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Journal of Chromatography A, 1009 (2003) 105–110
JOURNAL OF CHROMATOGRAPHY A
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Analysis of proteins in the spent culture medium of *Lupinus albus* by electrospray ionisation tandem mass spectrometry

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Abstract

Lupinus albus cell cultures secrete a large set of hydrolases into their medium with a small number of highly abundant proteins. We have investigated the protein composition of the medium with two different methods, two-dimensional gel electrophoresis–electrospray ionisation tandem mass spectrometry (ESI-MS–MS) and enzymatic analysis. The proteomic approach revealed the presence of several abundant proteins that had been overlooked using standard enzyme assays, e.g. subtilisin-like protease, glucan 1,3-β-glucosidase, α-amylase, chitinase, thaumatin-like protein, and a secretory pathogenesis-related protein. Several low-abundant proteins were readily detectable by enzymatic assays (peroxidases, phosphatase), but could not be found by ESI-MS–MS. Both data sets support the assumed lytic function of the medium, which appears to be similar to that of the plant vacuole.
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Keywords: *Lupinus albus*; Proteins

1. Introduction

Lupinus albus suspension cell cultures secrete hydrolytic enzymes into their culture medium. Since most of these enzymes are typically present in the vacuole, it was suggested that the medium functions as an additional lytic compartment [1–3]. In addition, a limited number of proteins could be identified in the medium by electrophoresis and Edman degradation [4,5].

In recent years, new techniques have been developed in protein chemistry. By using immobilised pH gradients (IPGs) in the first dimension, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has become a reproducible high-resolution method. Furthermore, new mass spectrometry techniques [matrix-assisted laser desorption ionisation (MALDI), electrospray ionisation tandem mass spectrometry (ESI-MS–MS)] allow the analysis and identification of separated proteins in nanogram quantities. Additionally, the human and other genome projects of recent years have enlarged protein databases to facilitate protein identification. As a result, many of the proteins from organisms whose genome has not yet been sequenced can be identified by homology criteria.

In order to further investigate the protein composition secreted into the medium of *Lupinus albus*, we separated the medium proteins by 2D- and 1D-PAGE. Via ESI-MS–MS analysis we could identify several plant defence-related proteins which typically accumulate in the vacuole and the extracellular space. Additionally, enzymes described to accumu-

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doi:10.1016/S0021-9673(03)00622-8

late in the spent medium were detected using *p*-nitrophenyl (PNP)-labelled substrates. By combining both methods the set of identified proteins which are secreted into the medium could be expanded. Since the newly identified proteins mainly exhibit antifungal activity, these data further support the predicted lytic function of the cell culture medium.

2. Experimental

2.1. Cell culture

Lupinus albus suspension cell cultures were grown in 50 ml/flask on PM6 medium in a 7-day cycle under constant light conditions and shaking [6]. The spent medium was harvested by filtration. Cell growth was monitored as the increase in fresh weight. Inorganic phosphate was determined after complex formation with ammoniummolybdate at 850 nm.

2.2. Electrophoresis

One-dimensional sodium dodecylsulphate (SDS)–PAGE was performed using a Hofer electrophoresis chamber (Amersham Bioscience, Freiburg, Germany) [7]. Proteins were precipitated from the medium by addition of 20% trichloroacetic acid. After incubation for 2 h at 4 °C, two acetone washing steps followed. Proteins were run in Laemmli buffer (50 m*M* Tris–HCl pH 6.8, 2% SDS, 25% glycerol and 0.01% bromophenol blue) at 15 mA (stack gel) and separated at 30 mA. For two-dimensional gel electrophoresis an IPGphor system (Amersham Bioscience) was used [8]. Precipitated proteins were redissolved in rehydration buffer {8 *M* urea, 2% 3-[(cholamidopropyl) dimethylamino]-1-propanesulfonate (CHAPS), 30 m*M* 1,4-dithio-DL-threitol (DTT), 0.5% IPG buffer and 0.01% bromophenol blue} and applied onto strips for rehydration (rehydration 12 h at 50 V). The sample was focussed under the following conditions: 200 V for 1 h, 500 V 1 h, 1000 V 1 h, gradient to 8000 V 1 h, 6 h 8000 V. IPG gels were equilibrated for the second dimension in 10 ml equilibration buffer (6 *M* urea, 50 m*M* Tris–HCl pH 6.8, 2% SDS, 30% glycerol) containing 2% DTT for 15 min. Reduced disulfide bonds were alkylated by a second 15-min equilibration step using 2.5% iodoacetamide in the absence of DTT. Fine chemicals were obtained from Sigma–Aldrich (Munich, Germany).

2.3. Tryptic digestion and mass spectrometry

Excised gel plugs were washed with 100 μl water, 100 μl 50% acetonitrile and subsequently shrank in 100 μl acetonitrile. Modified trypsin (Promega, Madison, WI, USA) (12 ng/μl) in 40 m*M* ammonium hydrogencarbonate buffer was added and incubated overnight at 37 °C. Custom-made chromatographic columns were used for desalting the supernatant of the tryptic digest. A column consisting of 20 μg Poros R2 material (50 μm bead size; PerSeptive Biosystems, Darmstadt, Germany) in 5 μl 75% methanol–1% acetic acid–24% water was packed in a constricted GELoader tip (Eppendorf, Hamburg, Germany). After equilibration with 1% acetic acid the sample was loaded, the column was washed with 4 μl 1% acetic acid and peptides were eluted with 0.5 μl 75% methanol–1% acetic acid directly into a precoated borosilicate nanoelectrospray needle (New Objective, Woburn, MA, USA). MS analysis was performed on a quadrupole time-of-flight (Q-TOF) mass spectrometer (PE Sciex, Weiterstadt, Germany) equipped with a nano-ESI ion source (MDS Protana, Odense, Denmark). A potential of 720 V was applied to the nanoelectrospray needle. The declustering potential and focusing potential was set to 40 and 100 V, respectively. Fragmentation of selected peptides (unit resolution) was usually performed at three different collision energies (22, 27 and 35 V). Amino acid sequences were deduced manually or by the Bioanalyst software (PE Sciex) and searched against the NCBI non-redundant database using Mascot (Matrix Science, London, UK).

2.4. Enzymatic analysis

The bicinchoninic acid method was used to measure the protein concentration (Pierce, Rockford, IL, USA). The activity of phosphatases, galactosidases, glucosidases and mannosidases was determined spectrometrically with *p*-nitrophenyl-labelled substrates (Sigma–Aldrich) as described [1–3]. Release of *p*-

Table 1
Composition of the spent medium of *L. albus*. Comparison of the enzymatic analysis and SDS–PAGE coupled ESI-MS–MS

Identified protein	Enzymatic analysis	SDS–PAGE with ESI-MS–MS	Catalysed reaction	Predicted function	Ref.
Phosphatase	++	−	P_i release	Lytic	[1–3,10]
Peroxidase	++	−	Peroxide reduction	Lytic	[1–3,11]
DNAse	+	−	DNA degradation	Lytic	[1–3]
α-Mannosidase	+	−	PS degradation	Lytic	[1–3]
α-Galactosidase	+	−	PS degradation	Lytic	[1–3]
α-Amylase	−[a]	+	Starch degradation	Antiviral?	[1,12]
β-Glucosidase	+	+	PS degradation	Antifungal	[1–3,14]
Subtilisin-like protease	−	+	Protein degradation	Antifungal	[15,16]
Acidic chitinase	−	+	Chitin degradation	Antifungal	[14,17]
Thaumatin-like protein	−	+	Unknown	Antiviral	[4]

PS, polysaccharide. +, Protein identified; −, protein not identified; ++, enzymes exhibiting highest activity.
[a] Identified after ion-exchange chromatography.

nitrophenol was measured at 405 nm after 30 min incubation at 37 °C. A molar absorption coefficient of 1.8 mM^{-1} cm^{-1} was used to determine the concentrations [9]. α-Amylase activity was analysed using starch as substrate. Release of maltose was measured using a colometric 3,5-dinitrosalicylic acid test. The activity of peroxidase and DNAse was detected spectrometrically using a standardised assay (Sigma–Aldrich) [1–3].

3. Results and discussion

Proteins secreted into the medium were identified and characterised using two different methods: enzymatic analysis and ESI-MS–MS following one- and two-dimensional gel electrophoresis. Results of both methods were compared with respect to the proteins identified (Table 1), the selectivity and the sensitivity of the methods (Table 2).

3.1. Enzymatic analysis of the spent medium

Several specific substrates were selected in accordance with the enzymes found in lytic compartments [1–3]. Enzymatic activity was detected in the spent medium without further purification or enrichment of proteins. The enzymes α-mannosidase, β-galactosidase, α-glucosidase, phosphatase, peroxidase and

Table 2
Overview of the selectivity and sensitivity of the mass spectrometry and enzymatic analysis

	Enzyme assay	SDS–PAGE+ESI-MS–MS
Information obtained	Activity	Sequence, molecular mass
Advantage	Identification directly from the medium, test in a physiological environment	No preselection of enzymes, identification of posttranslational modifications
Selectivity	Only proteins that exhibit catalytic activity	Only proteins abundant on gel
Sensitivity	Nine units (PNP substrate)	10 ng Protein (silver staining)
Instrumental requirements	Low	High

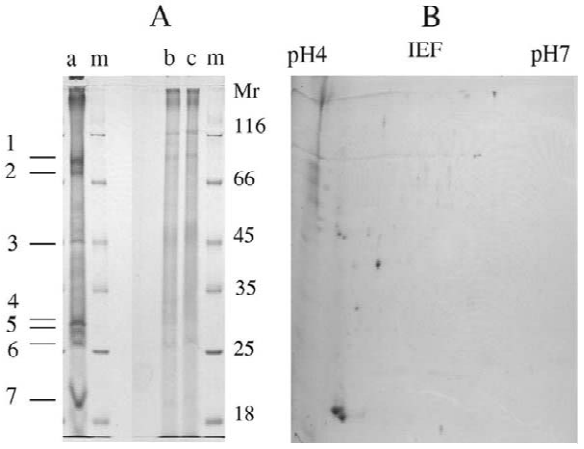

Fig. 1. One- and two-dimensional separation of extracellular matrix proteins. Medium proteins separated on two-dimensional gels at pH 4 to 7 (A) were silver stained, and the protein on 10% SDS–PAGE (B) was coomassie stained. Extraction of the TCA precipitation pellet after two acetone washing steps with water was sufficient to resuspend almost all proteins (A, line a). A further extraction with 2% SDS (A, line b) and 10% SDS (A, line c) shows that none of the proteins remain in the pellet. The highly abundant proteins marked (1–7) were excised from the gel and analysed further. Molecular masses ($\times 10^3$) were calculated according to the M_r of the marker proteins (A, line m). IEF, isoelectric focusing.

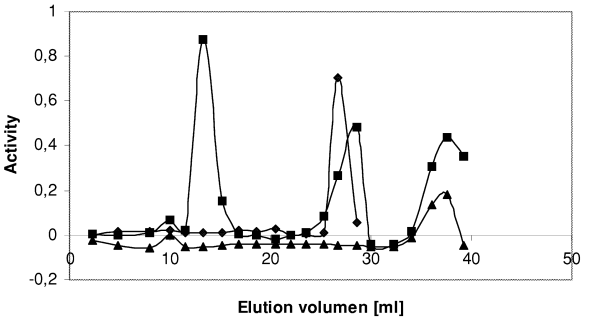

Fig. 2. DEAE-Sepharose ion-exchange chromatography of medium proteins. A desalted total protein extract from 100 ml medium (day 7) was bound to a DEAE column (bed height 12.5 cm) at pH 8.8. The flow through was discarded and bound proteins were eluted by a linear NH_4HCO_3 gradient (flow-rate 0.3 ml/min). Collected fractions were tested for (▲) amylase, (♦) phosphatase and (■) peroxidase activity. Fractions exhibiting high activity were pooled, TCA precipitated and further analysed by SDS–PAGE.

DNAse could be detected in the medium, supporting the suggested lytic function [1–3].

3.2. Analysis of extracellular proteins by gel electrophoresis and ESI-MS–MS identification

Proteins of the major bands of a 10% SDS–PAGE were excised and in-gel digested with trypsin. A two-dimensional separation of the concentrated protein of the spent medium results in a very small number of highly abundant proteins (Fig. 1). In the case of probes with low complexity, one-dimensional SDS–PAGE offers several advantages over 2D-PAGE (Table 3). Therefore, for further experiments one-dimensional SDS–PAGE was used.

All digests contained at least one peptide with an interpretable spectrum, allowing the unambiguous determination of an amino acid sequence. Six of seven proteins were identified from MS–MS spectra using the mascot database (Table 1). Proteins identified in the medium via ESI-MS–MS exhibit antimicrobial activity and accumulate in the vacuole and the extracellular space [14–16]. Additionally, an α-amylase was identified by ESI-MS–MS. The presence of α-amylase was confirmed enzymatically after DEAE anion-exchange chromatography (Fig. 2) and additionally with a specific substrate (amylopectin

Table 3
Advantages and limitations of 2D-PAGE and SDS–PAGE

	2D-PAGE	SDS–PAGE
Information obtained	Isoelectric point, M_r	M_r
Resolution	Up to 1000 proteins on one gel	Fifty distinct bands
Limitations	No extreme hydrophobicity, acidic, basic proteins	Only partial separation
Time requirement	2–3 Days	1 Day
Main application	Comparative studies	Fast separation

azur). The physiological function of extracellular α-amylase remains unclear, since starch accumulation and degradation take place in the chloroplast compartment. Viral induction of α-amylases has been reported recently, but other functions were also proposed [13,14].

Results from enzymatic analysis and ESI-MS–MS analysis overlap only partially (Table 4). The main reason for the difference observed is the differing selectivity of the two methods (see Table 1). This is particularly prominent in the case of phosphatases and peroxidases. Both enzymes exhibit high turnover numbers and therefore are readily detectable in enzymatic assays, although the respective proteins are present in low concentrations. All attempts to visualise the corresponding proteins by SDS–PAGE failed, even after enrichment and purification by ion-exchange chromatography (Fig. 2). As these results show, the traditional enzymatic analysis is an indispensable method for the detection of enzymes in low abundance.

In contrast, subtilisin-like proteases have a high cleavage specificity [18]. Therefore, the catalytic activity of a subtilisin-like protease is difficult to detect using standard protease assays and its presence can only be revealed by ESI-MS–MS.

The spent medium of plant suspension cell cultures is a good example of a low complex protein mixture with a large number of proteins in low abundance. In this case, both methods, enzyme assays and protein analysis, were found to complement each other. Each of the two methods itself supports the predicted lytic function of the medium. In combination, a more complex function of the medium, similar to that of the vacuole, becomes evident. Cytosolic proteins could not be detected in the spent medium, indicating that the proteins present are actively secreted and not due to release from dead cells [1–3].

4. Conclusions

Two methods with different selectivity have been used to investigate protein accumulation in a culture medium. Whereas enzyme analysis, based on high turnover rates, indicates a lytic function, ESI-MS–MS mainly identified highly abundant pathogen defence-related proteins. By combination of both data sets, a more precise view of the function of the medium was obtained. All identified proteins are known to accumulate in the vacuole or the extracellular space of plants in vivo. Therefore, our data support the predicted lytic function of the medium [1–3].

Acknowledgements

We are grateful to Andreas Wachter (Heidelberger Institut für Pflanzenwissenschaften, Heidelberg) and

Table 4
Tryptic peptides identified by mass spectrometric analysis. Spots are numbered chronologically with respect to their molecular mass (M_r). The score shown in parentheses displays the minimum significant score ($P<0.05$). Molecular masses of the listed proteins were calculated according to the marker proteins on SDS–PAGE

Spot number	Peptide sequence	Mascot score	Identified protein	Database accession	M_r ($\times 10^3$)	Calculated M_r ($\times 10^3$)
1	LGVQPSPVVAAFSSR	177 (74)	Subtilisin-like-protease	gi7435657	81	85
2	DLVTGFLK/IGAATALEVR	104 (40)	Glucan 1,3-β-glucosidase	gi11358952	68	77
3	GYAPSITK/LWDLDASK	65 (44)	α-Amylase	gi20336385	47	51
4	NDXPTQNSVGR	–	Unidentified	–		33
5	YGGVMLWDR/FNDXQSGYA[a,b]	63 (40)	Acidic chitinase	gi116330	32	29
6	TVDQXWQDYK[a]	104 (74)	Secretory pathogenesis-related protein	gi10140695	29	27
7	DVAAGTTQR[a]	–	Thaumatin-like protein	gi22830597	25	19

[a] Proteins identified via homology of peptides (maximum one variable amino acid).
[b] C-terminal peptide, partial sequence.

Jens Treutlein for their critical comments and Anita Ring for technical assistance.

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