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Preliminary communication

IDENTIFICATION OF HEAT SHOCK PROTEINS FROM BACTERIA BY ELECTROPHORETIC SEPARATION AND NANOFLOW LC-MS/MS

E. SZANICS[a]*, B. DEVREESE[b] and J. VAN BEEUMEN[b]

[a]Central Food Research Institute, H-1022 Budapest, Herman O. út 15. Hungary
[b]Laboratory of Protein Biochemistry and Protein Engineering, Ghent University, KL Ledeganckstraat 35, B-9000 Gent. Belgium

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Examination of heat shock and PR ("pathogenesis-related") proteins is of special interest in food science. Many food allergens have a similar or the same structure as PR proteins, which are produced in the plants as a response to pathogenesis or certain environmental stresses. The protein set of the psychrophilic bacterium *Shewanella hanedai* was studied by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Gel patterns from control and heat-treated bacteria were evaluated by PDQUEST software. The differentially expressed proteins were excised from the gel and digested by trypsin. The tryptic peptides were analysed by nanoflow LC-MS/MS. On the basis of amino acid sequences obtained by this method, the proteins were identified by similarity searching in the protein database. Using this proteomic approach a heat shock and a 50S ribosomal protein were identified as the major heat induced proteins in *Shewanella hanedai.*

Keywords: heat shock protein, psychrophilic bacteria, two-dimensional polyacrylamide gel electrophoresis, automated nanoflow LC-MS/MS

The dramatic developments in gene technology during the last two decades have also had a profound influence on the focus of protein chemistry. The human genome is fully sequenced and genomes from a number of other organisms are also currently being investigated. The next step is to establish the functions of the proteins to investigate their mechanisms of action. The development of a comprehensive strategy combining high-resolution two-dimensional polyacrylamide gel electrophoresis with highly sensitive biological mass spectrometry and the availability of rapidly growing protein databases have paved the way for high-throughput proteomics. "Proteomics" involves the analysis of the expressed part of genome, i.e. the complete protein content of a living cell or organelle as influenced by its environment. This kind of analysis allows linking a particular phenotype to different expression levels of particular proteins. The current methodology requires the separation of the protein extracts via two-dimensional polyacrylamide electrophoresis, visualization of the proteins, recognition of individual

* To whom correspondence should be addressed.
Fax: +36 1 214 1249; e-mail: e.szanics@cfri.hu

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proteins that are differently expressed under given cell conditions and their mass spectrometric identification. Most proteomics data describe the use of MALDI-TOFMS to identify the proteins observed on a 2D-PAGE gel. MALDI-TOFMS is mainly used for the peptide fingerprinting method (JUNGBLUT & THIEDE, 1997). It is not suitable for homology based search routines, which excludes the application of the method in the analysis of organism for which little genomic information is available. We have demonstrated that automated nanoflow LC-MS/MS allows de novo peptide sequence determination from tryptic fragments from 2D-PAGE separated proteins. This method can be useful for the study of organisms of which a genome sequencing project has not yet been set up (DEVREESE et al., 2001). Many of the known plant food allergens are homologous to pathogenesis-related proteins, proteins that are induced by pathogens, wounding or certain environmental stresses. Heat shock protein-related epitopes are common allergenic determinants for cereals. Electrophoretic methods and immunoblot techniques are used for identification of the allergenic determinants (MATUZ et al., 2000; BREITENEDER & EBNER 2000; CHIUNG et al., 2000). To evaluate the capabilities of our proteomics approach to analyse this group of proteins, we established a preliminary study on the heat shock response of a bacterium of which no genomics study has been performed.

1. Materials and methods

1.1. Bacterial growth and preparation of extracts

Shewanella hanedai was grown aerobically overnight in 200 ml Marine Broth medium at 16 °C. For heat treatment, overnight grown culture was incubated at 37 °C for 1 h. The cells were centrifuged and washed twice using a 50 mM Tris-HCl solution (pH 8). The bacteria were lysed using 8 M urea, containig 40 mM Tris, 4% 3-[(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.05 (m/v)% DNase I, after which the pellets were centrifuged at 13200 RPM.

1.2. 2D-PAGE

Hundred µl of the bacterial extract (±200 µg of protein as determined by the Bradford protein concentration test) was loaded on 18 cm immobilized pH gradient (IPG) strips, pH range 4–7 (Pharmacia) via the passive in-gel rehydration protocol (7 h) described by SANCHEZ and co-workers (1997). The isoelectric focusing (IEF) was performed using a Multiphor II system (Pharmacia) running a standard program as provided by the manufacturer. After completion of the IEF program, the strips were equilibrated in a 50 mM Tris-HCl solution (pH 8.8) containing 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS) and 1% dithothreitol (DTT) for 10 min, after which the solution was replaced with the same solution expect that DTT was replaced by 5% iodoacetamide. The strips were then loaded on the home-casted vertical SDS-PAGE gels and were subjected to electrophoresis in a Bio-Rad Protean system at 20 mA for

15 min, followed by a ±5 h run at 50 mA until the bromophenol blue front reached the bottom of the gel. After completion of the run, the gels were stained with Coomassie Brillant Blue.

1.3. In situ digestion

After the Coomassie-staining gel samples from control and heat-treated bacteria were evaluated by PDQUEST software. Proteins of which differential expression could be demonstrated, were cut from the gels. Protein digestion was performed using a slightly modified protocol from ROSENFELD and co-workers (1992). The gel pieces were destained by washing twice with 150 µl of 50 mM ammonium bicarbonate in 50% acetonitrile/water (20 min at 30 °C) and were dried at room temperature. The tubes were chilled on ice and 8 µl of digestion buffer (50 mM ammonium bicarbonate) containing 100 ng modified trypsin were added. The samples were kept on ice for 45 min to allow the enzyme to enter the gel. Twenty µl of digestion buffer were added and left at 37 °C overnight. The supernatant was recovered and the remaining peptides were extracted from the gels by washing twice with 60% acetonitrile/0.1% formic acid in water. The extracts were collected and the sample dried in a Speedvac. The samples were redissolved in 12 µl of 0.1% formic acid.

1.4. Nanoflow LC/MS/MS

Coomassie Blue stained spot was loaded on a commercial nano-HPLC system, i.e. an Ultimate Micro LC system combined with a FAMOS autosampler system (LC-Packings, Amsterdam, The Netherlands). The system contains a classical reciprocal pump system at 150 µl min^{-1} with a built-in flow splitting system to reduce the flow rate to 100 nl min^{-1}. The samples were loaded onto the column (PEPMAP, 75 µm i.d., 15 cm, LC-Packings) using on-line preconcentration step on a micro precolumn (800 µm i.d., 2 mm) cartridge. The washing step was performed using 0.1% formic acid/water delivered at 10 µl min^{-1} by a 130 A syringe pump (PE Biosystems, Foster City, CA, USA). After 10 min, valve A was switched to connect the precolumn to the separating column and the gradient was started. A linear gradient from 5% acetonitrile/0.1% formic acid in water to 80% acetonitrile/0.1% formic acid in water over a period of 30 min was applied. The outlet of the column was directly connected to a home-made electrospray ionization device that holds a New Objectives nanoelectrospray needle, fit on a Q-TOF mass spectrometer. This system allows automated MS to MS/MS switching upon entry of a peptide that produces a signal above a certain threshold.

1.5. Protein identification using database searching algorithms

The MS/MS spectra were interpreted manually. The derived sequences were used for similarity searching using the Blast algorithm (National Centre of Biotechnology Information (NCBI)).

2. Results and discussion

High resolution 2D-PAGE is very useful for separating complex protein mixture. Proteins extracted from *Shewanella hanedai* were separated by 2D-PAGE and detected by Coomassie Brillant Blue staining. Figure 1 shows the 2D-PAGE gel from a total protein extract from the control *Shewanella hanedai*. Figure 2 is the 2D-PAGE gel of a total protein from heat-treated bacteria. The proteins on the gels were evaluated with the PDQUEST software. Two major differentially expressed spots could be detected and these were submitted to identification. Figure 3 and Fig. 4 show the MS/MS spectra of a peptide obtained after tryptic digestion of the two individual spots, analysed via the nanoflow LC-MS/MS. A partial sequence was easily read from MS/MS spectrum, which was used for identification of the protein by similarity searching in the protein database. The determined partial sequences and the identification of the protein at spots are also included in Table 1. On the basis of comparison of control and heat-treated *Shewanella hanedai* bacterium a heat shock and a 50S ribosomal protein were identified as the major heat induced proteins. The heat shock protein is similar to the Hsp60 family of proteins which are known as major stress induced proteins and are chaperones involved in protein folding (CHIUNG et al., 2000). The allergenic character of these proteins is well established. This demonstrates that the used set-up and the nanoflow LC-MS/MS strategy are useful for the identification of food allergen, also from organisms of which little or no genomic information is available.

Table 1. Summary of MS/MS data obtained from two spots of 2D-gel of heat-treated *Shewanella hanedei*, and identification of the proteins

Spot number	Sequence deduced from spectra	Protein identity
1	YLYQGLAER	Heat shock protein
2	ANLGNLGDQVAVK	50S Ribosomal protein

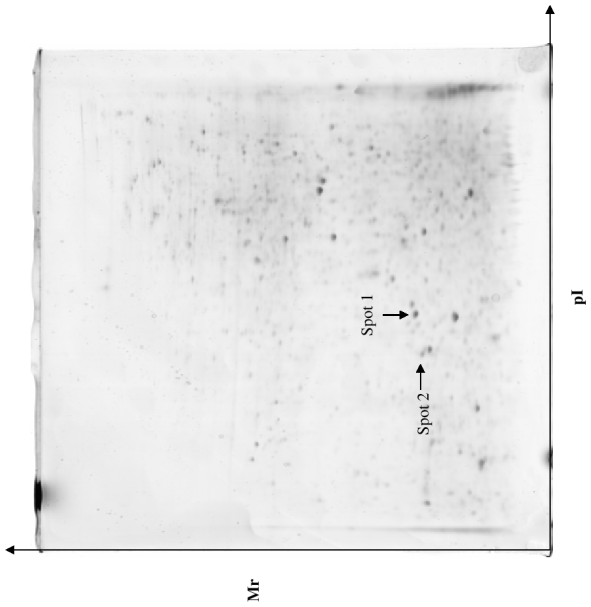

Fig. 2. 2D-PAGE gel of a protein extract from the heat-treated *Shewanella hanedai*

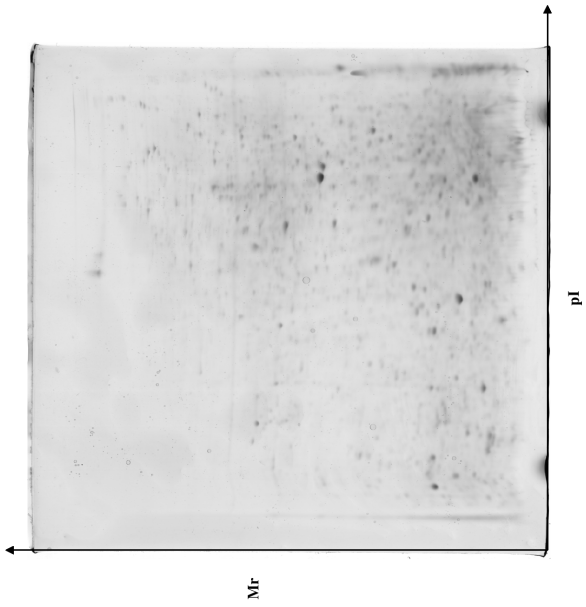

Fig. 1. 2D-PAGE gel of a protein extract from the bacterium *Shewanella hanedai*

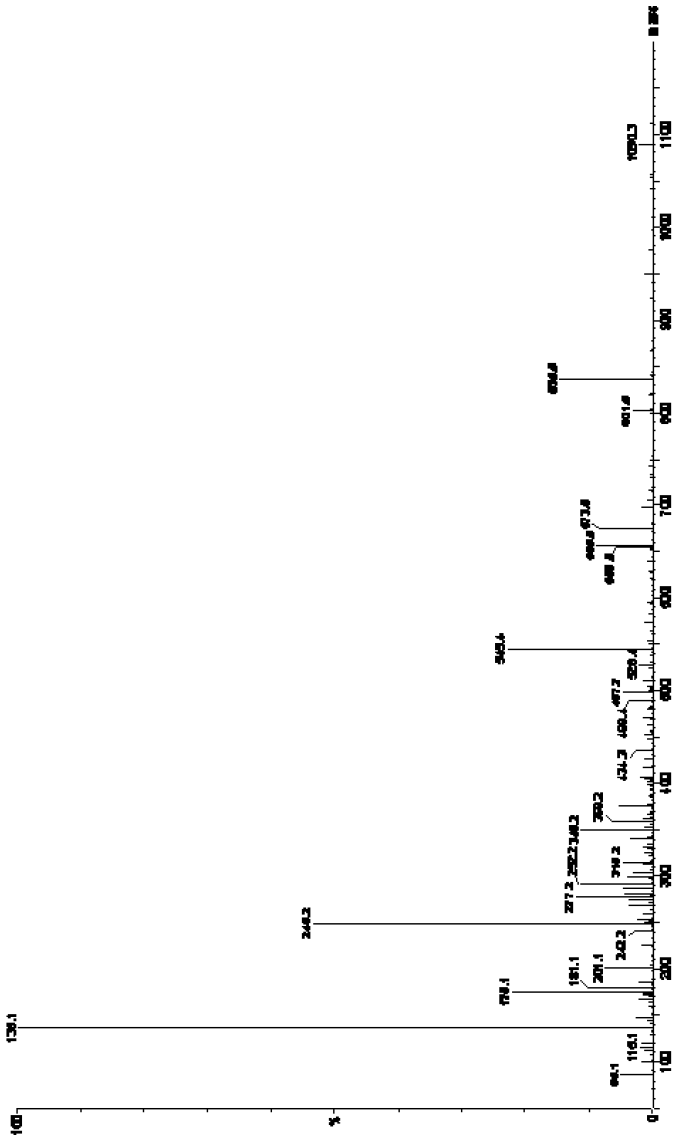

Fig. 3. MS/MS spectrum of the heat shock protein obtained by nanoflow LC/MS/MS from a tryptic digestion of spot 1

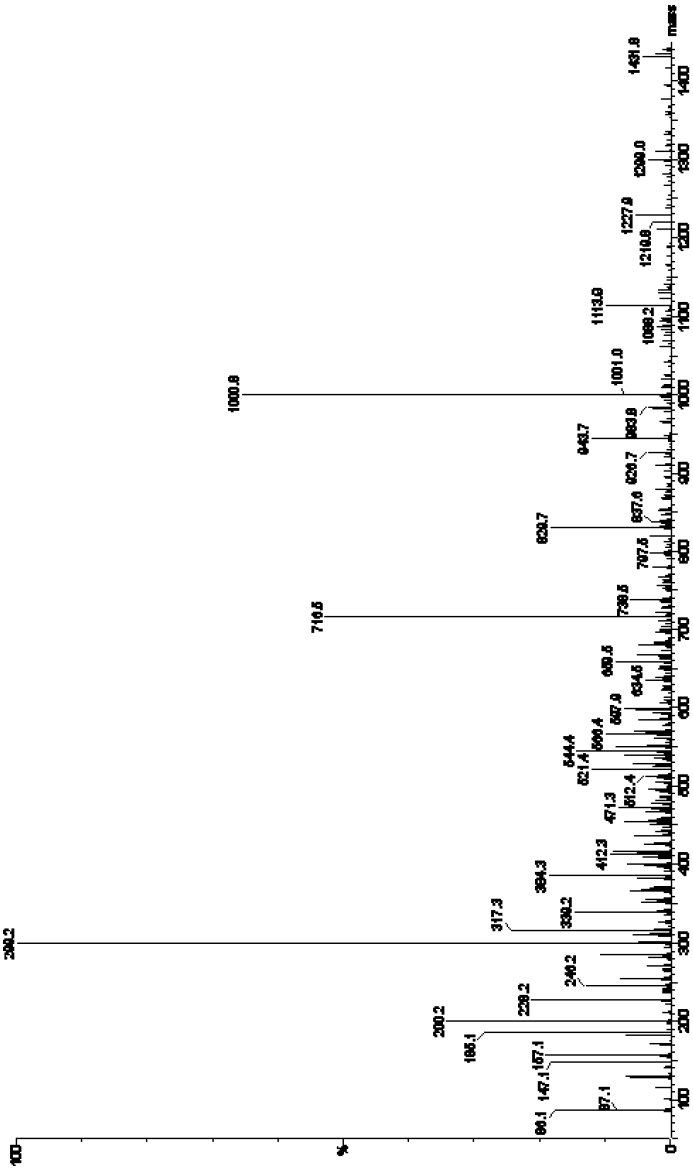

Fig. 4. MS/MS spectrum of the 50S ribosomal protein obtained by nanoflow LC/MS/MS from a tryptic digestion of spot 2

3. Conclusion

Two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients (IPG-DALT) has a unique capacity for the resolution of complex mixtures of proteins. In our paper, we employed this technique to study the protein set of *Shewanella hanedai*. The differentially expressed proteins were identified by nanoflow LC-MS/MS, which allows de novo peptide sequence determination. It is clear that the combination of high resolution 2D-PAGE and automated nanoflow LC-MS/MS is an extremely powerful tool in the enhancement of our knowledge of an ever increasing number of organisms.

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