**Collagenase as a useful tool for the analysis of plant cellular peripheries**

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**Abstract**

A technique for the selective loosening of the cell wall structure and the isolation of proteins permanently knotted in the cell walls was elaborated. Following treatment with collagenase, some proteins, such as calreticulin (CRT) and auxin binding protein 1 (ABP1) were released from purified cell walls, most probably through destruction of respective interacting proteins. The results were confirmed by the immunolocalization of the ABP1 and CRT with confocal and electron microscopy. On the other hand, potential substrates of collagenase, among them annexin 1 have been recognized. Mass spectra of annexin 1 obtained after collagenase digestion and results from analysis of potential cleavage sites suggested that the mechanism of enzyme cleavage might not depend on the amino acid sequence. Summarizing, collagenase was found to be a very useful tool for exploring molecules involved in the functioning of cellular peripheries.

**Keywords:** collagenase, cell wall, calreticulin, auxin-binding protein 1, annexin 1

# 1. Introduction

A technique for the selective loosening of the cell wall structure and the isolation of proteins permanently knotted in the cell walls was elaborated. Following treatment with collagenase, some proteins, such as calreticulin (CRT) and auxin binding protein 1 (ABP1) were released from purified cell walls, most probably through destruction of respective interacting proteins. The results were confirmed by the immunolocalization of the ABP1 and CRT with confocal and electron microscopy. On the other hand, potential substrates of collagenase, among them annexin 1 have been recognized. Mass spectra of annexin 1 obtained after collagenase digestion and results from analysis of potential cleavage sites suggested that the mechanism of enzyme cleavage might not depend on the amino acid sequence. Summarizing, collagenase was found to be a very useful tool for exploring molecules involved in the functioning of cellular peripheries. medium of cultured cells. The second set constitutes proteins that are entangled in the CW networks with ionic bonds. These can be extracted from the walls with salts. Both groups were recently reviewed (Albenne et al., 2013; Jamet et al., 2008; Rose and Lee, 2010). The third group comprises proteins that are more permanently associated with the walls either via covalent bonds or ‘‘caged’’ within the polysaccharide scaffold. These are usually poorly characterized due to their oxidative cross-linking under stress conditions, poor extractability and the need for special extraction methodologies.

The major assumption here is that some of the apoplastic proteins could be released from the walls by the action of collagenase. A previously developed methodology for selective digestion of various wall components (Wojtaszek et al., 2005, 2007) was used here to test this assumption. Collagenase, an enzyme not occurring and with no potential substrate in plants has been selected for such purpose on the basis of rare and scattered data. It was shown that exogenous application of collagenase inhibited cytoplasmic streaming in Vallisneria cells (Masuda et al., 1991), and disrupted graviresponses of *Chara corallina* cells (Wayne et al., 1992). Moreover, in some proteins, like wall-associated kinases 2 and 4, extracellular domains similar to collagen have been found (Anderson et al., 2001; He et al., 1996, 1999). It was also suggested that glycine-rich proteins (GRP) could be specifically cleaved by collagenase (Ringli et al., 2001). Here, we have utilized collagenase as a very useful tool for exploring molecules located at the cellular peripheries.

**2. Results**

**2.1. Cell wall proteins non-covalently associated with apoplastic compartments of plants and suspension-cultured cells**

As mentioned above, three types of CW proteins can be distinguished on the basis of their interactions with cell wall components (Jamet et al., 2008). Since collagenase digestion was used after isolation of non-covalently-bound proteins, in the first stage we wanted to know exactly which proteins comprise this group of proteins. In this way, we excluded the possibility of contamination of the covalently-bound protein group by the non-covalently bound proteins, and provided a reference for further experimental stages of this study. Non-covalently-bound proteins were extracted from type I (white lupine) and type II (maize) plant cell walls. Despite the lack of genomic data for lupine, the choice of this species is substantiated by the long history of research on lupine in our lab, and the availability of all the optimized protocols necessary to carry out this research.

In addition, proteins secreted into medium and CW proteins of the primary walls of Arabidopsis suspension-cultured cells were analysed. Respective protein fractions were separated electrophoretically, and the acquired protein profiles were compared. Selected apoplastic proteins were identified using mass spectrometry. As a result, a set of proteomic maps of the CW proteins was created revealing the qualitative and quantitative differences between them. In total, 59 CW proteins were identified and these are listed in Supplementary Data 1. An example of the 2D PAGE of the CW proteins that were extracted from the white lupin roots is also presented in Supplementary Data 1. This set of data was used to analyse the predictive potential of the available bioinformatics tools. Only 39% of predictions obtained with PSORT and Plant-PLoc servers were compatible with experimental data. These assessments are in agreement with our previous observations (Łuczak et al., 2009). Also others indicate that in silico approaches do not prove the extracellular location of proteins due to existence of unconventional protein secretion system (Ding et al., 2012; Jamet et al., 2008).

The 2DE proteomic maps obtained for the Arabidopsis cultured cells resolved the highest number of proteins, i.e. circa 65 proteins secreted into culture medium and approximately 110 proteins extracted from the walls. Twenty-eight of those proteins were identified (Supplementary Data 1). All of the identified proteins could be classified into three categories: (1) proteins that participate in the wall assembly and rearrangements, such as endoglucanases, exoglucanases, glucosidases, pectin esterases, expansin, xylanase inhibitors and hydrolases; (2) proteins related to stress response and defence, such as chitinases, oxalic acid oxidase, polygalacturonase inhibitor proteins, PR10 proteins, peroxidases, extracellular dermal glycoprotein (EDGP), HSP70, HSP90, berberine bridge enzyme-like protein and lectin-like protein; and (3) proteins that participate in signal transduction, such as subtilisin-like serine proteases, and leucine-rich repeat (LRR) proteins. Some of the identified proteins could be assigned to more than one category.

**2.2. Application of collagenase for studies of proteins from type I cell walls of white lupine**

It is normally assumed that via salt extraction one can isolate proteins that are associated with cell walls through non-covalent interactions. Another set of wall proteins can be isolated through either disruption of polysaccharide chains or polysaccharide interactions or through more or less selective digestion of peptidebonds. In this study collagenase was used to selectively digest wall proteins leading to the loosening of wall structure. This treatment shall release new set of wall proteins/peptides, most probably permanently embedded in the polysaccharide matrix. Purified white lupine cell walls used previously for salt extraction of CW proteins were subjected to collagenase digestion. Among many collagenase types commercially available, three types, namely IV, VII and XI, were used for this treatment. Pure buffer was used as a control. Proteins released into the solution were collected, separated by electrophoresis, and compared (Fig. 1). The patterns of protein bands allowed to characterize the level of enzymatic autodegradation. Type VII and XI collagenases were found to be easily degraded through autoproteolytic cleavage, while for type IV collagenase autodegradation occurred to much smaller extent. Similarly, in the latter case quite a few protein bands derived from lupine cell walls could be observed. Therefore, for all the following experiments only collagenase type IV was used.

Although several lupine protein bands were observed in the samples, only two proteins, released from the walls after collagenase digestion, were identified by LC-MS/MS. These were identified as (1) a homolog of calreticulin from *Arabidopsis thaliana* (five peptides) and (2) cp-wap13 from *Vigna unguiculata* (seven peptides) (Fig. 1; Table 1). To confirm the biochemical data, immunodetection with an anti-maize calreticulin (anti-CRT) antibody was performed. Because this protein often co-localizes with auxin binding protein 1 (ABP1; Baluška, personal communication), anti-maize ABP1 antibodies were also used. The results confirmed that both proteins were indeed released from white lupine walls treated with collagenase (Fig. 2a and b). Interestingly, control lanes revealed the presence of weak signals for CRT, but not for ABP1. These results confirmed the detection of a new CRT isoform in the fraction of proteins released by the collagenase which was not observed in the control lane (Fig. 2a).

To finally confirm the results above, three additional controls were also performed. Firstly, to exclude the unspecific binding of antibodies to the oligosaccharide side chains of the CW proteins, protein samples were deglycosylated before electrophoretic separation and immunodetection. Secondly, protein samples were digested with trypsin instead of collagenase. In the first case, anti-CRT and anti-ABP1 antibodies recognized the same protein bands (Fig. 2c and d). In the second, no signal was detected with both antibodies (Fig. 2a and b). As it was demonstrated previously that germin might be very similar to ABP1 (Woo et al., 2002), proteins released from the CW after collagenase treatment were also probed with anti-wheat germin antibodies as an additional control. Collagenase did not release the proteins that were recognized by the latter antibody. The only detected signal was in the control sample not treated with collagenase (Fig. 2e). Finally, CW proteins salt-extracted from lupine walls were also digested with collagenase. In that case, the majority of native proteins were completely resistant to collagenase activity, even after 12 h of digestion. How protein samples were deglycosylated before electrophoretic separation and immunodetection. Secondly, protein samples were digested with trypsin instead of collagenase. In the first case, anti-CRT and anti-ABP1 antibodies recognized the same protein bands (Fig. 2c and d). In the second, no signal was detected with both antibodies (Fig. 2a and b). As it was demonstrated previously that germin might be very similar to ABP1 (Woo et al., 2002), proteins released from the CW after collagenase treatment were also probed with anti-wheat germin antibodies as an additional control. Collagenase did not release the proteins that were recognized by the latter antibody. The only detected signal was in the control sample not treated with collagenase (Fig. 2e). Finally, CW proteins salt-extracted from lupine walls were also digested with collagenase. In that case, the majority of native proteins were completely resistant to collagenase activity, even after 12 h of digestion. However, protein denaturation before enzyme treatment seemed to increase the susceptibility of wall proteins to collagenase (data not shown).

**2.3. Utilization of collagenase to study proteins from type II cell walls of maize**

As the organization of cell walls in grasses is different from that of dicot plants, maize cell walls were used for the similar set of experiments with collagenase digestion. In addition, both antibodies used previously, namely anti-CRT and anti-ABP1 antibodies, were raised against maize proteins, and this allowed to study their specificity in a homologous system. Purified maize walls, after salt extraction of the CW proteins, were digested with collagenase type IV. The treatment released only few proteins (data not shown). Interestingly, anti-ABP1 antibody recognized only one specific protein band (Fig. 3a). No such signal could be seen in the fraction of total maize root proteins or in salt-extracted CW proteins. Analogous experiment with the use of trypsin led also to negative result. On the other hand, the use of anti-CRT antibody did not show such clear-cut results (Fig. 3b). The proteins recognized by anti-CRT were observed in the fraction of total root proteins, in salt extracted CW proteins as well as within the fraction of wall proteins released by collagenase treatment. However, when comparing with controls, some quantitative differences were noted with the intensity of the signals increasing 2.5-fold for proteins released by collagenase. Similarly, as with lupine proteins, digestion of the maize walls with trypsin did not reveal positive signals.

Finally, salt-extracted CW proteins of maize were subjected to collagenase digestion. Similarly as for lupine proteins maize CW proteins were susceptible to collagenase only after denaturation but regardless of the time of digestion (1 or 12 h) (Fig. 4). The protein within the bands that disappeared after collagenase treatment were identified by MS analysis as exoglucanase and glucan endo-1,3-b-glucosidase (Table 2). In lanes after digestion no peptide for both proteins were identified.

**2.4. Use of collagenase in studies of apoplastic proteins from A. thaliana suspension-cultured cells**

Cultured cells of A. thaliana were used to analyse the apoplastic proteins from primary walls. Intact cells or cells after CaCl2 wash were treated with collagenase at two concentrations: 2.5 and 5 g/mL for 4 and 8 h. Following treatment, the cells were reextracted and released CW proteins were analysed with anti-CRT and anti-ABP1 antibodies. In addition, proteins released into culture medium were also treated with collagenase. The results differed from those collected during the lupine and maize experiments (Fig. 5). The proteins recognized by the anti-ABP1 antibodies were found to be both secreted into medium and released from the walls by salt extraction. Collagenase digestion did not result in any additional protein signal. To the contrary, the immunodetection signal in the CW fraction disappeared after 4 h of treatment with 2.5 g/mL collagenase (Fig. 5a). The analogous signal in the culture medium proteins decreased 2-fold after 4 h of treatment with 2.5 g/mL collagenase, and completely disappeared after 8 h of 5 g/mL collagenase treatment (Fig. 5b). Respectively, anti-CRT antibodies recognized proteins both in culture medium and CW fractions (Fig. 5c). Following 2.5 g/mL collagenase digestion, CRT signals in the CW protein fraction decreased 2.2-fold after 4 h and disappeared completely after 8 h of treatment. Digestion with 5 g/mL collagenase led to the disappearance of signals in both the culture medium and CW fractions after 4 h (Fig. 5c and d). These data strongly suggest that the A. thaliana proteins recognized by anti-ABP1 and anti-CRT antibodies could be potential substrates for collagenase. Moreover, contrary to lupin and maize CW, time of collagenase incubation was in this case very important and resulted in obtaining different data.

Both proteins fractions, culture medium and CW proteins, collected before and after the enzyme treatment were electrophoretically separated, either 1D or 2D. The obtained protein profiles confirmed that, when compared with control samples, several protein bands/spots disappeared or decreased after enzyme digestion. These proteins were identified by MS/MS analysis and results are summarized in Table 3. The identified proteins were subjected to bioinformatics analysis as well as database and literature search to obtain more detailed information. Among the proteins secreted into culture medium, the relative abundance of isoforms of peroxidase and acidic endochitinase decreased (fold changes: -1.7 and -1.5, respectively). Analysis of wall proteins revealed more significant changes. When compared with the control, the relative abundance of several proteins was reduced. Most of the identified proteins were involved in the loosening of wall structure, and these included: endo-1,4-b-glucanase 17 (-4.1-fold), endo-1,4-b-glucanase 9 (-3.9-fold), b-glucosidase 15 (-3.2-fold) and a-expansin 4 (-3.7-fold). Two proteins disappeared completely following collagenase digestion: annexin 1 and cobalamin-independent methionine synthase 1. This result was very interesting because both of the proteins were previously identified in the apoplast or CW (Bayer et al., 2006; Bindschedler et al., 2008; Ge et al., 2011) while neither of them has a signal peptide.

**2.5. Specificity of collagenase digestion – a case of annexin 1**

To check if annexin 1 might be a potential substrate for collagenase, annexin 1 was obtained through overexpression in a bacterial system and digested in vitro. The control protein was collagen type IV, denatured prior to digestion. Annexin 1 was susceptible to collagenase digestion. The inhibition of the enzyme activity by EDTA or EGTA, used as inhibitors of metalloproteinases, blocked annexin 1 proteolysis, while serine protease inhibitors (TLCK or BHH) were not effective (Fig. 6). Therefore, we conclude that annexin 1 degradation

is the specific result of collagenase activity.

Utilization of model protein allowed also for the evaluation of potential cleavage sites. To this end collagenase digestion of annexin 1 was analysed using MALDI-TOF mass spectrometry. The mass spectrum of annexin 1 (Fig. 7a) revealed the presence of a 37.0 kDa molecular ion, which disappeared following the application of collagenase. The digestion resulted in large fragments with masses ranging from 6 to 17 kDa (Fig. 7b). As a controls, the spectra showing autodegradation of collagenase type IV were collected (Fig. 7c) and protein samples were digested with trypsin instead of collagenase (Fig. 7d). Collagenase digestion resulted also in small peptides with masses ranging between 800 and 3500 Da (Fig. 7e). Subtraction of those spectra revealed unique fragments specific for annexin 1 (Fig. 7b and e).

List of masses observed on MS spectra after ollagenase cleavage of annexin 1 are presented in Supplementary Data 2. Only fragments observed in all 4 replicates were subjected to approach similar to peptide mass fingerprinting: mass of peptides obtained after digestion were compared with calculated fragments of annexin 1. Calculations were carried out using Sequence Editor 3.2 software (Bruker Daltonics) and no strict rules for digestion were considered. It resulted in a high number of combinations, but among them only one fragment could be matched per given peptide mass. It can thus be concluded that these matches are probable and cleavage sites can be treated as putative sites of enzyme action. Identified fragments covered the annexin 1 sequence in 87.9%. Studying of cleavage sites did not give us a general idea how collagenase digests plant proteins. One cannot exclude that the mechanism of enzyme cleavage depends more on the spatial structure of the substrate than on the amino acid sequence (Ringli et al., 2001). However, we observed that in 13 out of 27 of predicted cleavage sites leucine or isoleucine was present. Masses and sequences of obtained fragments are presented in Table 4. Sequence of annexin 1 mapped with predicted collagenase cleavage sites is presented on Fig. 8.

**2.6. Immunolocalization of proteins recognized and digested by collagenase in A. thaliana cell suspension**

To verify the biochemical and MS data, A. thaliana suspension cultured cells were used for immunofluorescence localization of CRT and ABP1 proteins. The experiments were done on live cells iving the possibility to explore the native localization of those proteins. Therefore, to maintain proper osmotic conditions, all stages of the procedure were conducted in culture medium with constant shaking. The labelling of control cells with either anti-ABP1 or anti-CRT antibodies led to strong extracellular fluorescent signals (Fig. 9a and c), which took more punctate distribution within cell walls. Fluorescent labelling from both antibodies disappeared completely following application of 5 g/mL collagenase (Fig. 9b and d). No fluorescence signal from the CW was observed when primary antibody or primary + secondary antibodies were omitted from the solution (Fig. 9e and f).

Fluorescence labelling was also done using the cells subjected earlier to salt extraction of the CW proteins with 0.2 M CaCl2. In this case only weak extracellular signals from either anti-ABP1 or anti-CRT antibodies were observed (Fig. 10a and b). It should be mentioned, however, that after the application of CaCl2, the cells did not stain with Evans blue. As damaged cells in normal cell culture were also not stained by Evans blue, the physical conditions of the cells were assessed based on cell morphology. For comparative purposes, A. thaliana cells were also subjected to trypsin digestion. Here, application of proteolytic enzyme was not effective as the fluorescent signals from either antibody did not disappear (Fig. 10c and d). In addition, trypsin digestion was also more destructive to the cells. A large number of damaged cells with disturbed CWs was observed, and nearly half of the cells was stained with Evans blue.

Transmission electron microscopy and gold-conjugated antibodies were used to obtain more detailed information on the distribution of ABP1 and CRT. As previously, normal cells and cells subjected to CaCl2 wash were treated with collagenase, fixed and used for TEM analysis. The results confirmed earlier data from the confocal microscopy (Fig. 11). For both proteins, gold particles were found within the walls of Arabidopsis cells (Fig. 11a, b, e and f). The characteristic punctate and very regular arrangement of gold particles traversing the walls, particularly for ABP1, might suggest more localized distribution of the proteins. However, collagenase treatment changed the distribution of ABP1 and CRT dramatically. The enzyme decreased and even abrogated the number of gold particles for both proteins (Fig. 11c,d,g and h).

**3. Discussion**

The existence of a WMC continuum in plant cells was proposed several years ago (Wyatt and Carpita, 1993). However, very few molecules functioning as linkers within the continuum has been identified (reviewed in Baluška et al., 2003; Kasprowicz et al., 2011). Genomic data suggest that plants are devoid of proteins typically involved in the construction of complexes linking the interior of animal cells to extracellular matrix, such as integrins, filamin, or tensin (Hussey et al., 2003). This is rather unexpected situation as the formation and maintenance of WMC continuum seems to be essential for life and proper functioning of the cells (Brownlee, 2002; Ingber, 2003; Wojtaszek, 2001). Among the potential plant linker molecules one can find: wall-associated kinases (Anderson et al., 2001), arabinogalactan proteins, pectins and cellulose synthase (Kohorn, 2000), myosins class VIII (Wojtaszek et al., 2005), callose synthase (Delmer, 1999), lectin-receptor kinases (Gouget et al., 2006), K+ channel KAT1 (Homann et al., 2007), and formin 1 (AtFH1; Martiniere et al., 2011).

The biochemical nature of the WMC connections is not fully understood, and both protein-protein and protein-polysaccharide interactions are indicated. The major reason is methodological as any attempt to isolate potential linkers involves as a first step disruption of at least one element of WMC continuum, usually cell walls. Multi-network nature of cell walls make this issue even more difficult. Proteins that are permanently associated with the walls are the most difficult to isolate. During our previous research, we have elaborated a methodology to study the complexity of internal wall connections. It was based on modified technique for protoplast isolation and utilized various hydrolases to selectively severe cell wall-protoplast anchors (Wojtaszek et al., 2005). It was also used for the isolation of proteins that are strongly embedded in the walls. For example, application of enzymes digesting cellulose, hemicelluloses or pectins allowed for release of new wall proteins (Wojtaszek et al., 2007). For the purpose of this work, the use of another enzyme, collagenase, was optimized. Previous studies have shown that proper functioning of algal cells in Chara corallina (Masuda et al., 1991) and Vallisneria (Wayne et al., 1992) could be affected by collagenase digestion. In both cases, the disturbances evoked suggested that the anchoring of cytoskeleton in the walls was affected. Similar conclusion has been drawn from the studies on the role of myosin VIII in the amyloplast relocation in root cap cells (Wojtaszek et al., 2005). Therefore, we decided to use collagenase to shed light on intramolecular complexity of plant walls. Two possible approaches could be envisioned here. The direct digestion of putative collagenase substrates would lead to the their disappearance from the observed CW protein profile. On the other hand, collagenase digestion of potential substrates could destroy specific protein-protein interactions within the walls. Such indirect action would result in the appearance of new wall proteins (Wojtaszek et al., 2007).

Application of collagenase to various plant substrates led to interesting observations. Collagenase is a highly selective enzyme that under natural conditions recognizes the tertiary structure of proteins and to a lesser extent, the protein sequence (Fields, 1991). This high level of selectivity was demonstrated for the proteins secreted by Arabidopsis cells into culture media. Although all proteins were available to the enzyme, and digestion conditions were also favourable, only few selected proteins were degraded by collagenase (Table 3). Another level of specificity is indicated through comparison of collagenase digestions of plant (lupine, maize) vs. cultured cells material. Here, the differences in the mode of CW penetration by the enzyme could be observed. Plant tissues ould contain secondary cell walls, and therefore the proteins released or digested by collagenase are rather difficult to reach. To the contrary, Arabidopsis suspension cells are surrounded by primary walls, with their half-plasmodesmata open to the culture medium (Bayer et al., 2004), and the enzyme penetrates them relatively easily and quickly, releasing or digesting mainly ionically bound CW proteins. Interestingly, the treatment of cell suspension with enzyme caused visible changes in the content of proteins involved in loosening or remodelling of the wall structure (Table 3). When combined with the notion that the CaCl2 wash used to isolate wall proteins leads at the same time to increased pectin cross-linking, one can conclude that the cells may react to the collagenase treatment by reducing or completely inhibiting the secretion of cell wall modifying proteins. Finally, some of the obtained results suggest that collagenase might be useful for studies of proteins that are difficult to reach in other ways. Collagenase digestion of lupine roots resulted in the release of CRT and cp-wap 13 (Fig. 1; Table 1) for which plasmodesmata localization via relation to ER structures was earlier suggested (Baluška et al., 1999; Chen et al., 2005; Faulkner et al., 2005). Immunodetection experiments confirmed the presence of CRT and ABP1 in a pool of proteins released by the collagenase treatment both from lupine (Fig. 2) and maize (Fig. 3) cell walls. It can thus be concluded that collagenase digested proteins interacting directly with CRT or ABP1 allowing the disruption of bonds holding either protein in the walls. Reextraction of plant material led to their release from the walls. Different results were obtained for the primary walls of Arabidopsis suspension cells. Here, molecules recognized by anti-CRT and anti-ABP1 antibodies were present in the fractions of loosely bound wall proteins, and the collagenase treatment abrogated (Fig. 5a and c) or decreased (Fig. 5b and d) the intensity of the signals. Location of those proteins at cellular peripheries was also confirmed by immunolocalization experiments (Fig. 9). The use of intact living cells helped to avoid a strong fluorescence signal from the ER and revealed only information about extracellular labelling. Moreover, these clear extracellular signals completely disappeared after collagenase treatment but not after trypsin digestion. This conclusion was substantiated by TEM experiments and immunogold labelling (Fig. 11). Although the characteristic distribution of gold particles in specific areas of CW in cells that were not treated with collagenase (Fig. 11a and b) is very suggestive, the nature of those areas needs to be investigated further.

ABP1 was discovered some 40 years ago, but its location and mode of action remained elusive until very recently. It is one of three auxin receptor systems necessary for embryogenesis (Chen et al., 2001) and postembryonic shoot and root development (reviewed by Sauer and Kleine-Vehn, 2011; Scherer, 2011; Tromas et al., 2010). ABP1 regulates PIN-dependent auxin efflux from cells (Cˇ ovanova et al., 2013) as well as mediates effect of auxin on clathrin-dependent endocytosis (Robert et al., 2010). ABP1 is primarily observed in the ER, but evidence suggests that small amounts of this protein work at the cell periphery. Moreover, a possible function of ABP1 in the ER is not supported by any experimental data. ABP1 can easily be found in culture media of suspension cells (Jones and Herman, 1993). However, as ABP1 contains a canonical KDEL sequence, its targeting to the cell periphery remains unclear. Moreover, this protein does not show any similarity to known receptors and does not contain any hydrophobic domain that could bind to the PM (Timpte, 2001). Therefore, ‘‘docking hypothesis’’ has been formulated according to which ABP1 binds to the membrane via interactions of its C-terminus with membrane protein (Barbier-Brygoo, 1995). Such potential docking protein was identified as C-terminal peptide-binding protein

1 (CBP1) (Shimomura, 2006). CBP1 has been suggested to bind ABP1 and mask the KDEL signal enabling secretion of the complex to the outer face of the PM. Here, ABP1 is anchored via GPI tail of CBP1 to the membrane (Paulick and Bertozzi, 2008). However, the interaction and functional relationship between ABP1 and CBP1 have not been demonstrated. Recent suggestion that the C terminal ABP1 region may interact with co-receptor involved in the transmembrane transmission of the extracellular auxin signal (Shi and Yang, 2011) has been finally confirmed, and transmembranevkinase receptor-like kinases were identified as ABP1 interactors transducing auxin signal and activating plasma membrane associated Rho-like GTPases from plants (ROP; Xu et al., 2014). Our experiments confirm that ABP1 is located in the extracellular matrix of both plants and Arabidopsis suspension cells.

Application of collagenase to Arabidopsis suspension cells revealed the existence of other potential substrates: annexin 1 and cobalamin-independent methionine synthase 1. Both proteins do not have signal peptides, but both were previously identified in extracellular location (Fernandez-Calvino et al., 2011). Identification of annexin 1 among the salt-extracted wall proteins of white lupine (Supplementary Data 1) suggests that this relationship is not incidental. In cell suspensions, annexin 1 was also found in the pool of CaCl2-washed CW proteins, indicating its possible functions outside of the PM. Annexin 1 binds actin cytoskeleton or mediates the anchoring of the cytoskeleton to PM (Hu et al., 2000). Some data suggest also its possible involvement in the formation of ion channels (Mortimer et al., 2008). In plant cells annexin 1 co-localizes also with AGPs (Clark et al., 2005). Interestingly, reports of interactions between annexins and collagen have been described in animals. The collagen-binding anchorin CII was identified as annexin V (Hofmann et al., 1992). This protein, similar to plant annexins, is located in the PM or secreted outside the cell. It also binds native collagen in vitro, suggesting its function as a collagen receptor.

The susceptibility of annexin 1 to collagenase type IV was shown by *in vitro* experiments (Fig. 6) and MS analysis (Fig. 7). The action of the enzyme seemed to be very specific, and com- pletely different from trypsin digestion. Collagen is the most resistant extracellular matrix protein in nature due to its tertiary structure (Okuyama et al., 1981). The fragmentation spectra of annexin 1 confirmed its selective susceptibility to the enzyme activity. Trypsin digestion usually generated typical spectra with peptides between 1000 and 3500 kDa. Following collagenase treatment, large, unique fragments ranging in mass from 6 to 17 kDa (Fig. 7b) were also created. The appearance of such large fragments suggests that collagenase recognizes the tertiary structure of annexin 1 rather than its sequence. Interestingly, prediction of the cleavage sites in annexin 1 revealed that in almost half of these sites Leu or Ile were present. Mammalian collagenases bind collagen at unique cleavage sites that are characterized by Gly–Leu or Gly–Ile bonds that are followed by either Ala or Leu residues (Fields, 1991). Unfortunately, a comparison of the collagen type IV sequence with those of annexin 1, ABP1 or CRT did not reveal any specific similarities. It should be reminded here that a similar mechanism of digestion was previously suggested for GRPs using bioinformatics approaches (Ringli et al., 2001).

**4. Conclusions**

A method for the selective loosening of the CW structure and the isolation of proteins permanently entangled in the walls was elaborated. Collagenase was found to be a valuable tool for studying extracellular proteins and the complexity of protein-protein interactions in the walls. Potential substrates of collagenase or their protein interactors have been identified and their possible roles discussed. The biochemical data were confirmed by the immunolocalization of the ABP1 and CRT with confocal and electron microscopy. Finally, the mass spectra of annexin 1 obtained after collagenase digestion indicated that the mechanism of enzyme cleavage is highly specific, depending more on the spatial structure of the substrate than the amino acid sequence.

**5. Experimental**

**5.1. Plant material**

White lupin (*Lupinus albus* L. cv. Bac) seeds were treated with 70% (v/v) ethanol for 10 min, and sterilized with 10% (v/v) KOCl for 30 min. They were washed several times with deionized sterile water and grown on perlite beds (five seedlings per pot) under greenhouse conditions. For the analyses 21-day-old white lupin roots were used. Maize (*Zea mays* L. cv. Careca S230) were washed overnight with running tap water, and germinated vertically in wet paper rolls for 5 days at 26 °C in the dark. The primary roots were hen used for the extraction of wall proteins. A. thaliana cell culture (a kind gift from Rafael Pont-Lezica) was maintained in Murashige and Skoog medium (1962) supplemented with 3% (w/v) sucrose, 0.5 mg/L naphthaleneacetic acid and 0.05 mg/L kinetin at 22 °C and pH 5.8 in the dark. The suspension was subcultured every 9 days by transferring 15 mL of culture into 80 mL of fresh medium. The wall proteins were isolated from four-days-old cultures. To verify the culture conditions, viability test with Evans blue was performed using a Nikon Eclipse TE200 inverted microscope. All experiments and measurements were carried out at least in four replicates.

**5.2. Isolation of cell wall proteins from plant tissue**

Homogenization of plant tissue and purification of the walls was carried out according to Wojtaszek and Bolwell (1995). Proteins were isolated from the walls with two rounds of 1 M NaCl extraction for 60 and 90 min. The high-salt extracts from were combined and kept in 4 °C until further use. The CW pellet remained after the salt-extraction was normally used for enzymatic digestion. However, in some cases, it was used for additional extraction of proteins in HB buffer (Wojtaszek and Bolwell, 1995) with 1% (w/v) SDS at 70 °C for 1 h. After incubation, the pellet was centrifuged at 12,000g for 10 min, and the protein-containing supernatant was collected and prepared for digestion or electrophoretic separation.

When necessary, extraction of total proteins was also performed. Fifty milligrams of maize or lupin roots were ground into a powder in liquid nitrogen, and proteins were extracted using Laemmli solubilization buffer (Laemmli, 1970). Following centrifugation at 12,000g for 10 min, the protein-containing supernatant was collected and prepared for digestion or electrophoretic separation.

**5.3. Isolation of proteins from suspension-cultured cells**

Cell suspensions were filtered on a funnel through Miracloth. Culture medium, containing secreted proteins, was immediately centrifuged at 800g for 15 min, and kept at 4 °C until further use. The remaining cells were washed with ice-cold fresh medium, and the ionically-bound CW proteins were isolated with 0.2 M CaCl2 for 30 min (Wojtaszek et al., 1995a). Following extraction, the CaCl2 wash was prepared for electrophoretic separation, while the cells were used for enzymatic digestion.

**5.4. Preparation of CW proteins for electrophoresis or enzymatic digestion**

Cell wall protein extracts as well as the Arabidopsis medium before or after collagenase digestion were purified and concentrated by ultrafiltration on an Amicon (Millipore, USA) device using filters with a 10 kDa cut off. Buffer exchanges were performed

three times using Tris-buffered saline (TBS) pH 7.5. The protein concentration was determined using bovine serum albumin as a standard (Bradford, 1976). The samples containing the appropriate amount of proteins were precipitated with cold acetone and then centrifuged at 12,000g for 10 min. The supernatant was discarded, and the protein pellet was washed several times with 80% (v/v) cold acetone. After re-centrifugation, the pellet was lyophilized using a Christ Alpha 3.2 freeze dryer (Osterode, Germany). The proteins were separated by electrophoresis or directly digested with collagenase.

**5.5. Enzymatic hydrolysis of purified cell walls**

The purified plant cell wall pellet after the CW protein extraction was portioned (5 g fresh weight), washed several times with digestion buffer I (50 mM HEPES/NaOH, pH 6.8) and then digested (12 U enzyme in 50 mL of digestion buffer) with three types of collagenase: type IV (2 U/mg), VII (1.9 U/mg) and XI (1.8 U/mg) (Sigma–Aldrich) in digestion buffer I at RT for 1 h with stirring. Following treatment, the CW pellet was centrifuged at 1500g for 15 min and the supernatant was collected. The CW pellet was washed several times with digestion buffer I and then subjected twice to salt extraction with 1 M NaCl in HB buffer (15 and 90 min). All supernatants were prepared for electrophoretic separation.

Collagenase digestion was conducted on intact A. thaliana cells or cells after the salt extraction of the CW proteins. In the first pproach, collagenase type IV was added to 100 mL of intact cultures in fresh medium to reach a final concentration of 2.5 or 5 g/mL. The cells were cultured for a further 4 and 8 h. As a control, the cells not treated with collagenase were used. After treatment, the cells were centrifuged and the medium proteins were harvested. The cells were washed five times with fresh medium, and the proteins were re-extracted with 0.2 M CaCl2 for 30 min.

In second approach, A. thaliana cells were centrifuged, the medium was collected, and cells were washed with fresh medium before being subjected to salt extraction with 0.2 M CaCl2 for 30 min. Extracted CW proteins were collected, the cells were washed and then treated with collagenase (2.5 g/mL) for 30 min. After treatment and washing with fresh medium, the cells were subjected to a subsequent extraction with 0.2 M CaCl2 for 30 min.

As a control, the cells treated with trypsin or not treated with any enzyme were used. The collected enzyme mixture, control buffers and salt extracts from both approaches were prepared for electrophoretic separation. In every step, a portion of the cultures was collected, and the cells were subjected to immunolocalization experiments.

**5.6. Enzymatic hydrolysis of extracted CW proteins**

The lyophilized CW proteins (50 g) were dissolved in digestion buffer II (25 mM Tris/HCl, pH 7.5, and 60 mM CaCl2). Then, 5 L (25 g/mL) of collagenase type IV was added. The positive enzyme control was collagen type IV. For the negative control, the enzyme was inhibited by EDTA or EGTA (12.5 or 25 mM). To verify the enzyme specificity and exclude the possibility of non-specific digestion, the enzyme was incubated with serine protease inhibitors TLCK and BHH at 10 and 100 mM, respectively, before the enzyme addition to the digestion mixture. As an additional control, the CW proteins were in some cases treated with 0.2 g of sequencing-grade trypsin (Promega) instead of collagenase. The digestion of the CW proteins was carried out at 37 °C for 1–12 h. The reaction was quenched by adding the sample buffer (Laemmli, 1970). After digestion, the samples were frozen and maintained at 20 °C until further use.

In some cases, the CW proteins were denatured before collagenase digestion. A 20-g sample was treated with 0.5% (v/v) -mercaptoethanol and incubated for 5 min at 95 °C. For enzymatic deglycosylation, 20 g of proteins were incubated for 5 min at 95 °C and then treated with 5 U N-endoglycosidase F (Roche). The deglycosylation was carried out overnight at 30 °C in 20mM sodium phosphate, 0.1% SDS, pH 7.8, with constant stirring.

**5.7. Enzymatic hydrolysis of annexin 1**

For the experiment a recombinant annexin 1, obtained after expression in Escherichia coli strain BL21 (Gorecka et al., 2005) was used. Five microliters (25 g/mL) of collagenase type IV was added to 10 g of annexin 1 in digestion buffer II. The positive and negative controls were the same as in the experiment above. After digestion, samples were analyzed by MALDI-TOF or prepared for electrophoretic separation. For fragmentation profile comparison, digestion of annexin 1 with trypsin was also performed; 0.2 g trypsin was added to 10 g annexin 1 in 20 L of 25mM NH4HCO3. The digestion was performed overnight at 37 °C.

**5.8. Electrophoresis**

SDS–PAGE was performed according to Laemmli (1970). 2DE was performed according to Kalinowski et al. (2002). Following separation, the gels were fixed with 50% ethanol and 10% acetic acid and stained with either SYPRO Ruby (Molecular Probes), Blue Silver (Candiano et al., 2004), or subjected to Western blotting analysis. For the SYPRO Ruby staining, the images were captured using Typhoon 8600 (Amersham Pharmacia, USA). For the Blue Silver staining, the images were collected with Plustek OpticPro ST64. Semi-quantitative analyses of protein accumulation were carried out using Image Gauge software, version 4.0. Fold change were calculated between protein signal before and after collagenase digestion.

**5.9. Western blotting**

Electrophoretically separated proteins were transferred onto nitrocellulose membrane using wet transfer unit (Hoefer) in 10 mM CAPS/NaOH, pH 11, 10% methanol, and 0.1% SDS. Following transfer, membranes were gently rocked at RT in blocking buffer [3% (w/v) BSA in TBST buffer: 10 mM Tris/HCl, pH 8.0, 150 mM NaCl and 0.05% v/v Tween-20] for 1 h. The blots were then incubated overnight with rabbit anti-CRT (Baluška et al., 1999) and

anti-ABP1 (Venis et al., 1992) primary antibodies raised against maize proteins or anti-germin antibodies (Dumas et al., 1993) raised against wheat proteins at a 1:400 dilution with 1% BSA overnight at 10 °C. After washing, the membranes were incubated at RT in goat biotin-conjugated anti-rabbit antibody (1:10,000 dilution) for 1 h and washed again. Finally, the membranes were incubated with anti-biotin antibody conjugated with Cy3 dye at 1:1000 dilution for 1 h. The images were captured using Typhoon 8600 (Amersham Pharmacia) with 532 nm excitation and 580 nm emission wavelengths. Semi-quantitative analyses of protein accumulation were carried out using Image Gauge software, version 4.0.

**5.10. Immunolocalization experiments**

Intact Arabidopsis suspension cells as well as cells after the salt extraction of CW proteins were used for experiments. All washings were performed with fresh culture medium, and all experiments were repeated at least four times. Cells (0.5 mL) were incubated for 1 h with 1% BSA to block the non-specific binding sites. Cells were washed and incubated with the anti-ABP1 or anti-CRT antibodies (1:400 dilution) for 1 h with constant shaking in the dark. After washing, the cells were incubated with secondary antibodies conjugated with FITC dye in a 1:400 dilution. As a control, labelling without primary or without primary and secondary antibodies was used. The cells were observed using a Nikon Eclipse TE200 inverted microscope (Tokyo, Japan) or the confocal microscope MRC 1024 (Bio-Rad, USA). For confocal microscopy, the observations were carried out in three channels: for chlorophyll autofluorescence (filter 585 LP), for FITC (filter 540 DF30) with emission spectrum 488 nm for both filters, and for transmitted light. To convert .pic files into bit maps (.bmp), Confocal Assistant software, version 4.02, was used. This software was also used to overlay the images coming from individual channels.

For the transmission electron microscopy analysis, cultured Arabidopsis cells and cells after the salt extraction of the CW proteins were prefixed in 1% (v/v) glutaraldehyde in 0.05 M sodium cacodylate, pH 7.0 for 1 h, washed with the same buffer and embedded in 1% (w/v) low-melting-point agarose. Agarose cubes were incubated with 4% paraformaldehyde and 0.25% glutaraldehyde in 0.05 Msodium cacodylate, pH 7.0, for 2 h at 4 °C. The cubes were washed three times for 15 min with 0.05 M sodium cacodylate. Following dehydration in a graded ethanol series, the cubes were infiltrated overnight with ethanol/LR Gold (3:1), 48 h with ethanol/LR Gold (1:1) and 24 h with ethanol/LR Gold (1:3). Finally, the cubes were embedded in LR Gold and polymerized at RT with 2% benzoyl peroxide for 48 h. Ultra-thin sections were cut and collected on nickel grids. The sections were immunostained with the anti-ABP1 or anti-CRT antibodies in a 1:5 dilution following blocking the non-specific binding proteins with 5% BSA. The sections were washed five times with PBS and incubated with the secondary antibody (goat anti-rabbit IgGs conjugated to 10-nm gold particles, Sigma-Aldrich) for 1.5 h in a 1:10 dilution. As a control, labeling without primary antibodies was used. After washing, the grids were counterstained with uranyl acetate. The labeled sections were examined using a JEM1200 EX (JEOL USA) transmission electron microscope.

**5.11. Mass spectrometry**

Following electrophoresis, protein bands/spots were manually excised from gels, transferred to Eppendorf tubes and digested with trypsin as described (Łuczak et al., 2008). Peptide samples were desalted and concentrated with C18 ZipTips (Millipore) according to the manufacturer’s protocols and analyzed using LCMS/MS. All LC-MS/MS analyses were performed using an RP-C18 pre-column (180 m x 20 mm, 5 m, Waters) and a nano-UPLC column (BEH130 C18, 75 m x 150 mm, 1.7 mm, Waters) in a gradient of 5-30% ACN in 0.1% formic acid for 45 min (250 nl/min). LC (Waters) was coupled with a Q-TOF II MicroMass mass spectrometer (Waters). The MS/MS ion analyses were carried out using the MASCOT server. Protein identification was accomplished using the SwissProt/UniProt (2013; 542 324 sequences in total) and NCBI (2011; 13 366 630 sequences in total) databases. Database evaluation was performed using all entries. The protein search was performed using the following parameters: peptide mass tolerance of 0.25 Da, fragment mass tolerance of 0.25 Da, one allowed missed cleavage, allowed variable oxidation modifications (Met) and allowed fixed modifications of carbamidomethyl (Cys). The significance threshold (p < 0.05) was set using the Mascot algorithm. Proteins were identified on the basis of at least three unique peptides with peptide score higher than 40 (p < 0.05). The sequences obtained from the database searches were analyzed for the presence of the signal peptide and subcellular localization using the SORT (Nakai and Horton, 1999) and Plant-PLoc (Chou and Shen, 2008) servers.

For the MS analysis of annexin 1, the MALDI spectra were analyzed on an AutoFlex MALDI-TOF (Bruker Daltonics) mass spectrometer that was operated in reflector mode and used delayed ion extraction. Annexin 1 samples were concentrated with C18 ZipTips. Following digestion the samples were eluted directly with a-cyano-4-hydroxycinnamic acid (HCCA) or with sinapinic acid matrices on a MALDI AnchorChip target plate (Bruker Daltonics). The undigested annexin 1 samples were co-crystalized with sinapinic acid. The positively charged ions ranging from 500 to 3600 Da, 3 to 20 kDa, 12 to 45 kDa and 10 to 90 kDa m/z were analyzed. All of the spectra were evaluated using flexAnalysis and BioTools (Bruker Daltonics). All MS analyses were conducted with four replicates. Masses unique for products of collagenase digestion which were not observed in the spectrum of pure autodegraded collagenase were subjected to analysis of cleavage sites. Only fragments observed in each replication were analyzed. Prediction of the collagenase leavage sites in annexin 1 sequence was performed using Sequence Editor 3.2 software (Bruker Daltonics). Prediction was based on peptide masses obtained using MALDI-TOF mass spectrometry. The matching was performed using the peptide mass tolerance of 0.5 Da. Oxidized methionine was taken under consideration when matching protein fragments.

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**Figure captions**

Figure 1

A representative example of the 2D PAGE analysis of the CW proteins extracted from the white lupin roots. IEF was performed at pH 3-10.

Figure 2

The purified CW of the white lupin roots after the salt extraction of the proteins (C) were treated with collagenase types IV, VII and XI for 30 min, after which the proteins were re-extracted for 15 and 90 min (e.g., IV15’ and IV90’). The control sample was treated with buffer without enzyme and also re-extracted (C15’ and C90’). Coll IV, coll VII and coll XI indicate lines with pure enzymes. The arrows indicate the proteins likely released by collagenase type IV.

Figure 3

Western blotting analysis of the CW proteins of the white lupin roots. The purified CW after the salt extraction of the proteins (C) were treated with collagenase type IV for 30 min (coll30’), after which the proteins were re-extracted for 15 and 90 min (coll90’). The proteins were separated by SDS-PAGE and detected using the anti-CRT (A), anti-ABP1 (B) and anti-germin (C) antibodies.

Figure 4

Western blotting analysis of the CW proteins of the maize roots. The purified CW after the salt extraction of the proteins (C) were treated with collagenase type IV for 30 min (coll30’), after which the proteins were re-extracted for 15 and 90 min (coll90’). As a control, digestion with trypsin was used under the same conditions (tr30’ and tr90’). As a control, the total root proteins were also isolated using SB buffer, and SDS after the salt extraction of the CW proteins. The proteins were separated by SDS-PAGE and detected using the anti-ABP1 (A) and anti-CRT (B) antibodies. Coll - pure collagenase type IV, tr - pure trypsin.

Figure 5

SDS-PAGE of the CW proteins of the maize roots after *in vitro* collagenase digestion. The isolated and purified CW proteins were denatured or not and then collagenase-digested for 1 or 12 h at 37°C. After the SDS-PAGE separation, the proteins were stained with SYPRO Ruby. C: control CW proteins without digestion; -: without denaturation; +: after denaturation; coll- and coll+: CW proteins after collagenase digestion without and after denaturation, respectively; coll: pure enzyme.

Figure 6

Western blotting of the *Arabidopsis* suspension extracellular proteins detected with the anti-ABP1 (A,B) and anti-CRT (C,D) antibodies. The intact *Arabidopsis* cells were treated with 2.5 or 5 μg/ml collagenase type IV, and after 4 or 8 h of incubation with the enzyme, the medium proteins were collected, and the cells were subjected to salt extraction for 25 min. C4h, C8h -control CW or medium proteins without digestion; 2.5 and 5 μg/ml - collagenase concentration; 4 h and 8 h - time of treatment; coll - pure enzyme.

Figure 7

SDS-PAGE of annexin 1 after being subjected to collagenase type IV digestion. A - pure annexin; A+C - annexin 1 after being subjected to collagenase treatment; EGTA12.5, EGTA25, EDTA12.5, EDTA25 - annexin 1 after collagenase treatment in the presence of EGTA or EDTA in two (12.5 and 25 mM) concentrations; TLCK+BHH - annexin 1 after collagenase treatment in the presence of TLCK and BHH protease inhibitors; coll - pure collagenase.

Figure 8

MALDI ToF mass spectrum of pure undigested annexin 1 (molecular ion 37.02 kDa). Sinapinic acids were used as a matrix.

Figure 9

Comparison of the peptide mass spectra of trypsin- (A) and collagenase- (B) digested annexin 1. The arrows indicate the unique fragments of annexin 1 peptides likely present due to the collagenase digestion. HCCA was used as a matrix.

Figure 10

Comparison of the mass spectra of annexin 1 after digestion with collagenase type IV (A) and pure collagenase (B). The arrows indicate the unique fragments that were likely digested annexin 1 because they were not observed in the spectrum of pure autodegraded collagenase.

Figure 11

Confocal microscopy analysis of the *Arabidopsis* suspension cells before (A,C,E,F) and after (B,D) collagenase treatment. Immunolocalization was performed using the anti-ABP1 (A,B), anti-CRT (C,D) and FITZ-conjugated secondary antibodies. As a control, the experiment was performed without the primary antibody (E) or without either antibody (F).

Figure 12

Confocal microscopy analysis of the *Arabidopsis* suspension cells after the salt extraction of the CW proteins or after trypsin treatment using the anti-ABP1 (A,C), anti-CRT (B,D) and FITZ-conjugated secondary antibodies.

Figure 13

Electron microscopy analysis of the *Arabidopsis* suspension cells before (A,B) and after (C,D) collagenase treatment. Immunolocalization was performed using the anti-ABP1 and gold-conjugated secondary antibodies. The scale bar represents 100 nm.

Figure 14

Electron microscopy analysis of the *Arabidopsis* suspension cells before (A,B) and after (C,D) collagenase treatment. Immunolocalization was performed using the anti-CRT and gold-conjugated secondary antibodies. The scale bar represents 100 nm.

Table 1.

List of proteins identified as potential substrates for collagenase or proteins released from the CW after the enzyme treatment. The proteins were identified using MS/MS and/or western blotting.