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

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This paper forms part of a special issue of *Phytochemistry* dedicated to the memory and legacy of Professor (Godfrey) Paul Bolwell, MA DSc (Oxon). (1946–2012), internationally-recognised plant biochemist and Regional Editor of *Phytochemistry* (2004–2012). He is much missed by his friends.

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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.

1. Introduction

The plant cell wall (CW) is a multinetwork combination of proteins, polysaccharides and phenolic compounds. In recent years we have learnt a lot about the individual components of the walls. However, the details of the CW assembly and structural organization are recognized only fragmentarily (for review see Wojtaszek, 2000). The various wall components are interconnected by numerous covalent, ionic and hydrogen bonds, as well as by electrostatic and hydrophobic interactions, resulting in the formation of an extremely durable and multifunctional cell wall structure. The extent of intermolecular interactions directly affects their extractability from the walls. Looking closely at wall proteins in those respects, at least three broad, partly overlapping, sets can be distinguished. The first one includes proteins that are loosely associated with CW and easily released into the apoplast or into the 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 gravireponses 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)

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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 peptide bonds. 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,

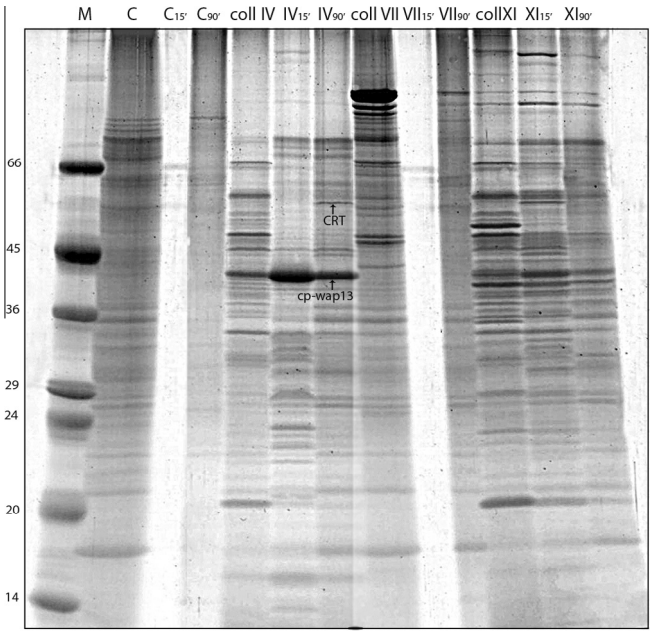

Fig. 1. The purified CW of the white lupin roots after the salt extraction of the proteins (C) were treated with collagenase types IV, VII and XI for 30 min, after which the proteins were re-extracted for 15 and 90 min (e.g., $IV_{15'}$ and $IV_{90'}$). The control sample was treated with buffer without enzyme and also re-extracted ($C_{15'}$ and $C_{90'}$). Coll IV, coll VII and coll XI indicate lines with pure enzymes. The arrows indicate the proteins likely released by collagenase type IV.