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Mass spectrometric analysis reveals a cysteine bridge between residues 2 and 61 of the auxin-binding protein 1 from *Zea mays* L.

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Abstract The major auxin-binding protein (ZmERabp1) from maize (*Zea mays* L.) has been structurally characterized. We determined the position of a disulfide bridge in ZmERabp1 by mass-spectrometric analysis. We show that Cys2 and Cys61 are covalently linked and that residue Cys155 bears the free sulfhydryl group. By making use of electrospray mass spectrometry, the molecular mass of ZmERabp1 was determined to be 20 243 Da comprising a sugar moiety of 1865 Da, corresponding to a high mannose-type glycan structure. Due to the high homology among all characterized ABPs, the information on the disulfide bonds will be important for functional analysis of recombinantly expressed ABP1. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Auxin-binding protein 1; Cysteine bridge; Maize; Electrospray mass spectrometry; Mass spectrometry

1. Introduction

The plant hormone auxin mediates a wide range of responses during plant life, but its molecular mechanisms of action and its site(s) of perception remain to be determined [1]. So far the only candidate for an auxin receptor is the auxin-binding protein 1 (ABP1), a protein originally isolated from maize coleoptiles [2–5]. Numerous homologs were identified in other plant species including tobacco, strawberry and *Arabidopsis* [6–8]. ABP1 is an interesting but puzzling protein. It is predominantly localized in the endoplasmic reticulum (ER), although its putative place of action was shown at the plasma membrane by electrophysiological methods [9–12]. Based on physiological evidence, it has been suggested that this protein has a receptor function. Anti-ABP antibodies block auxin-induced hyperpolarization of tobacco protoplasts probably by disturbing auxin binding or auxin-induced conformational changes [8,13]. Conversely, antibodies directed against a putative auxin-binding domain mimic auxin responses and induce membrane hyperpolarization [9,14–16]. Genetic and molecular-physiological approaches showed a role of ABP1 in auxin-induced cell expansion in embryos and protoplasts [17–19]. Nevertheless, to elucidate the way of auxin signal transduction, the perception mechanisms as well as downstream acting elements have yet to be identified.

Although ABP1 from *Zea mays* (ZmERabp1) has been isolated more than 10 years ago, only limited structural information on this protein has become available [15,16]. It is known that the protein contains three cysteine residues which are located at strictly conserved positions in every known ABP homolog. Although ZmERabp1 forms a homodimer, under native conditions it is assumed that no intermolecular disulfide bridge exist [2]. For future biochemical studies, e.g. refolding of recombinant ABP1 proteins or elucidation of its crystal structure, it is essential to determine the residues involved in disulfide bridge formation.

Already in 1977 it was demonstrated that specific binding of 1-naphthylacetic acid to maize microsomes was decreased by dithiothreitol, pointing to the presence of a reducible group [20]. Recently Leblanc et al. speculated about the presence of a disulfide bridge in tobacco (*Nicotiana tabacum*) ABP1 (NtERabp1) between Cys61 and Cys155 [21]. Their results were based on epitope mapping using monoclonal antibodies against peptides of NtERabp1 on recombinant NtERabp1. Applying mass spectrometric analysis, we directly determined the location of the putative disulfide bridge in ZmERabp1.

2. Materials and methods

2.1. Purification of ZmERabp1

Maize seeds (*Z. mays*, var. Mutin) were soaked in water overnight and then allowed to germinate on moist tissue paper for 3 days at 28°C. Coleoptiles, including the primary leaf, were harvested, frozen in liquid nitrogen and stored at −70°C. All following steps were performed at 4°C. Using a Polytron homogenizer at maximum speed, coleoptiles were grinded in buffer STC containing 250 mM sucrose, 100 mM Tris–citric acid, pH 8.0, 1 mM EDTA, 0.1 mM $MgCl_2$, 10 mM ascorbic acid, 4 mM diethylthiocarbamic acid, 3.5 mg/l trasylol and 100 μg/l leupeptin. After filtration through a nylon net (135 μm), the insoluble material was re-extracted in STC (1:1 v/v) and the combined homogenate centrifuged for 30 min at $6000\times g$. Membranes were precipitated with $CaCl_2$ at a final concentration of 15 mM and centrifuged for 20 min at $10\,000\times g$. The pellet was homogenized in buffer A (2 mM K_2HPO_4, 0.5 mM EGTA, pH 8.0) and the same volume *n*-butanol was added. After stirring for 20 min, the two phases were separated by centrifugation for 20 min at $2000\times g$. The lower aqueous phase was dialyzed overnight against buffer B containing 10 mM sodium citrate, pH 6.5, 5 mM $MgCl_2$ and centrifuged for 30 min at $20\,000\times g$. The supernatant was applied onto a DEAE Sepharose fast flow column (Pharmacia) equilibrated with buffer B. After washing the column with buffer B, the ABP1-containing fraction was eluted with 250 mM NaCl in buffer B. The eluate was dialyzed overnight against buffer C (10 mM sodium citrate, pH 6.0, 5 mM $MgCl_2$) and applied onto an affinity column consisting of 4-hydroxyphenyl-

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Abbreviations: ABP, auxin-binding protein; ER, endoplasmic reticulum; ESI, electrospray ionization; ES-MS, electrospray mass spectrometry; LC, liquid chromatography; MS/MS, tandem mass spectrometry; Nt, *Nicotiana tabacum*; Zm, *Zea mays*

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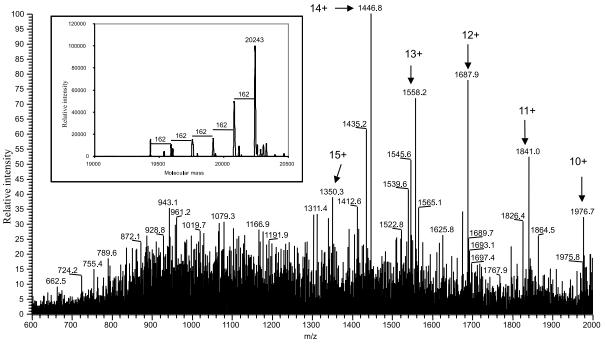

Fig. 1. ESI-MS analysis of purified ZmERabp1. The ZmERabp1 protein was purified to homogeneity and subjected to mass spectrometrical analysis. The numbers above the peaks represent the numbers of charges of the molecule. The insert shows the calculated molecular mass of ZmERabp1 after deconvolution. Besides the fully glycosylated protein, several glycoforms differing from the high mannose type by one or more sugar residues (Δ 162) can be detected.

acetic acid coupled to epoxy-activated Sepharose 6B (Pharmacia). The column was washed with buffer C and eluted with 50 mM NH_4HCO_3, pH 9.0. The eluate was lyophilized and stored at −20°C.

2.2. Alkylation

Lyophilized ZmERabp1 (100 μg) was dissolved in 0.3 ml 200 mM triethylamine/acetic acid, pH 10, 50% isopropanol (v/v), 4 M urea, to give a protein concentration of 1.67×10^{-5} M. To this solution 4-vinylpyridine was added to a 10^3 molar excess compared to three possible free sulfhydryls. The reaction mixture was incubated for 30 min at 37°C followed by an extensive dialysis against 50 mM NH_4HCO_3, pH 8.5. Urea and 1 M Tris/HCl, pH 8.6, were added to the protein solution to give a final concentration of 8 and 0.1 M respectively. Mercaptoethanol (14.3 μM) was added and after 1 h at room temperature (22–25°C) the solution was incubated with iodoacetamide (14.3 μM) for 30 min. By using an equimolar amount of iodoacetamide and mercaptoethanol, side alkylation reactions were kept to a minimum. After extensive dialysis against 50 mM NH_4HCO_3, pH 8.5, the sample was lyophilized and stored at −20°C.

2.3. Tryptic digestion

Lyophilized ZmERabp1, underivatized and derivatized, respectively, was dissolved in 50 mM NH_4HCO_3, pH 8.5, to give a final concentration of 50–100 μg/ml. Trypsin was added for a trypsin/ABP1 mass ratio of approximately 1:30. The digestion reactions were incubated overnight at 37°C, lyophilized and stored at −20°C.

2.4. Electrospray mass spectrometry (ES-MS) analysis of ZmERabp1 protein

Lyophilized ZmERabp1 (100 μg) was dissolved in solvent A (1% formic acid in water). The solution was directly injected into the mass spectrometer (2 μl/min) by infusion using a solvent flow of 60 μl/min (30% methanol in water with 1% formic acid). ES-MS was performed using a *ThermoQuest LCQ Classic* (Thermo Quest) equipped with electrospray ionization (ESI) source. The ion spray voltage was 4.2 kV. Fragments were assigned according to their molecular masses.

2.5. Liquid chromatography (LC)/ES-MS and tandem mass spectrometry (MS/MS) analysis of ZmERabp1 tryptic peptides

Lyophilized tryptic digest of ZmERabp1 was dissolved in solvent A (5% acetonitril in water containing 0.025% TFA). The peptides were separated on a BDS-Hypersil reversed phase C18 column (100×2.1 mm) and eluted with a linear gradient from 0 to 50% solvent B (5% water in 95% acetonitril containing 0.025% TFA) in solvent A within 60 min (0.3 ml/min). ES-MS was performed using a *ThermoQuest LCQ Classic* (Thermo Quest) equipped with ESI source by flow injection (0.3 ml/min). The ion spray voltage was 3.3 kV. Fragments were assigned according to their molecular masses. The MS/MS analysis was performed in a dependent scan, i.e. the most intensive ion was fragmented automatically.

2.6. Software

Mass spectra were analyzed using *Navigator* ver. 1.1 (Thermo Quest) and *BioMultiView* ver. 1.1.1. (Perkin Elmer). The fragment calculation was done with *GPMAW* ver. 3.13 (Lighthouse data).

Table 1
Theoretical and measured molecular masses of ZmERabp1

	Theoretical (Da)	Experimental data (Da)
ZmERabp1 polypeptide, reduced	18 379.86	
ZmERabp1 polypeptide, S–S bridge	18 377.84	
High mannose sugar ($GlcNAc_2Man_9$)	1 865.67	
Glycoprotein, reduced	20 245.53	
Glycoprotein, S–S bridge	20 243.51	20 243

The theoretical values were calculated from the deduced *Z. mays* amino acid sequence and the sugar moiety of the high mannose sugar type ($GlcNAc_2Man_9$).

3. Results and discussion

ZmERabp1 was purified to homogeneity and subjected to mass spectrometric analysis.

3.1. ES-MS analysis of ZmERabp1 protein

Isolated ZmERabp1 was analyzed by ES-MS. Several multiple charged molecules ($[M+10H^+]^{10+}$ to $[M+15H^+]^{15+}$) were detected (Fig. 1). From this spectrum, the molecular mass of

Table 2
Theoretical molecular masses predicted from the deduced *Z. mays* sequence for all possible tryptic fragments and the observed undigested fragment T11/12 as well as the disulfide-linked peptide T1/6.

Peptide	$[M+H]^+$	Sequence
T1	464.57	SCVR
T2	703.77	DNSLVR
T3	3043.45	DISQMPQSSYGIEGLSHITVAGALNHGMK
T4	1642.85	EVEVWLQTISPGQR
T5	623.73	TPIHR
T6	1292.50	HSCEEVFTVLK
T7	204.25	GK
T8	1094.32	GTLLMGSSSLK
T9	5431.04	YPGQPQEIPFFQNTTFSIPVNDPHQVWNSDEHEDLQVLVIISRPPAK
T9-Glyc	7297.71	
T10	2008.35	IFLYDDWSMPHTAAVLK
T11	1704.90	FPFVWDEDCFEAAK
T12	376.39	DEL
T11/12	2062.29	FPFVWDEDCFEAAKDEL
T1/T6	1754.04	SCVR-HSCEEVFTVLK

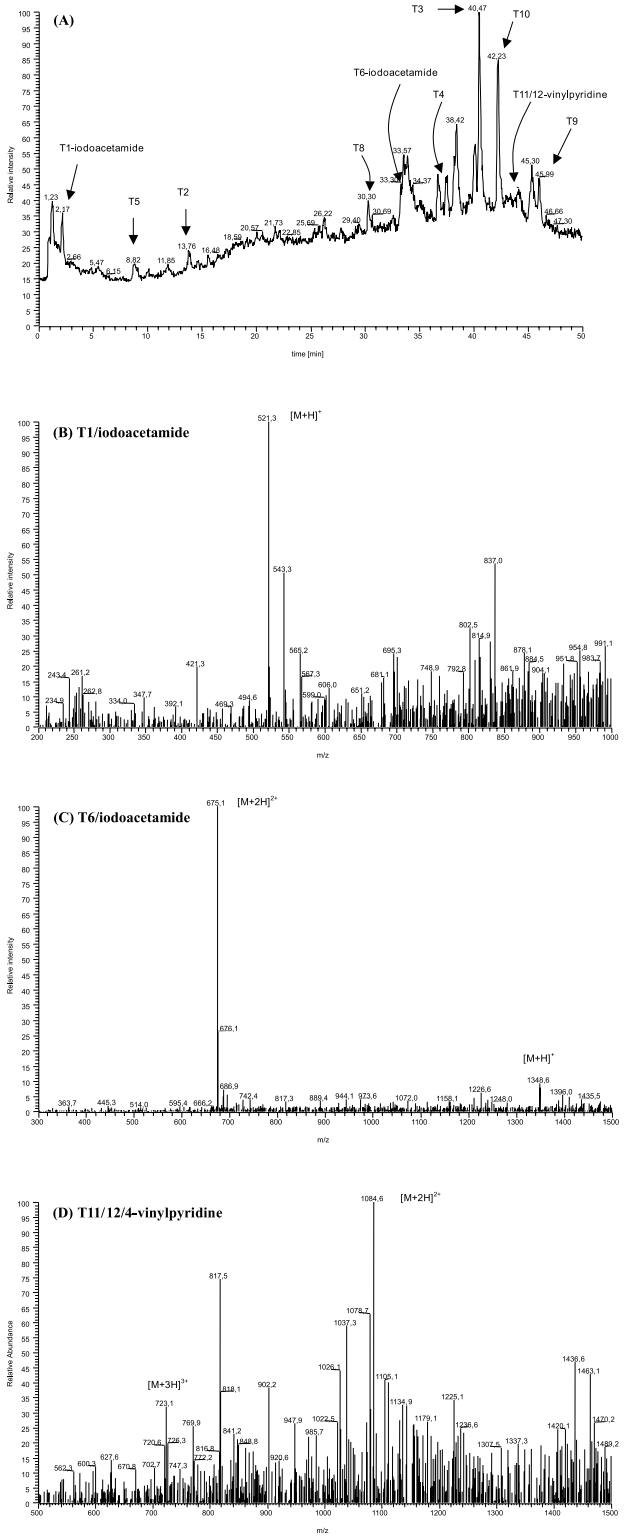

Fig. 2. ESI-MS analysis of a tryptic digest of specifically labeled ZmERabp1. The ZmERabp1 protein was purified to homogeneity and free sulfhydryls were derivatized with 4-vinylpyridine. After reduction, the protein was labeled with iodoacetamide. The tryptic digest was separated on HPLC and analyzed by MS. A chromatographic trace detected as total ion current (TIC) is shown in panel A. The MS scans of the derivatized peptides T1/iodoacetamide, T6/iodoacetamide and T11/12/4-vinylpyridine are shown in panels B, C and D, respectively.

the protein was calculated to 20 243 Da including the glycan residue (insert Fig. 1). The theoretical value of the molecular mass can be calculated based on the deduced amino acid sequence to 18 379.86 Da for the reduced form and to 18 377.84 Da assuming the presence of an intramolecular disulfide bridge. To determine the molecular mass of the glycan residue, the high mannose-type sugar predicted from earlier results was considered ($GlcNAc_2Man_9$, 1865.6 Da) [4]. The calculated mass of the oxidized form fits better (Δ 0.5 Da) with the measured value than the reduced form (Δ 2.5 Da) (Table 1). Therefore, already the determination of the molecular mass implies the presence of a disulfide bridge.

ZmERabp1 was not dimerized via an intermolecular disulfide bridge but formed a monomer under the conditions used in this analysis (compare to [2]). Additionally, the existence of a high mannose-type sugar was proven in accordance with earlier studies [4].

Five other significant molecular species are observed between 19 400 and 20 100 Da. These signals correspond to the presence of several glycoforms of ZmERabp1 (Δ 162 = mannose). These different glycosylation species reveal that at least a small portion of ZmERabp1 differs from the predicted high mannose type. Typically, glycan residues are modified in the Golgi giving support to earlier suggestions that ABP1 is entering this compartment probably on its way to the plasma membrane (reviewed in [22]).

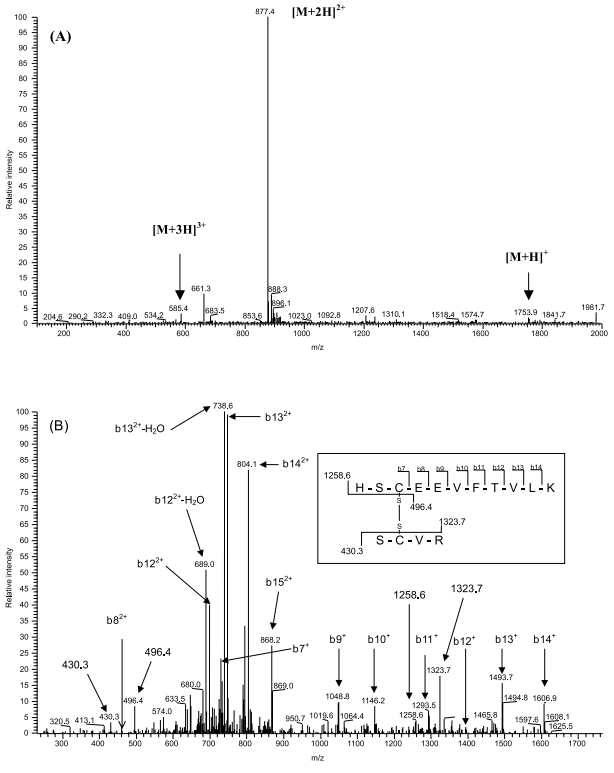

Fig. 3. ESI-MS and MS/MS analyses of disulfide-linked peptides T1 and T6. The ZmERabp1 protein was purified to homogeneity and subjected to tryptic digestion. The peptides were separated on HPLC and analyzed by ESI-MS. Panel A shows the single MS analysis of T1/T6. In panel B, the MS/MS scan depicts the fragmentation of T1/T6 which is schematically presented in the insert. The b-fragmentation series (b14-b7) proves the identity of the linked peptides.

Table 3
Molecular masses of tryptic fragments of ZmERabp1

Peptides	$[M+H]^+$ theoretical	$[M+H]^+$ found	Reduction time (min)	Remarks
T1	464.57	521.3	2.01–2.24	labeled with iodoacetamide (Δ 57)
T2	703.77	703.4	13.7–13.84	
T3	3043.45	3042.7	40.42–40.56	
T4	1642.85	1641.8	37.36–37.56	
T5	623.73	623.5	8.68–8.91	
T6	1292.50	1348.6	33.19–33.33	labeled with iodoacetamide (Δ 57)
T7	204.25	–	–	dipeptide, too small for detection
T8	1094.32	1093.5	30.23–30.33	
T9	7297.71	7296.6	45.92–46.09	high mannose type ($GlcNAc_2Man_9$)
T10	2008.35	2007.6	42.14–42.29	
T11/12	2062.29	2168.2	43.49–44.10	T11/12 labeled with 4-vinylpyridine (Δ 105)

The isolated protein was alkylated with 4-vinylpyridine (Δ 105) to label the free cysteine residue. Reduction was followed by derivatization with iodoacetamide (Δ 57) and tryptic digestion. After separation on HPLC the peptides were investigated by MS.

3.2. LC/ES-MS analysis of ZmERabp1 tryptic peptides

Firstly, the cysteine residues were specifically labeled according to their redox state: ZmERabp1 was subjected to alkylation of putative free sulfhydryls with 4-vinylpyridine. After reduction, disulfide-linked cysteines were tagged with iodoacetamide prior to tryptic digestion. The identity of peptides was assigned by comparison of the observed molecular masses to the theoretical values predicted from the deduced *Z. mays* amino acid sequence for all possible tryptic fragments (Table 2). The spectra of the peptides are presented in Fig. 2 and summarized in Table 3. In all studies we observed an incomplete digestion between peptides T11 and T12 resulting in a larger peptide of 2061 Da. Due to its small size, tryptic fragment T7 (204.25 Da) was not detected in all measurements because of short retention time on the high performance (HP)LC (Table 4).

The mass spectrometrical analysis revealed that the tryptic fragment T11/12 carried the 4-vinylpyridine residue resulting in a mass shift of 105 Da. This finding demonstrates that Cys155 is the only free sulfhydryl residue in ZmERabp1. In line with this observation was the finding that the tryptic peptides T1 and T6 showed a mass shift of 57 Da due to the covalently linked iodoacetamide residue.

Other possible species, i.e. peptides differing in the described derivatization pattern could not be found. Therefore, it is unlikely that a disulfide shuffling occurred during isolation which can occur at high pH.

To confirm the localization of the disulfide bridge between Cys2 and Cys61, tryptic digests obtained from isolated ZmERabp1 were analyzed by LC/ES-MS. We observed that the combined tryptic fragments T1 and T6 had a molecular mass of 1753.7 Da clearly demonstrating linkage via a disulfide bridge (Fig. 3A). No other putative dipeptide (T1/T11/12, T6/T11/12) reflecting another putative disulfide bridge between Cys2 and Cys155 or Cys61 and Cys155, respectively, could be detected. In an MS/MS experiment of the tryptic digest, all peptides theoretically predicted could be identified by fragmentation except fragment T11/T12 or T11 or T12 alone (data not shown). As a confirmation, the MS/MS scan of the cysteine-linked peptide T1/T6 is depicted in Fig. 3B. The fragmentation data are summarized in Table 5 giving direct proof for the presence of a disulfide bridge between Cys2 and Cys61. The nature of the covalent bond was proven by detection of two fragment pairs (*m/z* 1258.6 and 496.4 as well as 1323.7 and 430.3) typically found for disulfide-linked peptides.

In the case of ABP1, early experiments indicate that a disulfide bridge is necessary for auxin binding [20]. The finding of a precisely localized disulfide bond within ABP1 could indicate either an extracellular or a compartimented residence for ABP1. The analysis of proteins having one or more intracellular disulfide bridge typically points to an extracellular localization [23]. Based on the results presented here, the following conclusions can be drawn:

1. Due to the high homology and the conservation of all three cysteine residues, a general structural role for these residues in all ABP1s is likely.
2. In refolding experiments of any recombinantly expressed ABP1 species, the correct disulfide linkage has to be carefully checked since this represents a prerequisite for correct protein folding.

Table 4
Molecular masses of tryptic fragments of ZmERabp1

Peptide	$[M+H]^+$ theoretical	$[M+H]^+$ found	RT (min)	Remarks
T1/T6	1754.07	1753.7	22.02–22.11	T1 linked to T6 via disulfide bridge
T2	703.77	703.4	9.63–9.98	
T3	3043.45	3043.4	31.57–31.83	
T4	1642.85	1641.8	28.71–29.06	
T5	623.73	623.4	4.71–4.97	
T7	204.25	–	–	dipeptide, too small for detection
T8	1094.32	1093.5	22.21–23.08	
T9	7297.71	7295.4	37.79–37.88	confirmation of high mannose type ($GlcNAc_2Man_9$)
T10	2008.35	2007.8	33.5–33.75	
T11	1704.90	–	–	not detected
T12	376.39	–	–	not detected

Isolated ZmERabp1 was digested with trypsin, separated on HPLC and investigated by MS.

Table 5
Tandem mass spectrometrical analysis of the disulfide-linked peptides T1/T6

Sequence		$[M+H]^+$ / $[M+2H]^{2+}$ theor.	$[M+H]^+$ / $[M+2H]^{2+}$ det.
SCVR | HSCEEVFTVLK	T1/T6	1754.0 877.5	1753.9 877.4
SCVR | HSCEEVFTVLK	b_{15} b_{15}^{2+}	1736.0 868.5	n.d. 868.2
SCVR | HSCEEVFTVL	b_{14} b_{14}^{2+}	1607.9 804.4	1606.9 804.1
SCVR | HSCEEVFTV	b_{13} b_{13}^{2+}	1494.7 747.9	1493.7 747.6
SCVR | HSCEEVFT	b_{12} b_{12}^{2+}	1395.6 698.3	1394.4 698.0
SCVR | HSCEEVF	b_{11} b_{11}^{2+}	1294.5 647.7	1293.5 647.9
SCVR | HSCEEV	b_{10} b_{10}^{2+}	1147.3 574.1	1146.2 574.0
SCVR | HSCEE	b_9 b_9^{2+}	1048.1 524.6	1048.8 n.d.
SCVR | HSCE	b_8 b_8^{2+}	919.0 460.0	919.3 460.4
SCVR | HSC	b_7 b_7^{2+}	789.9 395.5	789.9 n.d.
SCVR (– H_2S)		430.5	430.3
SCVR | SSH		496.6	496.4
HSCEEVFTVLK (– H_2S)		1257.5	1258.6
SSH | HSCEEVFTVLK		1322.6	1323.7

Depicted is the b-fragmentation series (b15-b7) as well as the cleavage at the thioether bond. theor.: theoretical; det.: determined; n.d.: not detected.

3. It is very likely that ABP1 is either localized in a intracellular compartment (ER-lumen, vacuole) or at the plasma membrane due to the reducing environment of the cytosol.
4. In general, free sulfhydryls are very reactive and sensitive to oxidation. Very likely, the C-terminus with its free cysteine residue is protected within the protein structure or within the protein structure of other interacting partner proteins.

A putative role for ABP1 in auxin signal transduction has been proposed, but remains to be elucidated. Until now the only way to study the correct folding status of heterologous expressed ABP1 was the use of auxin-binding assays. The position of the intramolecular disulfide bridge gives another valuable information about the native structure of ABP1. Therefore, our results presented here will be important for further functional studies of recombinantly expressed ABP1.

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