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ScienceDirect
Phytochemistry 68 (2007) 1612–1622
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Proteomic analysis of *Chelidonium majus* milky sap using two-dimensional gel electrophoresis and tandem mass spectrometry

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Received 30 December 2006; received in revised form 29 March 2007; accepted 30 March 2007
Available online 18 May 2007

Abstract

Milky sap, a milky-like orange fluid, isolated from the Greater Celandine (*Chelidonium majus* L.), family Papaveraceae, serves as a rich source of various biologically active substances such as alkaloids, several flavonoids, phenolic acids and proteins. The objective of this study was to separate *Ch. majus* milky sap extract proteins using two-dimensional gel electrophoresis (2-DE) to demonstrate for the first time the protein composition in the sap and to identify them using liquid chromatography-tandem mass spectrometry analysis (LC-ESI-MS/MS). It was possible to identify 21 proteins, which comprise disease/defence-related, signalling, Krebs cycle, nucleic acid binding and other proteins. The majority of the identified proteins can be linked to direct and indirect stress and defence reactions, e.g. against different pathogens. The specific protein composition of the milky sap suggests an important role of these proteins for the whole plant physiology and development.

Keywords: *Chelidonium majus*; Papaveraceae; Greater Celandine; Milky sap; Plant defence; Proteomics; Two-dimensional gel electrophoresis; Tandem mass spectrometry; LC-ESI-MS/MS

1. Introduction

Greater Celandine (*Chelidonium majus* L.) belongs to the family Papaveraceae and is a rich source of various biologically active substances (Colombo and Bosisio, 1996). Our interest in the Greater Celandine originated from the traditional use of fresh plant extracts against warts and condylomas, which are a visible effect of papillomavirus infection. Plant extracts were also used to treat liver disorders and fight fever. *Ch. majus* preparations have frequently been prescribed to treat gastric and biliary disorders (Benninger et al., 1999). It has also been found that they have antimicrobial, antitumor, anti-inflammatory, antifungal, and fungistatic properties (Colombo and Bosisio, 1996). The medicinal and pharmaceutical interest in this plant is based on its synthesis of pharmaceutically important compounds, such as alkaloids, flavonoids or phenolic acids (Hegnauer, 1990; Tome and Colombo, 1995). All of them occur in the milky sap – a milky-like orange fluid, similar to the latex isolated from the opium poppy (*Papaver somniferum*) (Decker et al., 2000).

Our previous study showed that the biological activity of *Ch. majus* sap may depend not only on its alkaloidal content but also on the presence of two biologically active proteins, which possessed lectin CM and DN-ase activity (Fik et al., 2000). Lately using affinity purification on HT Heparin column (GE Healthcare), one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (1-D SDS–PAGE) and liquid chromatography-tandem mass spectrometry analysis (LC-ESI-MS/MS), we have identified a novel extracellular peroxidase (CMP) in the milky sap of *Ch. majus* L., which was accompanied by two nucleases (CMN1 and CMN2) (Nawrot et al., 2007). This finding supports the assumption, that the biological

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doi:10.1016/j.phytochem.2007.03.039

activity and curing properties of this plant's sap may be also connected with the presence of proteins.

The objective of this study was to separate *Ch. majus* milky sap extract proteins using two-dimensional gel electrophoresis (2-DE) to give a premier overview of the protein composition in the sap and to identify them using mass spectrometry.

2. Results and discussion

2.1. Separation of Ch. majus milky sap proteins

Ch. majus L. milky sap was collected in each month during the whole vegetation period from May to October from different locations. The stems of adult *Ch. majus* plants were cut and the exuding orange milky sap was collected and separated by centrifugation. To visualize the protein composition of *Ch. majus* sap, extracts were subjected to SDS–PAGE and stained with silver according to Heukeshoven and Dernick (1985) (Fig. 2). The analysis showed a similar pattern of protein bands in all extracts, suggesting the presence of complex mixture of different proteins in the samples. To increase the resolution achieved by 1-D SDS–PAGE, a 2-DE protein separation system was employed to differentiate proteins present in extracts.

The 2-DE protein profile of *Ch. majus* milky sap proteins was preliminarily analysed in 3–10 pH range and proteins were detected by silver staining according to Heukeshoven and Dernick (1985). The protein pattern was reproducible among technical replicates of the same sample but not among replicates from independent extractions of the milky sap. The proteins from different 2-D gels differed in the number of proteins and their quantity (Fig. 1). To obtain a better resolution of the 2-DE protein profile, extracts were analysed on 3–7 pH gradient strips as the first dimension and stained with silver according to Shevchenko et al. (1996), the method compatible with MS. Analysis of the 2-D gels revealed changing pattern of about 20 proteins ranging from p*I* 3–7 and molecular weight of 2–65 kDa (Figs. 2 and 3).

Although 2-DE is an established and powerful technique for analysing complex mixtures of proteins, it has been criticized for its low reproducibility due to indeterminate errors that have two sources: analytical and biological. The origin of analytical variation is generally associated with the many experimentally complex techniques including protein extraction, IEF, SDS–PAGE, staining, destaining and imaging that contribute to variations in the gel patterns, spot intensities, and protein expression quantification. The origin of biological variance is different and arises from natural quantitative variations in protein levels associated with different plants. Biological variance may arise from small differences in microenvironments. An example would be shading of one leaf by another or different soil conditions resulting in different expression of proteins in different plants. Another source of biological variation would encompass small genotypical variations within a heterozygous organism resulting in differential protein expression, and post-translational modifications (PTMs) of some proteins (Asirvatham et al., 2002; Jorge et al., 2005).

Milky sap was collected from *Ch. majus* plants which grew in their natural habitats in different seasons of the year. The milky sap samples were collected from *Ch. majus* plants which grew in several locations, repeatedly every two weeks from each location, from adult *Ch. majus* plants

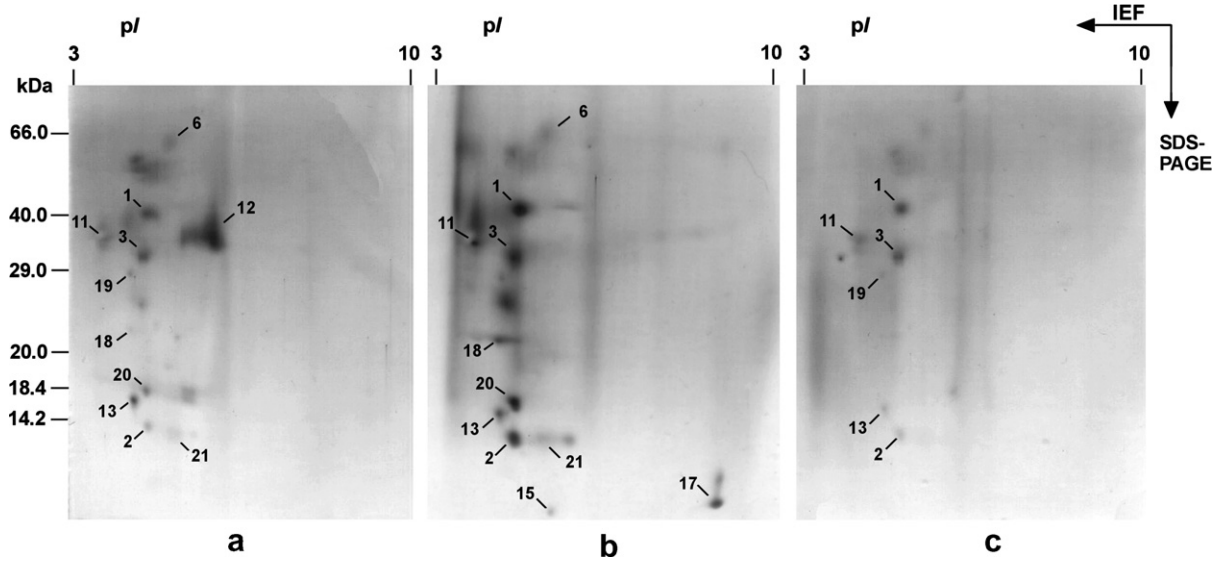

Fig. 1. 2-DE protein profiles of *Chelidonium majus* milky sap extracts collected at the end of May (a), at the end of May (from different plant), 10× concentrated (b) and at the beginning of October (c). Proteins were analysed by 2-DE on 7.5 cm, 3–10 pH gradient strips as the first dimension and 10% SDS–polyacrylamide gel as the second dimension and detected by silver staining (Heukeshoven and Dernick, 1985). 50 ng of the total protein content in the applied sample was separated in (a) and (c) and 500 ng in (b). The protein pattern was reproducible among technical replicates of the same sample but not among replicates from independent extractions of the milky sap. Extracts collected in autumn seem to be less complex than those collected in spring.

of similar developmental stage. Chosen 2-D gels are representative to periods in which protein extracts of the milky sap were the most abundant in proteins.

2-DE protein profiles of the extracts collected contain many differences which are discussed below.

Two different 2-DE protein profiles of the same extract collected at the end of May (Figs. 1b and 2), which was 10× concentrated, showed different polypeptide patterns. This could be the result of the fact, that the extract in Fig. 1b was separated in different pH range during the first dimension (IEF) of 2-DE than the same extract presented in Fig. 2. Differences between 2-D protein profiles of the extracts in Figs. 2 and 3 possibly resulted from biological variation, especially from differences in microenvironments, because presented extracts were collected from different plants in different times – at the end and in the middle of May, respectively. The same reason probably contributed to differences between extract collected at the end of May (Fig. 1a) and the extract collected at the beginning of the month (Fig. 4a). Both extracts contain spots 1, 3, 12 and 20, but extract presented in Fig. 1a contains much more proteins. On the other hand, extract present in Fig. 4a contains protein spots 8 and 9, which are not present in Fig. 1a. These proteins show DN-ase activity (Fig. 4b) and could be members of pathogenesis-related (PR) protein family, which are induced in pathogenical or related situations. The inducible character of CMN1 nuclease, which corresponds to spots 8 and 9, could be the reason of their absence in different extracts.

Additionally, the protein profile of the extract collected at the end of May shows the presence of spot 12, representing CMN2 nuclease (Fig. 1a), which is not present in concentrated extract also collected at the end of May, but from different plants (Fig. 1b). This might resulted from differential expression pattern of this protein in different plants.

2.2. Identification of separated proteins by tandem mass spectrometry

Twenty-two stained with silver (Shevchenko et al., 1996) protein spots were excised from the gels (Figs. 2–4) and subjected to LC-ESI-MS/MS analysis. In order to confirm that proteins with similar positions among gels are truly identical, we analysed most of the spots from two different gels and obtained similar MS/MS results.

It was possible to identify 21 proteins from the milky sap of *Ch. majus*. One of the analysed spots produced insufficient information for its identification (spot 8). Three of the spots produced only single peptide match and relatively low score results (spots 7, 10, 12), nevertheless they were assigned as identified due to a good correlation between experimental and theoretical p*I*/molecular mass (spots 10, 12) or previous results obtained from 1-D SDS–PAGE gels which were stained with silver and analysed using LC-MS/MS (data not shown), suggesting the presence of such protein with high probability (spot 7).

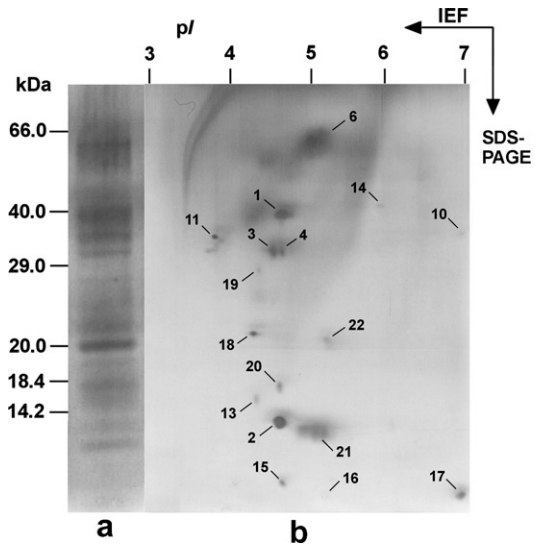

Fig. 2. (a) 1-D SDS–PAGE protein profile of silver-stained (Heukeshoven and Dernick, 1985) *Ch. majus* milky sap proteins collected at the end of May (100 ng of the total protein content in the applied sample was separated). (b) 2-DE protein profile of silver-stained (Shevchenko et al., 1996) milky sap proteins from *Ch. majus* collected at the end of May, 10× concentrated. Proteins (500 ng of the total protein content in the applied sample) were separated on a first dimensional pH 3–7 linear IEF gels and second dimensional 10% vertical slab gels. The relative M_r is given on the left, while the p*I* is given at the top of the figure. Symbols indicate spots which were excised from the gel and subjected to LC-ESI-MS/MS.

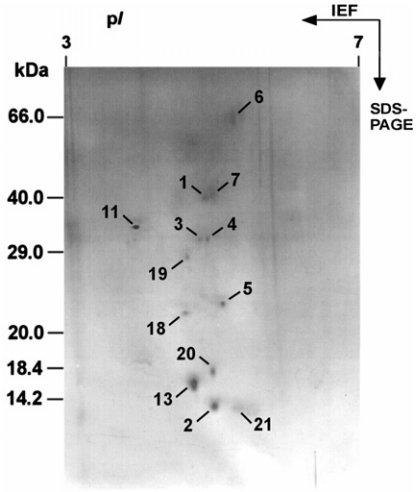

Fig. 3. A 2-DE protein profile of the extract collected in the middle of May (50 ng of the total protein content in the applied sample was separated). The extract contained protein spots 5 and 7.

Table 1 shows identified proteins and organisms from which they originate, together with the sequence coverage and the values for the experimental and theoretical p*I* and molecular masses. The number of matched peptides was between 1 and 13, with up to 45% sequence coverage. The nine spots (spots 2, 3, 4, 5, 10, 12, 18, 21, 22) showed a good correlation between experimental and theoretical p*I*/molecular mass. The remaining 12 proteins were identified by comparison with sequences from different organisms, showing more variable score results and bigger differences

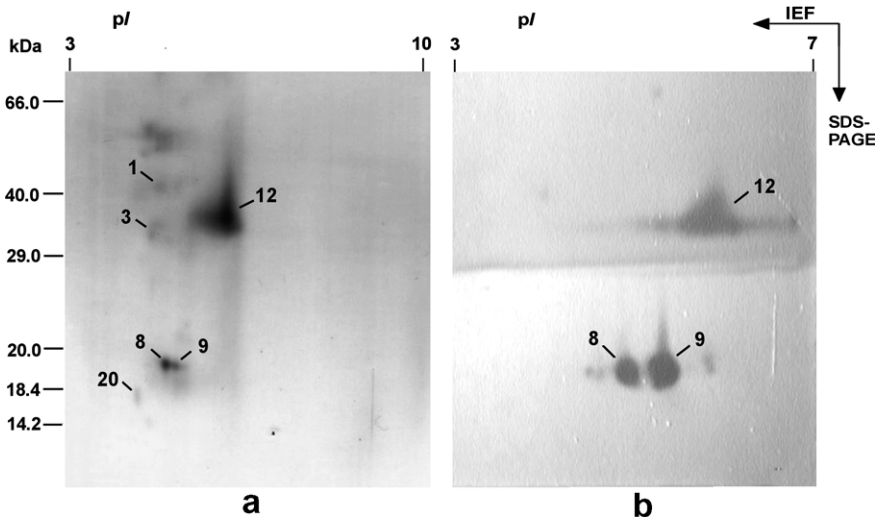

Fig. 4. A 2-DE protein profile of the extract collected at the beginning of May (a), compared to 2-D in-gel DN-ase assay of the same extract, showing DN-ase activity of three proteins (b). One of them is CMN2 nuclease (spot 12), of M_r about 36 kDa and a p*I* around 6.0. Two other activities represent CMN1 nuclease (spots 8 and 9), which was separated as two proteins of about 20 kDa. They differ only by a p*I* of 5.1 and 5.3, respectively. (a) and (b) are of different pH ranges, but similar molecular weights of separated proteins allow to compare the 2-D gel stained with silver (a) to 2-D in-gel DN-ase assay (b). 50 ng of the total protein content in the applied sample was separated both in (a) and (b).

between theoretical and experimental p*I* and molecular mass values (Table 1). This is a result of a general problem observed for species like *Ch. majus*, from which genomic sequence information is rarely available.

Some of the identified proteins were present in more than one spot (e.g. spots 1 and 2). This could arise from several causes. One of them are post-translational protein modifications (PTMs), the other could be proteolytic degradation of the protein, or the reason that these proteins are products of different genes (Jorge et al., 2005). These assumptions underline the complex composition of the milky sap.

2.3. Protein diversity observed in Ch. majus milky sap

Identified proteins were classified according to their functions in three general categories: disease/defence-related, nucleic acid binding and general cell metabolism (metabolism, energy producing, signal transduction, protein destination and storage, secondary metabolism).

Ch. majus milky sap exudes when the plant is injured. It is the protoplasm of specialized cells termed laticifers (Mahlberg, 1993; Kekwick, 2001). These cells, which transverse walls are more or less resorbed, are located in phloem areas of the vascular system (Fairbairn and Kapoor, 1960; Chauveaud, 1891) and form an internal, articulated, and fused cell system throughout the whole plant (Mahlberg, 1993). It was also shown that during cell differentiation of laticifers, a lot of vesicular organelles develop in their protoplast (Thureson-Klein, 1970; Dickenson and Fairbairn, 1975). The vesicles are the major site of alkaloid accumulation. For *P. somniferum* latex ca. 90% of the total alkaloid content was found in the vesicle fraction after centrifugation, comparing to ca. 70% for *Ch. majus* latex, possibly due to the greater fragility of *Chelidonium* vesicles (Roberts et al., 1983; Fairbairn et al., 1974). On the other hand, most of the proteins are the component of the cytoplasm (up to 95% within the *P. somniferum* latex) (Nessler et al., 1985).

Nevertheless the complexity of proteins in *Ch. majus* milky sap (21 identified proteins) seems to be lower than in the latex of opium poppy (*P. somniferum*), where 75 spots in the serum fractions were separated (Decker et al., 2000), and much lower than in the phloem sap of *Brassica napus* (Giavalisco et al., 2006), where identification led to more than a 100 different proteins. Phloem of higher vascular plants is the major route for the translocation and distribution of organic metabolites assimilated during photosynthesis (Kehr, 2006). Recent findings show that sieve elements are not only component for photosynthate allocation, but also transport information substances and large molecules like proteins or even mRNAs (Kehr, 2006; Walz et al., 2004).

2.3.1. Proteins involved in disease and defence responses

As a general reaction to environmental and biotic stresses, such as salt, light, drought, wounding, and pathogen attack, plants produce reactive oxygen species (Walz et al., 2002). The main function of superoxide dismutases (SODs; E.C. 1.15.1.1) is to scavenge O_2^- radicals to prevent oxidation of biological molecules (Karpinska et al., 2001). In the milky sap copper/zinc superoxide dismutase (spots 1 and 2) and manganese superoxide dismutase (spot 5) were identified. These proteins belong to two distinct groups of SODs – Mn-SOD appear to be closely related both in structural and evolutionary aspects to Fe-SOD, but Cu/Zn-SOD has no sequence similarity to them and appears to have evolved separately in eukaryotes (Chen et al., 2002). Cu/Zn-SOD is a component of an antioxidant

Table 1
Proteins identified in *Chelidonium majus* milky sap using LC-ESI-MS/MS

Spot[a]	Identified protein[b]	Accession No.[c]AGI code[d]	Matched peptides[e]	Score[f]	Mol. mass (kDa) theoret./ exp.[g]	p*I* theoret. (calc.)/ exp.[h]	Sequence coverage (%)[i]
Disease/defence responses							
1	Superoxide dismutase (Cu–Zn) (*Oryza sativa*)	gi|538430	5	262	15.3/40.0	5.71/4.7	23
2	Copper–zinc superoxide dismutase (*Nelumbo nucifera*)	gi|58616003	4	205	15.5/14.0	5.67/4.6	23
3	Hypothetical protein – wild cabbage (fragment), region: glyoxalase (lactoylglutathione lyase) (*Brassica oleracea var. gemmifera*)	gi|7488556	3	200	29.0/30.0	4.94/4.5	15
4	Putative lactoylglutathione lyase (*Arabidopsis thaliana*)	gi|15810219 At1g11840	5	365	32.0/30.0	5.11/4.7	13
5	Manganese superoxide dismutase/metal ion binding/ superoxide dismutase (*Arabidopsis thaliana*)	gi|15228896 At3g56350	1	85	26.9/25.0	6.25/4.9	6
6	FAD linked oxidase, N-terminal (*Medicago truncatula*)	gi|87240745	2	70	59.7/65.0	8.73/5.1	1
Nucleases/nucleic acid binding							
7[j]	ATGRP2B (glycine-rich protein 2B); DNA binding/ nucleic acid binding (*Arabidopsis thaliana*)	gi|15226451 At2g21060	1[j]	62[j]	19.4/40.0	6.29/4.8	9
9	Nucleic acid binding (*Arabidopsis thaliana*)	gi|15241136 At5g56140	2	50	34.0/20.0	7.88/5.3	7
10[j]	RNA binding/catalytic/ribonuclease H (*Arabidopsis thaliana*)	gi|18400719 At2g25100	1[j]	50[j]	33.5/34.0	6.28/7.0	3
11	HTR12; DNA binding (*Arabidopsis thaliana*)	gi|18378832 At1g01370	2	60	19.8/35.0	11.57/3.8	5
12[j]	RNA binding/catalytic/ribonuclease H (*Arabidopsis thaliana*)	gi|18400719 At2g25100	1[j]	48[j]	33.5/36.0	6.28/6.0	3
13	Nucleic acid binding (*Arabidopsis thaliana*)	gi|15241136 At5g56140	4	72	34.0/16.0	7.88/4.4	8
General metabolism							
14	Malate dehydrogenase (*Arabidopsis thaliana*)	gi|18404382 At1g53240	6	139	36.0/41.0	8.54/6.0	16
15	Acyl-CoA binding protein (ACBP) (*Digitalis lanata*)	gi|6002104	8	240	10.1/9.0	5.84/4.7	43
16	Polyubiquitin (*Populus tremula* × *Populus tremuloides*)	gi|7862066	4	246	17.2/8.6	5.77/5.2	22
17	Ubiquitin (*Hordeum vulgare subsp. vulgare*)	gi|167073	7	261	17.9/8.5	9.83/7.0	45
18	FQR1 (flavodoxin-like quinone reductase 1) (*Arabidopsis thaliana*)	gi|15239652 At5g54500	13	401	21.8/23.0	5.96/4.3	27
19	ATP binding/kinase/ protein serine/ threonine kinase (*Arabidopsis thaliana*)	gi|15227808 At2g23300	2	64	84.5/29.0	6.88/4.4	2
20	Serine/threonine protein kinase, active site (*Medicago truncatula*)	gi|92899288	2	59	68.4/18.0	9.37/4.6	2
21	Rubber elongation factor (REF) (Allergen Hev b 1) (*Hevea brasiliensis*)	gi|132270	5	135	14.7/13.5	5.04/5.0	44
22	'Putative 1,4-benzoquinone reductase' (*Oryza sativa* (*japonica cultivar-group*))	gi|53749369	2	156	21.6/21.5	6.30/5.2	10
8	Not identified						

[a] Assigned spot numbers as indicated in Figs. 1–4.
[b] Identified homologous proteins and organism from which it proceeds.
[c] Database accession numbers according to: NCBInr (nr); trEMBL (trm).
[d] Arabidopsis genome initiative (AGI) locus codes for *A. thaliana* proteins (where appropriate).
[e] Number of matched peptides with Mascot search data (www.matrixscience.com).
[f] Mascot search probability based Mowse score. Ions score is $-10*\log(P)$, where P is the probability that the observed match is a random event. Individual ions scores >48 indicate identity or extensive homology ($p < 0.05$).
[g] Experimental and theoretical mass (kDa) of identified proteins. Experimental values were calculated with standard molecular mass markers. Theoretical values were retrieved from the protein database.
[h] Experimental and theoretical p*I* of identified proteins. Experimental values were calculated with Image Master 2-D Elite software. Theoretical values were retrieved from the protein database.
[i] Amino acid sequence coverage for the identified proteins.
[j] Identifications with only single peptide match and low score results were assigned as identified due to a good correlation between experimental and theoretical p*I*/molecular mass (spots 10, 12) or previous results obtained from 1-D SDS–PAGE gels which were stained with silver and analysed using LC-MS/MS, suggesting the presence of such proteins (spot 7) (data not shown).

defence system of pumpkin (*Cucurbita maxima*) and cucumber (*Cucumis sativus* L.) phloem sap (Walz et al., 2004), but it does not include Mn-SOD. The occurrence of Cu/Zn-SOD in two different spots, however, is surprising. It is probable that one of the spots 1 or 2 contains modified or possibly degraded Cu/Zn SOD. These

proteins, combined with previously identified CMP peroxidase (Nawrot et al., 2007) and glyoxalase (spots 3 and 4), are components of the enzymatic antioxidant system of the milky sap. Glyoxalase I (lactoylglutathione lyase) [EC 4.4.1.5] is an enzyme of the lyase class that catalyzes the condensation of methylglyoxal (MG) and glutathione (GSH) to form lactoylglutathione as a step in the conversion of MG to lactic acid (Yadav et al., 2005). Recent studies in plants showed that a different kind of stress conditions, like salt and metal stress, resulted in enhanced expression of glyoxalase I (Veena et al., 1999). The protein was also found in the opium poppy latex (Decker et al., 2000), and in the phloem sap of cucumber and pumpkin (Walz et al., 2004). Substantial number of experiments have demonstrated that plant reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), which comprises a plasma membrane-bound heterodimeric flavoprotein (spot 6), is an important source of ROS in plants under stresses (Hancock et al., 2002; Hao et al., 2006). It is well known that NADPH oxidase is involved in plant growth and development, in plant defence reactions to pathogen or elicitor attack and in response to various abiotic stresses (Hao et al., 2006). But such protein was not identified in the phloem sap of any species, not even in the opium poppy latex.

2.3.2. Nucleases/nucleic acid binding proteins

Six milky sap proteins were identified as a members of nucleic acid binding category. One of them (spot 7) belongs to glycine-rich (GRP) proteins, which possess RNA-binding properties. In most cases it has been shown that they are accumulated in the vascular tissues and that their synthesis is part of the plant's defence mechanism (Mousavi and Hotta, 2005). GRPs may have diverse localization and functions. The only common feature among all different GRPs is the presence of glycine repeat domains, which are highly flexible and may act in the protein–protein interaction (Sachetto-Martins et al., 2000). Their mRNA levels responded to different stress and hormone treatments like exposure to cold, wounding, acute hypersensitive response, salicylic acid treatment, abscisic acid treatment, or water stress (Nomata et al., 2004). Pathogenic attack has also been shown to modulate GRP expression (Sachetto-Martins et al., 2000). They have been found in *B. napus* as abundant class of phloem proteins (Giavalisco et al., 2006) and in *Ricinus communis* phloem sap (Barnes et al., 2004).

Spots assigned as 9, 10, 11, 12 and 13 were identified as nucleic acid binding, DNA binding, or RNA-binding proteins. RNA-binding protein, CmPP16, was found in the phloem sap of *C. maxima* (Xoconostle-Cazares et al., 1999). Spots 9 and 12, which represent CMN1 and CMN2 nucleases, respectively, were additionally shown to have DN-ase (Fig. 4) and RN-ase activity (data not shown). Enzymes of nucleolytic activity were not found in the phloem sap and in the latex of any other species. Nucleases are defined as a group of enzymes that are capable of hydrolyzing the phosphodiester linkages of nucleic acids. Ribonucleases (RN-ases) and deoxiribonucleases (DN-ases) are not only involved in nucleic acid metabolism, but also play important roles in many cellular processes. Moreover, nucleases are involved in host defence against foreign nucleic acid molecules (Hsia et al., 2005). In order to determine DN-ase activity of *Ch. majus* milky sap, extracts collected from May to October were separated using in-gel DN-ase assay. CMN1 nuclease of 20 kDa is present in extracts collected in May, and CMN2 of 36 kDa in extracts from the whole vegetation period (data not shown). Both proteins exhibit the highest activity in Ca^{2+} buffer, pH 8.0, so they belong to the Ca^{2+}-dependent nucleases class (Sugiyama et al., 2000). To increase resolution of protein separation, a 2-D in-gel DN-ase assay was performed. Gels after electrophoresis in the second dimension and ethidium bromide staining were compared to previous 2-D gels stained with silver (Fig. 4). 2-D gel of the extract collected in May showed DN-ase activity of three proteins. One of them is CMN2 nuclease (spot 12), of M_r about 36 kDa and a p*I* around 6.0. Two other activities represent CMN1 nuclease (spots 8 and 9), which was separated as two proteins of about 20 kDa. They differ only by a p*I* of 5.1 and 5.3, respectively (Fig. 4). In addition, despite higher quantity than in spot 9, spot 8 produced insufficient information for its identification. It could possibly resulted from some post-translational modifications, which made produced ion series difficult to identify. The both studied nucleases CMN1 and CMN2 display DN-ase and RN-ase activities (data not shown) and probably are a part of *Ch. majus* defence system. Nucleolytic activity of one of the studied nucleases, CMN1 (Fig. 4), could be associated with pathogenesis-related (PR) proteins, which are induced in pathogenical or related situations. PR-10 family comprises intracellular defence-related proteins with structural homology to ribonuclease (Park et al., 2004). Our results indicate, that CMN1 of 20 kDa might belong to RN-ase class II, which comprise nucleases of pH optima between 6 and 7, molecular masses between 17 and 21 kDa, and low sensitivity to EDTA (Yen and Green, 1991). Possibly also CMN2 nuclease could be engaged in PR-10 activity. Some PR-10 proteins display constitutive expression patterns unrelated to the pathogenic response (Liu and Ekramoddoullah, 2006), but this assumption needs further investigation.

2.3.3. Proteins involved in general metabolism

The third group of proteins identified in *Ch. majus* milky sap comprise proteins involved in general cell metabolism (metabolism, energy producing, signal transduction, protein destination and storage, secondary metabolism). Spot 15 was identified as acyl-CoA binding protein (ACBP). Cytosolic acyl-CoA binding proteins are small, highly conserved proteins that bind long-chain acyl-CoAs (Chye et al., 1999). These proteins could be involved in long distance signalling (Walz et al., 2004). Although this kind of proteins are mostly functioning in developmental processes (Hills et al., 1994), it has been supposed that they partici-

pate in the regulation of the acyl-pool of the plasma membrane, involved in acyl-CoA signal transduction (Chye et al., 1999). Acyl-CoA binding protein was identified in *P. somniferum* latex (Decker et al., 2000), and is also present in sieve-tube exudates of *C. maxima*, *B. napus* and *Cocos nucifera*. Moreover, it is a major phloem sap protein in rice (*Oryza sativa* L.) (Suzui et al., 2006). The presence of ubiquitin (spots 16 and 17) in phloem sap is well known (Schobert et al., 1995; Haebel and Kehr, 2001; Giavalisco et al., 2006), but there is no evidence that it is present in latex of any species. Malate dehydrogenase (spot 14) is one of the enzymes of the Krebs cycle. It was found as well as in *P. somniferum* latex (Decker et al., 2000) and in phloem sap of different plants, which contain this enzyme and also substrates of the citrate cycle, like malate, citrate, succinate and fumarate (Walz et al., 2004). Citrate cycle reactions in the phloem sap could provide carbon source for synthesising different amino acids (Walz et al., 2004). Flavodoxin-like quinone reductase (spot 18) might also play a role in stress response. It catalyzes the transfer of electrons from NADH and NADPH to several substrates and exhibits quinone reductase activity. It is supposed that auxin-inducible glutathione *S*-transferases and quinone reductases found in plants may play a role of detoxification enzymes, possibly to protect against auxin-induced oxidative stress (Laskowski et al., 2002). NAD(P)H quinone reductase, enzyme similar to 1,4-benzoquinone reductase (spot 22), was identified in the latex of *Hevea brasiliensis* (Chareonthiphakorn et al., 2002). It was found that it possess a supportive role in stabilization of the colloidal latex through its action in maintaining particle stability, particularly the integrity of the vessel's membrane (Chareonthiphakorn et al., 2002). Similar enzyme was also found in the latex of opium poppy (Decker et al., 2000), but not in the phloem sap of any species. Serine/threonine protein kinases (spots 19 and 20) phosphorylate many different proteins and are involved in many different processes for cell viability, including the phosphorylation of stress-induced proteins and also seed storage proteins (Irar et al., 2006). Different kinases were identified in the opium poppy latex (e.g. phosphoglycerate kinase involved in glycolysis pathway) (Decker et al., 2000) and in *R. communis* phloem sap (Barnes et al., 2004).

Ch. majus milky sap contains also the protein identified as the rubber elongation factor (REF) of *H. brasiliensis* (spot 21). REF is an enzyme involved in rubber biosynthesis, an isoprenoid polymer with no known physiological function to the plant, but commercially used as the raw material of choice for heavy duty tires and other industrial uses requiring elasticity and flexibility (Oh et al., 1999). In *H. brasiliensis*, rubber synthesis takes place on the surface of rubber particles suspended in the latex, which is the cytoplasm of laticifers (Han et al., 2000). Moreover, REF belongs to the group of *H. brasiliensis* latex allergens (Allergen Hev b 1) (Czuppon et al., 1993). Other findings show that also Mn-SOD identified in *H. brasiliensis* latex acts as a cross-reactive allergen (Hev b 10) (Wagner et al., 2001). It was hypothesized that other defence-related proteins of the plants could act as latex allergens (Yagami, 1998). Enzymes similar to REF seem to be specific to the latex of different plants, they were not identified in the phloem sap of any species.

2.4. Differences between proteins present in Ch. majus milky sap and phloem sap and latex of different plants

During sample collection stem of the plant was cut. Despite our best efforts, it cannot be excluded that *Ch. majus* milky sap, which was collected in the study, could be in fact a mixture of a prominent *Ch. majus* latex with some quantities of phloem and xylem sap of the plant. Many of the proteins identified in this study from *Ch. majus* milky sap have been previously detected in the latex of other plants, like *P. somniferum* and *H. brasiliensis* (glyoxalase – spots 3 and 4, acyl-CoA binding protein – spot 15, malate dehydrogenase – spot 14, quinone reductases – spots 18 and 22, kinases – spots 19 and 20, rubber elongation factor – spot 21). But we also detected several proteins that were not expected in this compartment, like glycine-rich proteins (spot 7) or ubiquitins (spots 16 and 17). They could be contaminations from surrounding tissues or companion cells reaching the sap during sampling. Therefore identified proteins are not necessarily associated only with laticifers.

The protein composition of the latex and the phloem sap of different species is closely related, but there are also apparent differences. To date, there is no evidence for the presence of nucleases in the phloem sap of any plant species. In the phloem sap of pumpkin and cucurbit (Walz et al., 2004) other members of pathogenesis-related protein family were identified (e.g. β-glucosidase, lipoxygenase), but not PR-10 family. Present data also show that phloem sap does not contain any oxidases (spot 6) or Mn-SOD (spot 5), which are present in *Ch. majus* milky sap. Also enzymes like REF (spot 21) or quinone reductases (spots 18 and 22), which have a role in latex stabilization, seem to be specific to the latex of different plants. Nevertheless major latex proteins (MLPs), first identified in the opium poppy (Nessler et al., 1985), were also reported in *B. napus* phloem sap (Giavalisco et al., 2006).

On the other hand, some very prominent and abundant phloem sap proteins known from many other plants are apparently not found in laticifers. For example, there is no evidence for the presence of proteins connected to amino acid metabolism in the latex of any species, as well as in *Ch. majus* milky sap. *B. napus* phloem sap contains a wealth of amino acid metabolism enzymes, like cystine lyase, methionine synthetase, S-adenosylmethionine synthetase, glutamine kinase and adenosine kinase (Giavalisco et al., 2006). Other group of proteins, which are present in the phloem sap, but were not identified in the present study, comprise calcium-related proteins, like annexins and calmodulins. Ca^{2+} elevations upon stress have been reported in a number of different systems, and annexins

are a class of proteins that can sense Ca^{2+} signals to further activate different downstream signaling pathways (Giavalisco et al., 2006). Plant calmodulins are involved in a range of responses to different environmental stimuli and biotic stresses and can modulate the activity of variety of target proteins (Giavalisco et al., 2006). Both protein classes were identified in *B. napus* and *R. communis* phloem sap (Giavalisco et al., 2006; Barnes et al., 2004). Annexins were also found in the latex of *P. somniferum* (Decker et al., 2000), suggesting that calcium-realted system exists in the latex of other plants. Phloem exudate of *B. napus* contains components of myrosinase system, which is best studied metabolic defence system in plants (Giavalisco et al., 2006). None components of such system were identified in the latex of any plant so far. Well-known class of probable defence and wound-induced proteins in sieve elements are phloem lectins, identified in *B. napus*, cucurbit, pumpkin and *Arabidopsis thaliana* (Dinant et al., 2003; Walz et al., 2004; Giavalisco et al., 2006). These proteins were not identified in the present study in *Ch. majus* milky sap, but were reported previously (Fik et al., 2000). Another class of well-known defence proteins are protease inhibitors, which are widespread in phloem sap of different plant species (Kehr, 2006), but were not identified in latex of any species. It should be also mentioned, that opium poppy latex and phloem sap of *B. napus* contain other antioxidant enzymes connected to glutathione metabolism, like glutathione-*S*-transferase or dehydroascorbate reductase and chaperones, like heat shock proteins (Decker et al., 2000; Giavalisco et al., 2006), which were not detected in the present study.

Comparing 2-DE protein profiles of *Ch. majus* milky sap collected in May to those collected in October (Fig. 1), it should be noted that October extract is clearly less complex. In general, our results showed that extracts of *Ch. majus* milky sap collected in autumn seemed to be less complex than those collected in spring and summer (data not shown), possibly due to forthcoming winter season. Nonetheless further research is necessary to understand seasonal changes in protein composition of *Ch. majus* milky sap.

3. Conclusions

To our knowledge, the present study demonstrates for the first time the protein composition of the Greater Celandine milky sap. It contains a diversity of defence-related and pathogenesis-related (PR) proteins. Results of this study show that the protein content of *Ch. majus* milky sap is similar in composition to proteins identified in the latex of opium poppy (*P. somniferum*), but in lower complexity (Decker et al., 2000). Some of the identified proteins were also found in the phloem sap of other plant species, e.g. pumpkin (*C. maxima*) and cucumber (*C. sativus* L.) (Walz et al., 2002, 2004; Kehr, 2006). The specific protein composition of the milky sap suggests an important role of these proteins for the whole plant physiology and development.

The results indicate that the majority of identified proteins are involved in stress and defence responses against different pathogens. Further functional analyses are important to confirm these results.

4. Experimental

4.1. Plant material

Ch. majus plants were collected in the neighbourhood of Poznan in different seasons of the year, from the early summer until autumn. A voucher specimen is deposited in the Department of Molecular Virology, Faculty of Biology, Adam Mickiewicz University in Poznan. The milky sap samples were collected from *Ch. majus* plants which grew in several locations, representing similar shady, humid, ruderal environments. Samples of the milky sap were collected repeatedly every two weeks from each location from adult *Ch. majus* plants of similar developmental stage (height of the plant ca. 50 cm). Each milky sap sample was taken from different *Ch. majus* plants. The stems of adult *Ch. majus* plants were cut, the surface was dried with filter paper, and the exuding orange milky sap was collected using plastic micropipettes (Eppendorf, Hamburg, Germany). Depending on the plant and the sampling site, up to 500 μl sample was collected from one stem using this method. The samples were directly dissolved in 0.1 M Tris–Cl buffer, pH 8.0, containing 10% glycerol (sap: buffer ratio was 1:2). The milky sap (33% v/v) samples were separated into a supernatant, referred to as a protein extract, and a pellet fraction by centrifugation at 12,000 rpm for 20 min at 4 °C as described in Fik et al. (2000), with modifications. Supernatants were stored at −20 °C for further analysis. Each sample was analysed at least twice by subsequent 2-DE. Protein concentration in the milky sap was determined according to Lowry et al. (1951).

4.2. 2-DE

Preparation of the gels and electrophoresis was performed according to Kalinowski et al. (2002). For the polymerization of polyacrylamide gels for the first dimension (IEF) glass tubes (1.2 × 75 mm) were used. Polyacrylamide column gels were prepared according to Mulcahy et al. (1981). Each column gel was 60 mm long and contained a mixture of Servalytes pH 3–7 and pH 3–10 (4:1 v/v). A 7 μl aliquots (ca. 0.5 μg protein) of the samples were loaded onto the first dimension gel. After IEF (3 h, 50–300 V), the column gels were transferred for 2 min to the buffer (0.1 M Tris–Cl, pH 6.8) with 0.1% w/v SDS and then placed on the top of the polyacrylamide slabs (60 × 60 × 1 mm). Vertical electrophoresis was conducted at 4 °C (15 mA/150 mm^2). In the second dimension, running gels containing 10% acrylamide and 50 mM Tris–glycine buffer, pH 8.9, containing 2 mM EDTA with 0.1% w/v SDS as a running buffer were used.

Separation of the proteins in the second dimension was conducted under 34 V for up to 22 h.

4.3. 1-D SDS–PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in a slab mini-gel apparatus according to Laemmli (1970), using 10% polyacrylamide as the separating gel and 5% polyacrylamide as the stacking gel as an initial analysis of the milky sap protein composition. The proteins were reduced by heating them to 100 °C in the presence of 2-mercaptoethanol for 5 min. Gels after SDS–PAGE were fixed and stained with silver according to Heukeshoven and Dernick (1985).

4.4. Staining

1-D and 2-D gels were stained with silver according to Shevchenko et al. (1996), which is compatible with MS. Initial 2-D gel stains were performed according to Heukeshoven and Dernick (1985) to better visualize all the spots in the samples. Images were digitalized with a SHARP JX-330 scanner using the LabScan software. The data processing was carried out using Image Master 2-D Elite (Amersham Biosciences) software. Experimental M_r values were calculated by mobility comparisons with protein standard markers (Fermentas).

4.5. Nuclease activity assay

An in-gel DN-ase assay was performed according to Thelen and Northcote (1989), with modifications (Ito and Fukuda, 2002). The milky sap protein extracts were dissolved in SDS–PAGE sample buffer without a reducing agent, incubated at 37 °C for 10 min, and subjected to 1-D SDS–PAGE on 10–12% polyacrylamide gel containing denatured calf thymus DNA (40 μg/ml). After electrophoresis and removal of SDS, gels were washed with reaction buffer (10 mM Tris–Cl, pH 8.0, containing 10 mM $CaCl_2$). DN-ase activity was visualized by staining the gel with ethidium bromide.

For 2-D in-gel DN-ase assay a 7 μl aliquots (ca. 0.5 μg protein) of the samples were loaded onto the first dimension gel. After IEF (3 h, 50–300 V), the column gels were transferred for 2 min to the buffer (0.1 M Tris–Cl, pH 6.8) without SDS and then placed on the top of the polyacrylamide slabs ($60 \times 60 \times 1$ mm). Vertical electrophoresis was conducted at 4 °C (15 mA/150 mm^2). In the second dimension, running gels containing 10% polyacrylamide gel with denatured calf thymus DNA (40 μg/ml) and 50 mM Tris–glycine buffer, pH 8.9, containing 2 mM EDTA with 0.1% w/v SDS were used as a running buffer. After separation and removal of SDS, gels were washed with reaction buffer (10 mM Tris–Cl, pH 8.0, containing 10 mM $CaCl_2$). DN-ase activity was visualized by staining the 2-D gel with ethidium bromide.

RN-ase activity was assessed as described by Yen and Green (1991), with some modifications (Bantignies et al., 2000).

4.6. LC-ESI-MS/MS analysis

Stained protein spots were excised from the gel and analysed by liquid chromatography coupled to mass spectrometer in the Laboratory of Mass Spectrometry, Polish Academy of Sciences, Warsaw, Poland. Samples were concentrated and desalted on RP-C18 precolumn (LC Packings, UK) and further peptide separation was achieved on a nano-HPLC RP-C18 column (LC Packings, 75 μM i.d.) of an UltiMate nano-HPLC system, using a 50 min linear acetonitrile gradient. Column outlet was directly coupled to Finnigan Nanospray ion source of LTQ-FT (Thermo, USA) mass spectrometer working in the regime of data dependent MS to MS/MS switch. An electrospray voltage of 1500 V and a cone voltage of 30 V were used.

4.7. Data analysis

Due to the poor protein and DNA sequence database coverage for *Ch. majus* obtained peptide data from the analysed spots were analysed automatically by database matching against the NCBInr protein database (NCBI, Bethesda, USA) with a *Viridiplantae* filter, using the MASCOT database search engine (Matrix Science, London, UK; www.matrixscience.com) (Perkins et al., 1999).

Acknowledgement

This work was financed by the Polish State Committee for Scientific Research (KBN) Grant No. 2P05F 041 27.

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