions yields fragments A and B, whereas a similar treatment of products from the reduced Fuc-9 yielded fragments U1–U6.

matched, for the most part, the initial hypothesis supporting the existence of only one free Cys residue (127) and two disulfide bonds (Cys64-Cys109, Cys162-Cys169 pairings) (Figure 2, Table 1). Nevertheless, we couldn't avoid by-product formation of fragments C and D (Table 1) which not only could be by-products of the reaction owing to the alkaline conditions, but are also consistent with

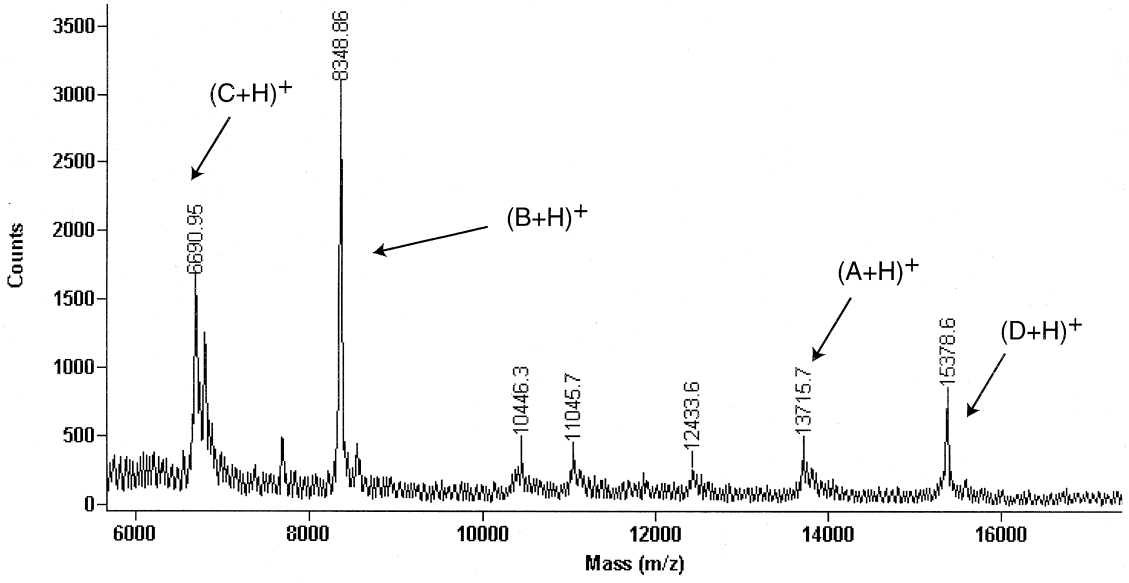

Figure 3 MALDI-TOF spectrum of the purified reaction product of Fuc-9 with NTCB, followed by specific cleavage at alkaline conditions. A [m/z $(M+H)^+$ 13 716 Da] and B [m/z $(M+H)^+$ 8348.9 Da] are the expected products, assuming Cys127 as the only free cysteine. Byproducts C [m/z $(M+H)^+$ 6690.9 Da] and D [m/z $(M+H)^+$ 15 379 Da] are formed during the alkaline treatment step.

Table 1 Theoretical and Experimental Average m/z $(M+H)^+$ Obtained from Reaction of NTCB with Fuc-9 Before (Fragments A–D) and After (Fragments U1–U6) Reduction with TCEP

Fragment	Theoretical m/z (Da)	Experimental m/z (Da)	Residues[a]
A	13 714	13 716	1–126
B	8346.3	8348.9	127–202
C	6687.5	6690.9	1–63
D	15 379	15 379	64–202
U1	6687.5	6691.8	1–63
U2[b]	5036.9	5036.6	64–108
U3[b]	2044.4	2044.5	109–126
U4[b]	3738.3	3738.2	127–161
U5	716.34	–	162–168
U6	3964.3	3964.1	169–202

[a] Numbers denote amino acid position, as found in the sequence of recombinant Fuc-9.
[b] m/z obtained after the loss of one molecule of water $(Ui-H_2O+H)^+$.

Fuc-9 with free Cys64 instead of Cys127. Our attempts to reduce these by-products by: (a) using less NTCB; (b) elimination of the excess of reagent before incubation at pH 9 (with repeated washings with 0.1% NH_4OH in a set-pack column); (c) shortening the incubation times; or (d) carefully controlling the pH, were unsuccessful. As described by Wu *et al.* [11], in most cases, disulfide bond exchange cannot be completely avoided. Consequently, the NTCB method cannot be employed as a unique test for the assignment of the disulfide bond pairing patterns.

Reaction of denatured (6 M GuHCl) Fuc-9 with 4-vinylpyridine or NTCB resulted in the reaction of one single molecule of derivatizing agent per molecule of protein (data determined by MALDI-TOF MS, see 'Materials and Methods'). This result confirms that there is only one free thiol per Fuc-9 molecule. On the other hand, the theoretical location of the five Cys residues in Fuc-9 was confirmed

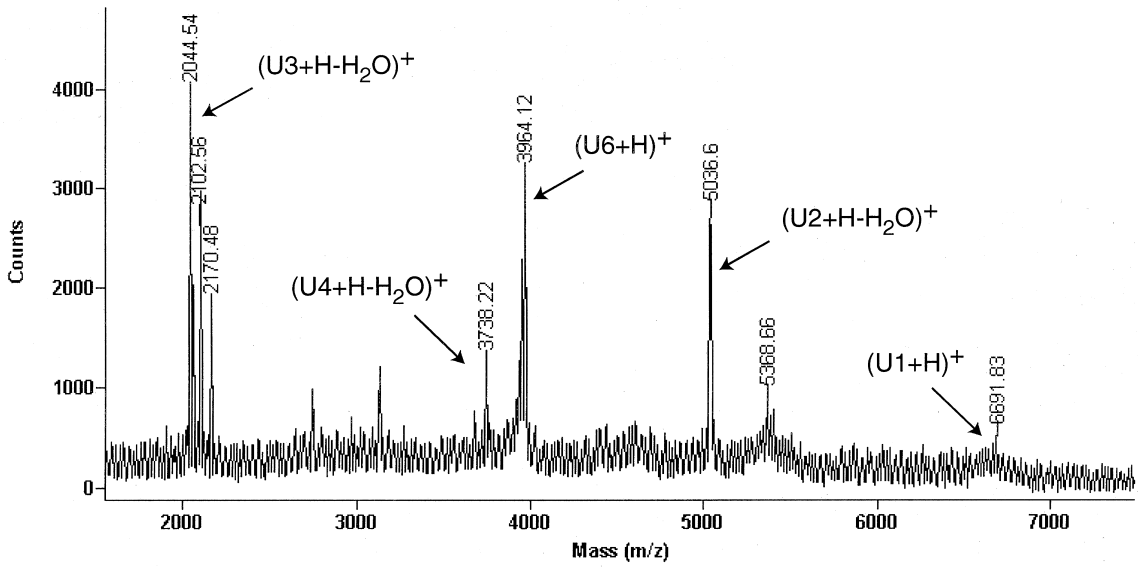

Figure 4 MALDI-TOF spectrum of the purified reaction product of reduced Fuc-9 with NTCB, followed by specific cleavage at alkaline conditions. U1 is the *N*-terminal cleavage product. U2–U6 appear as ITC fragments. Some of them can only be found after the loss of a water molecule $(Ui+H-H_2O)^+$. U5 fragment could not be found due to its low molecular weight (theoretical m/z 716.34 Da).

by the results obtained from an identical experiment involving the NTCB cleavage, but with the protein previously reduced with TCEP (Figures 2 and 4, Table 1). Taking together the results from the experiments described so far, we can conclude that at least a major population of protein had only Cys127 as a thiol free residue. However, the presence of products C and D in the crude from the NTCB treatment leads us to doubt the existence of a minor population of free Cys64. As we report below, a second experimental strategy for the elucidation of the disulfide structure of Fuc-9 has allowed us to exclude this last possibility. This strategy consisted of tryptic digestion of the denatured protein followed by: (1) reduction with DTT and alkylation with a 1:1 mixture of unlabeled acrylamide and deuterium labeled acrylamide [(2,3,3′-D_3-acrylamide]; or (2) alkylation with the latter mixture without previous DTT reduction [10] (Figure 5). Without prior separation, peptide mixtures have been analysed by MALDI-TOF MS. The Cys content in each fragment has been deduced from their isotopic distribution signature derived from the mass difference (3 Da) of the alkylating reagents. Comparison between results from reduced and non-reduced samples allowed us to locate disulfide bonds and free Cys residues. Figure 6 and Table 2 show the theoretical cleavage sites for trypsin in Fuc-9 and the calculated monoisotopic molecular mass of Cys- and cystine-containing fragments before and after acrylamidation.

First, the pH denatured Fuc-9 was digested with TPCK-trypsin and the crude mixture analysed by MALDI-TOF MS. A Zip-Tip C_{18} was used to clean and concentrate the sample before spotting it onto the plate, as described in the former strategy. As

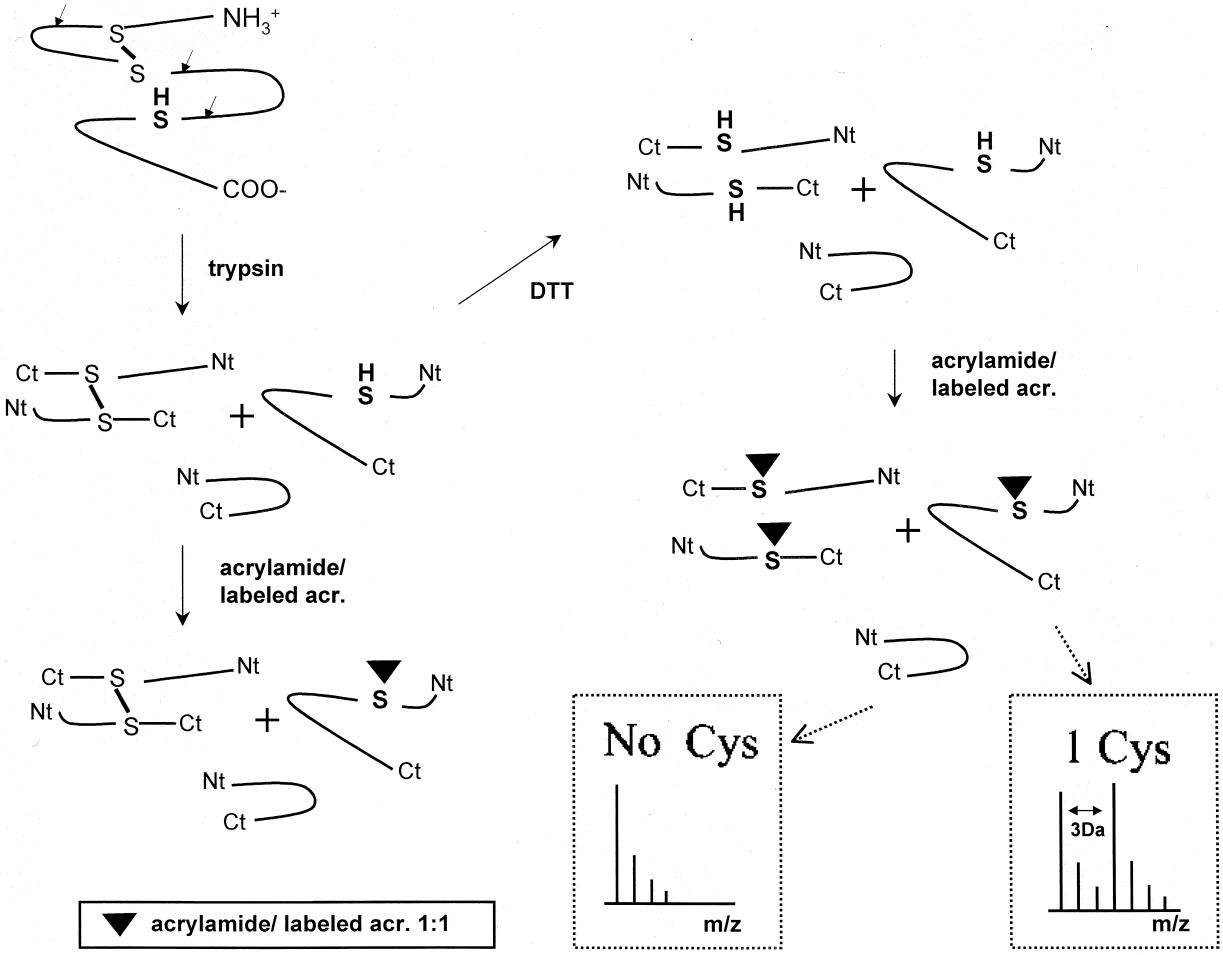

Figure 5 Schematic representation of our second strategy: trypsin digestion of a protein; DTT disulfide reduction of one fraction of the crude digest; and, finally, acrylamidation of both crude digest and reduced crude digest. A theoretical MALDI-TOF spectrum of a peptide without Cys residues (left) is compared with a spectrum of a peptide with one alkylated (with acrylamide/deuterium labeled acrylamide) Cys (right).

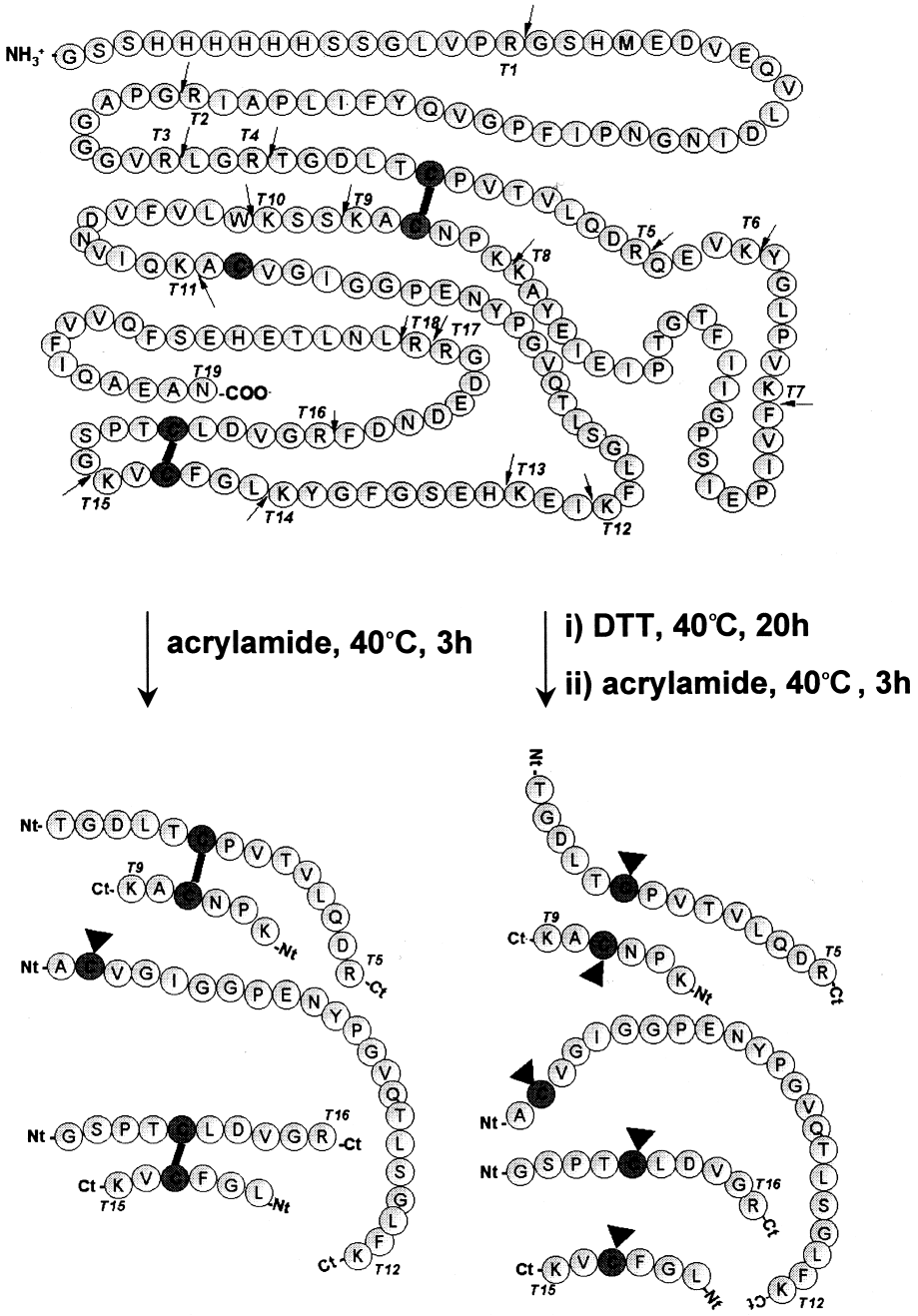

Figure 6 Schematic representation of the expected results from Strategy 2 applied to Fuc-9. Cleavage sites for trypsin (top). Acrylamidated Cys containing peptides resulting from derivatization of digested peptide mixture (left) and from derivatization of peptides previously disulfide reduced with DTT (right).

shown in Figures 7 and 8, the two theoretically expected disulfide linked peptides (T5–T9 and T15–T16) were observed with experimental masses $(M+H)^+$ of 2177.3 Da and 1667.4 Da, respectively. No other signals corresponding to fragments with different Cys pairings were detected. Peptide T12 could not be observed either in the crude mixture.

Second, the crude digest was Cys alkylated with the 1:1 mixture of unlabeled and deuterium labeled acrylamide, with the aim to selectively derivatize the theoretically unique free-Cys containing fragment. The corresponding alkylated fragment

Table 2 Monoisotopic m/z $(M+H)^+$ of Fuc-9 Cysteine Containing Tryptic Fragments Before and After Acrylamidation

Tryptic fragment[a]	Theoretical m/z (Da)	Experimental m/z (Da)	Residues[b]
T5–T9	2176.5[c]	2177.3	59–72/106–111
T15–T16	1669.0[c]	1667.4	159–164/165–174
T5	1517.7	–	59–72
T9	660.34	–	106–111
T12	2207.1	2207.1	126–147
T15	666.36	665.82	159–164
T16	1004.5	–	165–174
T5+Acr/Acr*	1588.7/1591.8	1589.8/1592.8	59–72
T9+Acr/Acr*	731.38/734.40	731.60	106–111
T12+Acr/Acr*	2278.1/2281.2	–	126–147
T15+Acr/Acr* (–NH_2)	721.38/724.40	721.89/724.83	159–164
T16+Acr/Acr*	1075.5/1078.6	1075.5/1078.5	165–174

[a] Tryptic peptides are labeled according to the theoretical tryptic digest, starting from the *N*-terminus of the sequence (Figure 6).
[b] Numbering corresponds to the sequence of recombinant Fuc-9.
[c] Average mass.

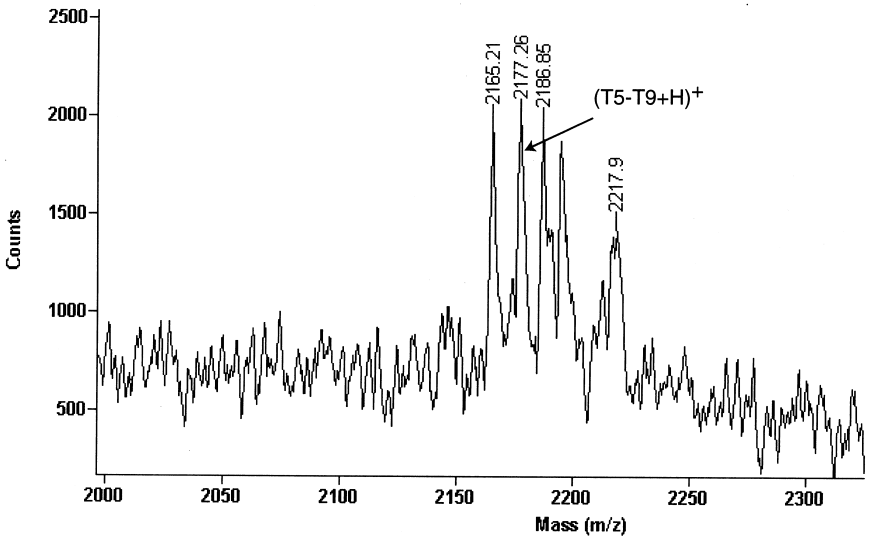

Figure 7 Region of a MALDI-TOF spectrum showing the experimental m/z $(M+H)^+$ of 2177.3 Da obtained for the first pair of peptides linked by a disulfide bond (T5–T9).

(T12 + Acr/Acr*) could not be detected by MALDI-TOF MS directly from the crude sample, but HPLC fractionation allowed the detection of fragment T12 (m/z $(M+H)^+ = 2207.1$ Da, Figure 9). Although the corresponding acrylamidated fragment was not observed, probably owing to the lack of reactivity of fragment T12 with the alkylating reagents, this result confirmed the presence of Cys127 as a free Cys in Fuc-9.

Third, the crude digest was reduced with DTT, alkylated with the mixture of unlabeled and deuterium labeled acrylamide, and directly analysed by MALDI-TOF MS. Figure 10 shows the MALDI-TOF spectra corresponding to the reduced and alkylated sample. We observed fragments T5 + Acr/Acr* [m/z $(M+H)^+$ 1589.8 Da, 1592.8 Da] and T16 + Acr/Acr* [m/z $(M+H)^+$ 1077.4 Da, 1080.4 Da]. T5 and T16 are generated from the reduction of the disulfide

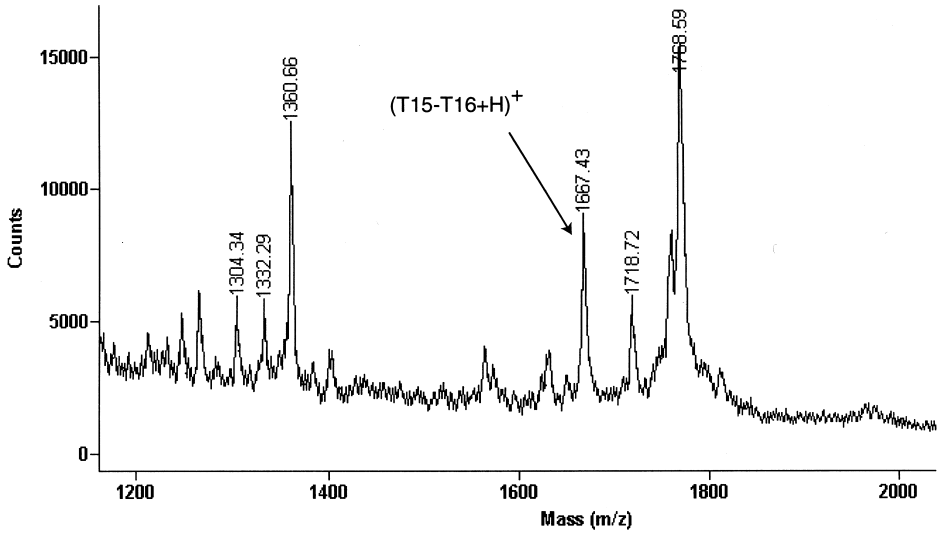

Figure 8 Region of a MALDI-TOF spectrum showing the experimental m/z $(M + H)^+$ of 1667.4 Da obtained for the second pair of peptides linked by a disulfide bond (T15–T16).

linked peptides T5–T9 and T15–T16, respectively. The isotopic distribution signature in each fragment gives evidence to their Cys content. The two lower molecular weight peptides [T9 (theoretical m/z 659.3 Da) and T15 (theoretical m/z 665.3 Da)], generated from the reduction of fragments T5–T9 and T15–T16, could only be detected by MALDI-TOF MS after HPLC separation, and subsequent collection of fractions. Their identity was confirmed by their isotopic distribution signature as well as by post source decay (PSD) [14]. As is shown in Figure 11, instead of the theoretical $(M + Acr/Acr^* + H)^+$ (m/z 736.4/739.4 Da), corresponding to fragment T15, the signal detected by MALDI-TOF MS was $(M + Acr/Acr^* + H\text{-}NH_2)^+$ (m/z 721.9/724.8 Da). Any doubt about the identity of this peak could be dispelled by looking at its isotopic distribution as well as applying PSD [a derived ion T15 at m/z $(M + H)^+$ 665.8 Da could be detected by PSD].

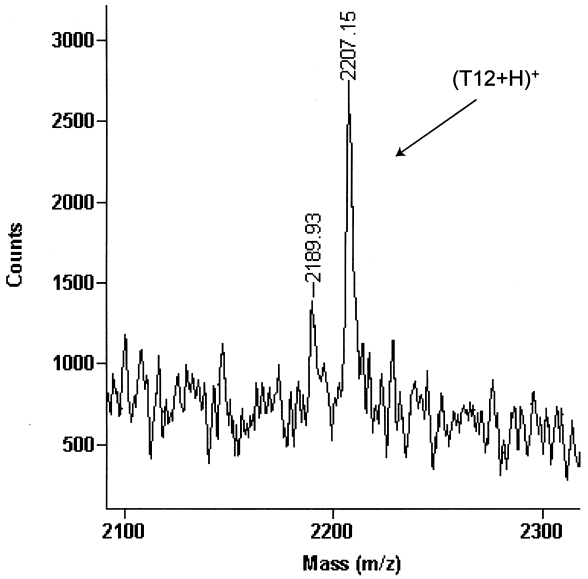

Figure 9 Region of a MALDI-TOF spectrum showing the experimental m/z $(M + H)^+$ of 2207.2 Da obtained for the free Cys containing peptide (T12).

CONCLUSIONS

We conclude that the disulfide pairings in the recombinant mature form of pea α-L-fucosidase are Cys64-Cys109 and Cys162-Cys169. Therefore, the *in vitro* folding of the Fuc-9 enzyme resulted in the same general covalent topology that was obtained for the proenzyme.

We have shown that even for large (>20 kDa) proteins, extensive HPLC separation is not an absolute requirement for elucidating, by MALDI-TOF MS, the disulfide topology of the protein. This result is in contrast with the well-established procedures of using HPLC-ESI-MS of proteolytic fragments. This last approach had been applied successfully in our laboratory for locating the disulfide bonds in the α-L-fucosidase proenzyme [3], but it is a much more tedious procedure that the one described in this work.

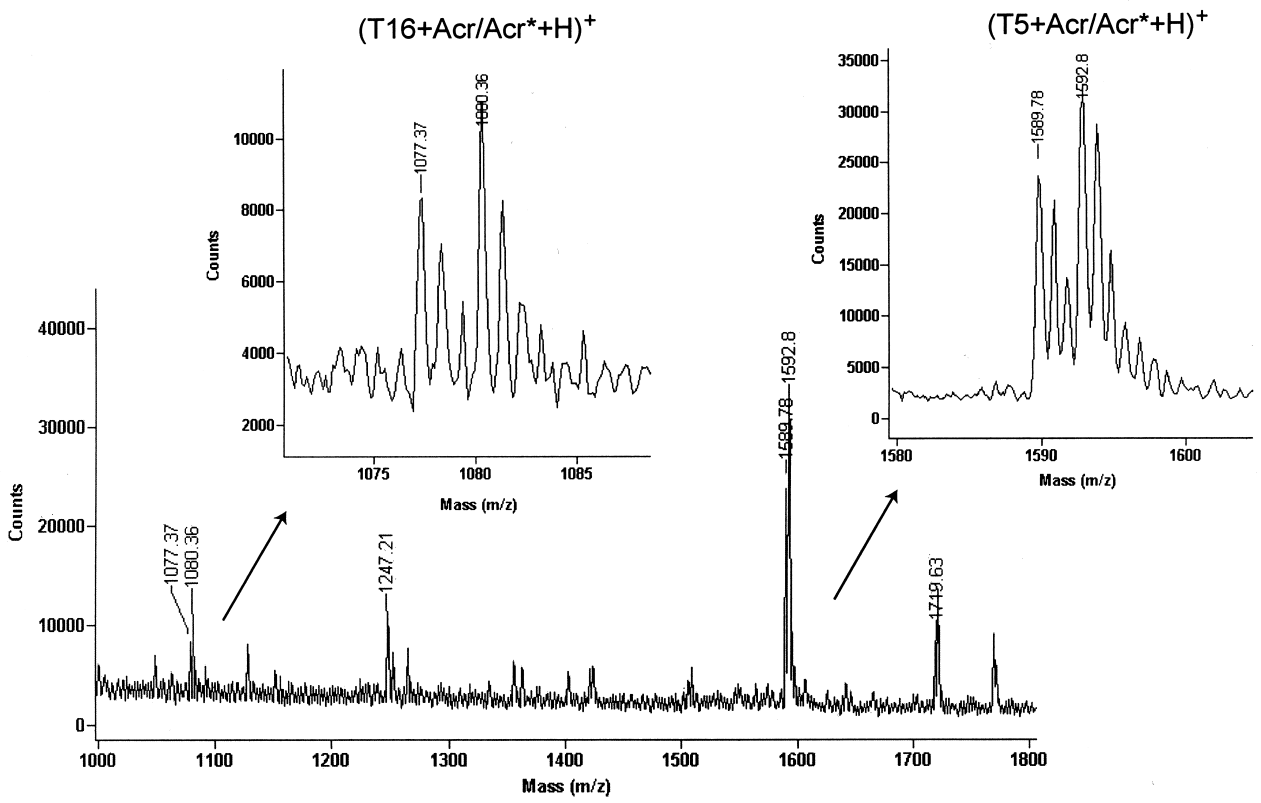

Figure 10 MALDI-TOF spectrum showing the experimental m/z $(M + H + Acr/Acr^*)^+$ of 1589.8/1592.8 and 1077.4/1080.4 Da obtained for the Cys containing peptides T5 and T16, respectively. It shows the difference in m/z (3 Da) between peptides derivatizated with acrylamide and those that reacted with deuterium labeled acrylamide.

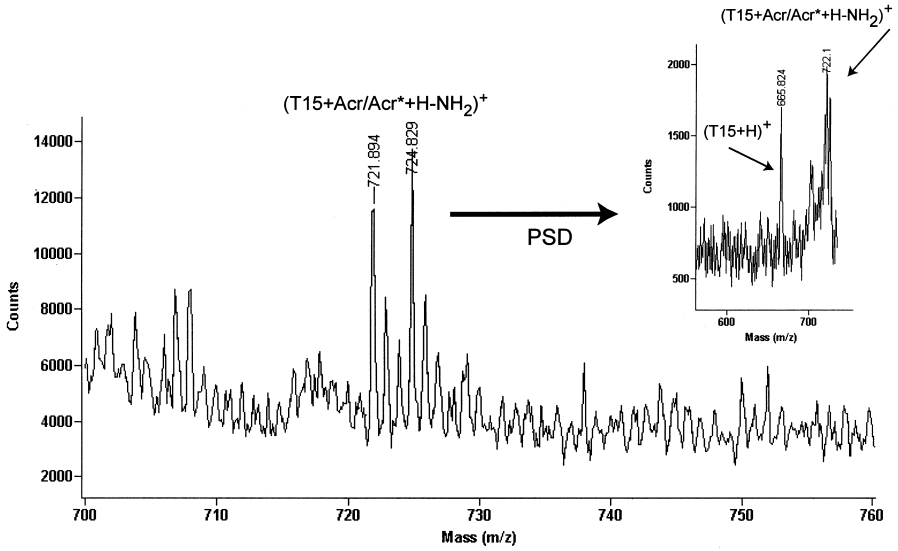

Figure 11 MALDI-TOF spectrum showing the experimental m/z $(M + Acr/Acr^* + H\text{-}NH_2)^+$ of 721.9/724.8 Da obtained for alkylated peptide T15. 665.8 Da is the m/z $(M + H)^+$ of this peptide after carrying on PSD of 721.9/724.8 Da m/z.

From an experimental point of view, we have shown that crude peptides could be directly analysed by MALDI-TOF MS with minimal clean-up using Zip-Tips C_{18}, and with sample concentrations of 4 pmol/μL. In exceptional cases, we have solved the lack of sensitivity owing to ion suppression in MALDI-TOF MS by analysing peptide digest mixtures by a partial separation of the peptides by HPLC, collection of fractions, and subsequent analysis by MALDI-TOF MS. Finally, one important advantage of our approach is that, in several instances, it provides redundant information. These redundancies can be considered as a cross-check to increase the reliability of the results.

Acknowledgements

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NOTES

1. In the case of the protein subjected to NTCB or 4-vinyl pyridine procedures, the crude mixtures were washed up with 0.01% NH_4OH to remove excess of NTCB and other salts.

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