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Hydrazine toxicity in rat hepatocytes studied by proteome analysis

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Title (English)

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Abstract

Hydrazine (HY) is a hepatotoxin. It has been shown to affect numerous crucial cellular processes. Here, the mechanism behind the toxicity of HY was investigated by proteomic technologies. Rat hepatocytes were isolated and incubated with various concentrations of HY (0, 1, 5, 10 mM) for 24 hours. The proteins were extracted from the cells and separated by two-dimensional (2-D) gel electrophoresis. The 2-D gel patterns were compared to each other. 62 proteins were found with altered expression upon stimulation with HY. Three of these were successfully identified as Erp29, transthyretin and thioredoxin peroxidase using mass spectrometry or matching. The exact role of these three proteins in HY hepatoxicity remains to be elucidated.

Keywords
Hydrazine, proteomics, 2-D gel electrophoresis, mass spectrometry, primary hepatocytes

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SUMMARY IN SWEDISH

Populärvetenskaplig sammanfattning

Hydrasin (HY) är en giftig substans, men är trots det en vanligt förekommande kemikalie. Den används bl a som raketbränsle, färgämne och för att förhindra rost. HY är också en av nedbrytningsprodukterna till två vanliga läkemedel. HY har visat sig vara hepatotoxiskt, dvs det har en skadlig effekt på levern. ATP-nivå, proteinsyntes och lipidmetabolism är några av de cellulära processer som påverkas. Den bakomliggande orsaken till HY:s toxicitet är dock fortfarande okänd. I detta examensarbete har därför HY:s toxicitet valts att studeras närmare.

Proteiner från leverceller (hepatocyter) behandlade med olika doser av HY extraheras fram och separeras i två dimensioner på en gel. Proteinerna bildar prickmönster på gelen. Intensiteten på en prick motsvarar mängden av protein i just den pricken. Genom att jämföra prickmönstret från behandlade celler med mönstret från obehandlade celler kan man få en uppfattning om vilka proteiner som upp- eller nedregleras till följd av HY-behandlingen. Dessa proteiner kan sedan identifieras med hjälp av masspektrometri och förhoppningsvis fås en indikation om vilka proteiner som är involverade i HY:s toxicitet.

Resultaten visar att 62 proteiner förändrade uttrycksnivå efter behandling med HY. Tre av dessa var Erp29, transthyretin och thioredoxin peroxidas. För att utröna rollen av dessa proteiner i hydrasins toxiska mekanism krävs ytterligare experiment.

Helena Persson

Examensarbete 20 p i Molekylär bioteknikprogrammet
Uppsala universitet, april 2002

CONTENTS

ABBREVIATIONS

ATP	adenosine triphosphate
BVA	biological variation analysis
DTT	dithiothreitol
2-DE	two-dimensional electrophoresis
DIA	difference in-gel analysis
ER	endoplasmatic reticulum
HCCA	α-cyano-4-hydroxycinnamic acid
HSP	heat shock protein
HY	hydrazine
IEF	isoelectric focusing
kDa	kilo Dalton
MALDI	matrix assisted laser desorption ionisation
MS	mass spectrometry
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
pI	isoelectric point
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
TOF	time of flight
TH	thyroid hormone
TP	thioredoxin peroxidase

INTRODUCTION

Even though hydrazine (HY) has been shown to be toxic, it is a commonly used chemical with a broad range of applications. It is used for instance as rocket fuel, corrosion inhibitor, dye and photographic chemical, as well as in the treatment of terminally ill cancer patients [1]. HY is also a metabolite of two commonly prescribed drugs, isoniazid and hydralazine. Isoniazid is used to treat tuberculosis, while hydralazine is a blood pressure reducing agent [2]. HY is known to be hepatotoxic, i.e. it has a toxic effect on the liver. However, the underlying mechanism is still not fully understood.

The main aim of this Degree project was to investigate the toxicity mechanism of hydrazine by proteomic technologies. The approach used is based on the observation that proteins, whose expression is strongly affected by a certain drug, can provide clues about a possible mechanism of drug action [3]. Just as abnormal changes in protein expression can be the cause of some diseases, it has been suggested that the up- or down-regulation of some proteins may in fact constitute the drug mechanism itself. Since HY is a hepatotoxin, a model system with primary hepatocytes (liver cells) was established. The proteomic analysis was performed using two-dimensional gel electrophoresis in combination with mass spectrometry and data base searching.

A more long termed goal of this study is to find markers of toxicity, which on an early stage can predict mechanisms and side effects of future drug candidates. Preliminary results suggest that this may be a good working strategy, since drugs with similar mechanisms have been shown to produce similar protein expression patterns [3]. When a large enough library of proteomics patterns has been collected for compounds of known toxicity, it will be possible to use it to find the toxicity of novel compounds. This goal is however beyond the scope of this project.

This report is written to be easily understood by people who have not previously been introduced to proteomics. In the following background section the current status of HY research is reviewed and the concept of proteomics as well as the basics of the different techniques used are described.

BACKGROUND

HYDRAZINE

HY (NH_2-NH_2) affects crucial cellular processes. It has been shown to cause an increase in the accumulation of fat in the liver as well as causing liver necrosis [4]. The mechanism behind HY's hepatoxicity is still unknown, but it is believed to involve depletion of ATP and inhibition of protein synthesis [5, 6]. HY has also been seen to cause a decrease in glutathione levels and changed levels of NADH and NADPH have been observed [2, 4].

Although well documented at a physiological level, information on the effects of HY on a protein level is limited. Some work on individual liver proteins have been made. Dilworth and co-workers [4] have studied the effect of HY on two different heat shock proteins, Hsp 25 and Hsp 72/3. However, HY did not give any significant increase in any of these stress proteins. A more global approach has been attempted by a research group at the University of Southern Denmark [7]. Proteomics was applied on a human liver cell line. Preliminary results show that HY causes changes in a number of different proteins. Down-regulated proteins included proteins involved in protease inhibition, protein degradation, amino acid synthesis, carbohydrate metabolism and protein synthesis. The up-regulated proteins included proteins involved in lipid metabolism, e.g. apolipoprotein E. Apolipoprotein E plays a crucial role in lipid metabolism and its elevated expression is believed to be one of the reasons of the accumulation of fat in the liver, which is observed after HY treatment. Different proteins involved in energy production were found to be both up- and down-regulated.

These observations can partly help explain the hepatoxicity mechanism of hydrazine, but the picture is far from being complete.

PROTEOMICS

Proteomics is the study of the proteome, the total sum of all proteins expressed by a genome at any given time [8]. Just as genomics intends to map all the genes present in a genome, proteomics aims to map all proteins expressed in a given cell, tissue or organism. Proteomics includes the isolation, separation, identification and functional characterisation of the proteome. Proteomics is based on the idea that changes in the abundance of proteins will allow us to observe what they are doing. It provides a 'snap-shot' on what is going on in the cell. By

understanding how protein expression is regulated, under e.g. drug treatment or during a certain disease, researchers hope to get information about the involved proteins in a drug response or a disease mechanism [9].

Proteomics vs. Genomics and Transcriptomics

The flow of genetic information in a normal cell is from DNA via mRNA to protein, as illustrated in Figure 1.

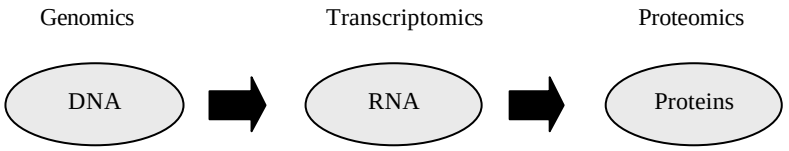

Figure 1. Flow of genetic information in a normal cell.

The genome is, unlike the proteome, rather static and essentially identical to all cells in an organism. It does not by itself reveal what proteins that will be expressed. The knowledge of the presence of a certain gene does not tell whether that gene will be transcribed and translated. By using the genomic information differently, one genome can give rise to many different proteomes depending on e.g. environment (stress, drugs, developmental stage, etc.) or type of tissue. In addition, there are many more proteins in a proteome, than genes in a genome due to splicing and post-translational modifications (phosphorylation, glycosylation, methylation, etc.). There are more than 200 amino acid modifications known to occur *in vivo* [10]. In many human diseases it is an incorrect modification of a protein that gives rise to a disease. Most of these modifications cannot be deduced by studying the genome [11]. Another reason for studying the proteome rather than the genome is that the targets for nearly all drugs used are proteins [12].

The protein expression can also be studied by looking at the mRNA levels. It could be expected that mRNA expression would reflect exactly the same as the proteome. However, the mRNA levels do not always show good correlation with its corresponding protein [13]. A protein cannot be synthesised without its mRNA being present, but a protein can be present in the cell without its messenger being present and vice versa.

Theory of Methods

Two-dimensional electrophoresis (2-DE) in combination with mass spectrometry and data base searching is currently the most common way of doing proteomic analysis [9]. The basic workflow used in this study can be seen in Figure 2.

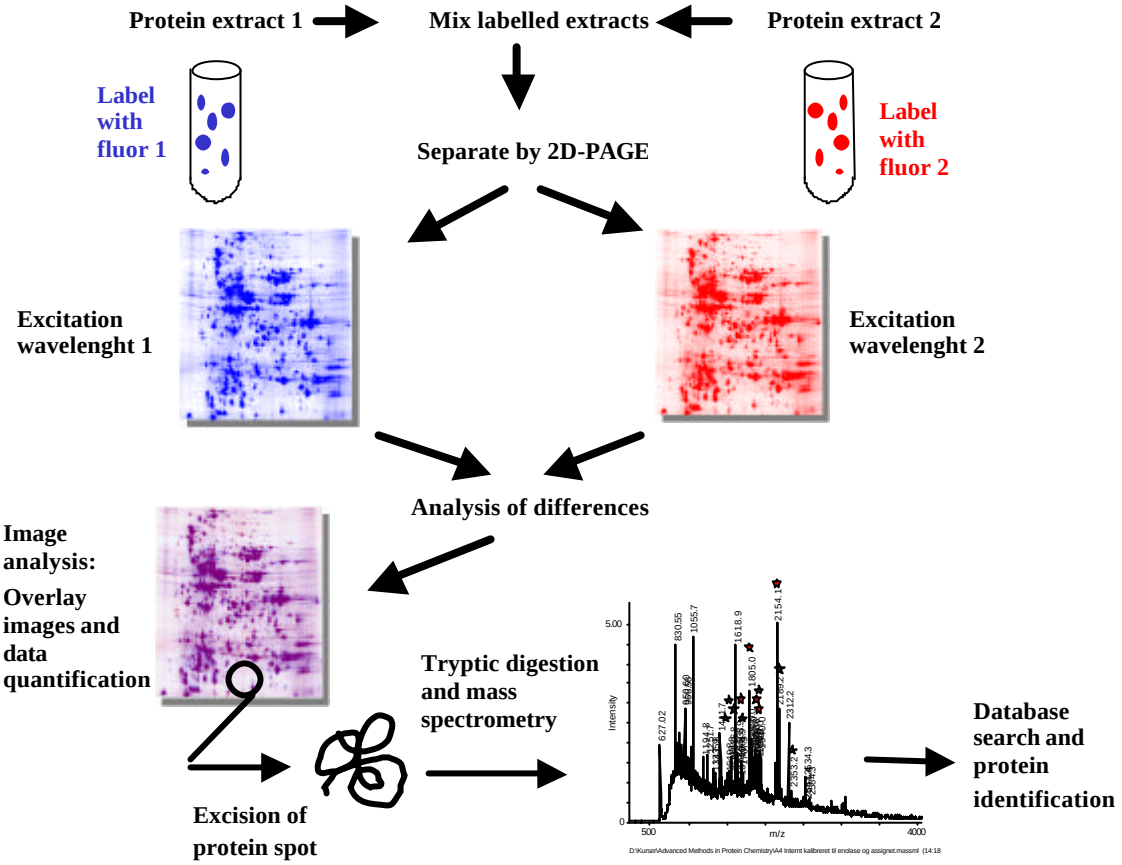

Figure 2. The basic workflow of this study.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)

2-D PAGE is used for separating and displaying components of complex protein samples, such as whole cell lysates [14]. As unveiled by the name, 2D-PAGE, the separation takes place in two dimensions, perpendicular to one another. In the first dimension separation is performed by isoelectric focusing, while the second dimension is performed by SDS polyacrylamide gel electrophoresis.

Sample Preparation

Pre-treatment of samples for 2-D PAGE involves cell lysis to free the proteins, solubilisation, denaturation and reduction to completely break up the interaction between the proteins, and labelling in order to visualise the proteins on the gel.

Isoelectric Focusing (IEF)

IEF is an electrophoretic technique that separates proteins according to their isoelectric point (pI), the pH at which their net charge is zero. The net charge of a protein is the sum of all negative and positive charges of its amino acid side chains and amino- and carboxyl-termini. The protein sample gets loaded to a pre-casted gel strip with a certain pH gradient. As the proteins enter the gel, the net charge of the proteins will change depending on the pH of their surroundings. Proteins are positively charged at pH values below their pI and negatively charged at pH above their pI. When the net charge of a protein is zero the electrophoretic mobility of that protein is also zero. Thus, a protein will move until it reaches the position on the gel where the pH corresponds to the pI of that particular protein. IEF can resolve proteins that differ in pI by as little as 0.01, which means that proteins differing by only one net charge can be separated [15]. However, the degree of resolution depends on the slope of the pH gradient.

SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

After IEF follows SDS PAGE. SDS PAGE separates polypeptides on the basis of their molecular size. The proteins are first dissolved in a solution of SDS. SDS is an anionic detergent that breaks almost all non-covalent interactions. It eliminates all tertiary and secondary structures and makes the proteins highly negatively charged. Dithiothreitol (DTT) is added to reduce disulfide bridges. Since the proteins will have almost the same form and mass-to-charge density, separation will be performed by molecular weight only. Small proteins move rapidly through the gel, while large ones stay at the top.

Visualisation of Proteins

The proteins are seen as spots on the gel. Ideally, each spot corresponds to a single protein. However, in order to be visible the proteins have to be labelled.

In this study, two kinds of gels are used, analytical and preparative gels. On the analytical gels, three spectrally distinct fluorescent dyes (Cy-dyes) are used, which allows the co-separation and visualisation of three different protein samples on the same gel. Each of the samples is labelled with a different fluorophore before mixing the samples together and separating them on the same 2-D gel. The samples are then imaged separately, giving rise to three different images. Since these images originate from the same gel, they can be overlaid and compared directly, making the analysis fast and accurate. However, when conventional dying techniques e.g. silver staining or Commassie blue® are used or a bigger experiment is run (comparison of more than three protein samples), images from different gels have to be compared. Unfortunately, there are often a lot of variations between gels and no two gels are directly super-imposable, making the analysis much more complicated (see below).

Another advantage of Cy-dyes, except that it simplifies the analysis and greatly decreases the number of gels that need to be run, is that it allows the use of one protein sample as internal standard.

However, Cy-dyes are not very well suited for protein identification by mass spectrometry. Cy-dyes cause a shift in the mass dimension by binding covalently to the lysine residues of the proteins. The labelling ratio is normally less than 5 %, i.e. more than 95 % of the protein sample is unlabelled [16]. A shift will thus be problematical when the protein is to be excised from the gel for MS analysis, since the visible protein spot does not correspond to the place where most of the protein is and the success of MS identification is highly dependent on sample amount. To solve this problem, SyproRuby together with larger sample amounts are used on preparative gels. SyproRuby is also a fluorescent dye, but since the proteins are stained after the separation shifting is not a problem. For this reason, Cy-dyes are used on analytical gels for identifying protein differences, while SyproRuby is used on preparative gels for spot excision and protein identification.

Pros and Cons of 2-D PAGE

For the past two decades 2-D PAGE has been the predominant technique of proteomics [14]. The reason for this is mainly its high resolution. On a typical gel around 2,000 protein spots can be separated, while the ‘best’ gels can separate over 10,000 proteins [8]. 2-DE also allows detection of different post-translational modifications due to shift in pI or/and molecular weight.

Fey *et al.* [17] argue that there may be more than five modification variants of each protein and that each of these variants plays specific roles. By studying these modification patterns the regulation of cellular activity can be investigated.

However, analyses of proteins by 2-DE is far from being trouble free. The major problem is that it is difficult to array all proteins of a complex sample on one gel. 2-D gels of crude samples fail in the ability to detect and analyse low-abundance proteins and proteins at the extreme of both pI and molecular weight. The detection of proteins expressed at low levels may be over-shadowed by high abundance proteins, which can be present at 10,000 times higher amount. Low abundance proteins are considered an important group since it includes receptors, regulatory proteins, and signal transduction proteins [8, 17]. Pre-fractionating cells into their various organelles before running the gels may help enrich for the desired protein. However, introducing additional steps may increase the experimental variation. It also makes the procedure much more complicated [17].

Protein analysis is technically more complicated than nucleic acid analysis. DNA and RNA are built up from four different nucleotides, while proteins have a basic alphabet of 20 amino acids. This makes proteins a very inhomogeneous group. They show widely different structure and chemical properties (e.g. polarity). As a consequence, it is almost impossible to find a single technique that can solubilise and separate all the different protein forms in a complex sample [18]. Especially the hydrophobic membrane proteins cause a lot of troubles. About 30 % of all proteins are estimated to be membrane proteins, but only about 1 % of these are actually resolved on the current 2D gels [17]. It is necessary to find a solution to this problem since many of these proteins, as well as the low abundance proteins, are common targets for drug development [8, 17].

Furthermore, proteins larger than 200 kDa are rarely seen on standard 2-D gels. Because of their size they do not readily enter the first dimension gel [19]. Proteins with very close pI and molecular weight may lead to co-migrating and overlapping spots. This can be partly overcome by running narrow and overlapping pH and molecular weight regions.

Another drawback of 2-DE is that it is not very reproducible between labs [12,14]. This makes it difficult to compare gels from different labs, something that could have simplified the analysis of the gels considerable.

Currently there is a lot of research going on trying to replace the 2-D gel technology. Protein arrays and capillary isoelectric focusing to separate proteins are a few techniques where a lot of work is done. However, none of these techniques currently have the resolution or the sensitivity of 2-D gels [14, 20].

Analysis of Gels

There are a number of different software programs available when it comes to analysis of two-dimensional gels. Analysis of the images in this study was carried out using DeCyder software (Version 3.5, Amersham Biosciences, Uppsala, Sweden). DeCyder consists mainly of two programs, DeCyder DIA (difference in-gel analysis) and DeCyder BVA (biological variation analysis).

The first step of the analysis is carried out in DIA. DIA automatically detects and quantifies protein spots. Boundaries are set around each spot that the program recognises as an individual protein. A filter can be applied to help the program distinguish proteins from noise, e.g. dust particles. Dust particles typically have a very high slope compared to proteins, as illustrated in Figure 3. By setting the program to detect peaks with less than a certain slope value, the dust particles can be ignored. In addition, minimum values of height, area and volume can be set. Even though a filter is used, artefacts often slip through. The spot finding therefore has to be manually edited so that any detected noise is removed. The DIA can then be used to compare differences of specific protein spots of images within a gel.

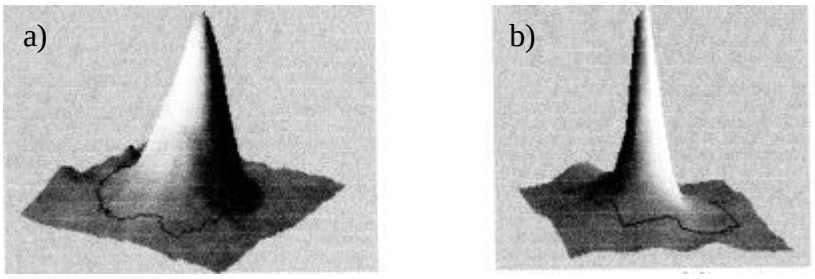

Figure 3. A protein spot usually has a smooth profile (a), whereas a dust particle will have a very steep slope (b).

Once a bigger experiment (more than one gel) is performed, BVA has to be used. BVA is unlike DIA capable of comparing many gels to each other. The DIA-processed images are imported into BVA. A master image is chosen and all other images are compared to this one. Approximately 50 spots common to the two gels (master and one other) being compared are assigned as landmarks or anchors and used to align and match the gel patterns. The program is then automatically set to match the rest of the spots with the help of the landmarks. This is however not straightforward as the automatic spot detection is far from being error-free. To ensure correct matching, matches therefore have to be manually edited. Another limitation with this program is that spot boundaries are not always the same on images from different gels. One protein spot in one gel may be divided into many small spots in another gel (Figure 4). Unfortunately, there is no function to manually change these borders.

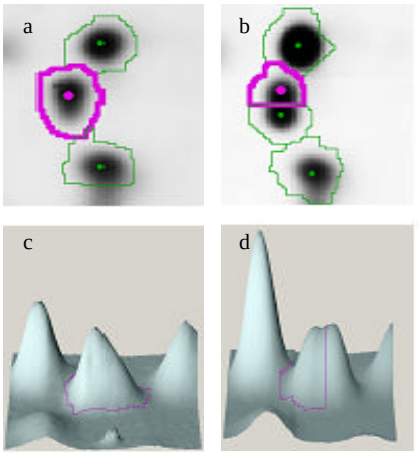

Figure 4. The upper two pictures show the same part of two different gels. In a, the middle spot is considered to be a single protein, while the same spot is split into two in b. 3-dimensional views of the spots are seen in c and d, representing a and b respectively.

A successful analysis of several gels requires the use of an internal standard. An internal standard helps to remove gel-to-gel variations seen between gels, which are not caused by the 'true' difference in protein concentration seen as a result of e.g. drug treatment. The internal standard is run on all the different gels and typically consists of a mixture of all the different protein samples being analysed.

The last step of the analysis is to identify the proteins of interest on the gel, e.g. find those proteins that are expressed differently in treated compared to non-treated cells. For changes in protein spots to be considered different, different criteria can be applied, e.g. the fold change in

protein abundance have to be above a certain value and have a certain statistical significance. These parameters can be found in a large list of variables that the software generates and the position of the interesting proteins can be obtained.

Even though a lot of the steps in the analysis process are automated, DeCyder still involves a lot of manual handling. All these manual steps make the process less robust. It increases user-to-user variability and errors are more easily introduced. The manual handling also makes the analysis a very time-consuming process and it is often considered the major bottleneck of proteomics [19].

Protein Identification by Peptide Mass Fingerprinting

Once proteins have been separated, visualised and quantified, it is time for identification. The method of choice in proteomics for identifying and characterising proteins is mass spectrometry combined with data base searching [17].

Digestion of Proteins

The spots are cut out from the preparative gel and the proteins are digested into peptide fragments by specific proteases. A commonly used enzyme is trypsin, which cleaves at the C-terminal side of arginine and lysine. The fragments are then analysed by MS.

Mass Spectrometry

There are a large number of different mass spectrometers. Although the principles of how they function and the types of samples that can be run on them differ greatly, they all share the ability of producing gaseous ions and separating them according to their mass-to-charge ratio (m/z). All mass spectrometers consist of three main components: ionisation source, m/z analyser, and detector.

In the following discussion focus will be on the type of mass spectrometry that is used in this study; matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS). The principle of the apparatus is illustrated in Figure 5.

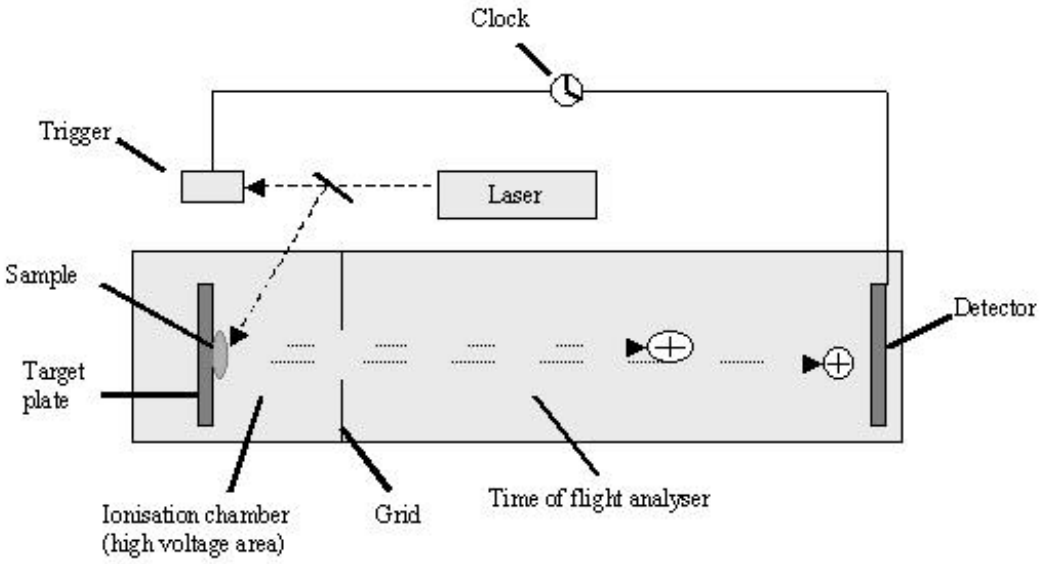

Figure 5. Schematic diagram of a MALDI TOF mass spectrometer.

The ionisation step starts with the mixing of the protein sample with a matrix solution. The functions of the matrix are to have a strong absorbance of energy at the laser wavelength and to isolate analyte molecules from each other to reduce intermolecular forces. The mechanism of ionisation is not totally understood [21]. However, the most accepted hypothesis suggests the following: a pulsing laser is used to irradiate the matrix-embedded sample. The absorbance of the laser energy by the matrix leads to the ejection of matrix particles. Clusters of single analyte molecules surrounded by neutral and charged matrix molecules are sputtered in the vacuum of the ionisation source. Proton transfer occurs between the photo-excited matrix and the analyte compound. The matrix molecules are evaporated and only ionised gaseous analyte molecules remain. The high voltage that exists between the target plate and the grid causes repulsion and acceleration of the positive charged peptides, which are drawn into field free drift tube (time of flight analyser) [21-23].

MALDI is a soft ionisation technique and it produces almost exclusively single charged ions. Since the following relationships hold:

$$E_{kin} = z \cdot e \cdot U \qquad (1)$$

(where z is the charge of the peptide, e is the elementary charge constant, and U is the voltage of the ionisation chamber) all the peptides will have the same kinetic energy as they pass the grid.

The kinetic energy can also be written as:

$$E_{kin} = \frac{m \cdot v^2}{2} = \frac{m \cdot \left(\frac{l}{t}\right)^2}{2} \qquad (2)$$

(where m is the mass of the analyte molecule, v is its velocity, l is the length from the target plate to the detector, and t is the time it takes for a particle to travel the distance l).

If equations 1 and 2 are combined m/z can be solved for:

$$\frac{m}{z} = 2 \cdot e \cdot U \cdot \left(\frac{t}{l}\right)^2 \qquad (3)$$

Since MALDI gives a z value of 1, the arrival time at the detector will depend only on the mass of the peptide. Smaller ions will travel faster than larger ions. A detector, which gets triggered by the laser pulse, records the time. The mass can then be calculated using equation 3 and the flight time of compounds of known masses, i.e. external or internal standards.

MALDI-TOF MS is extremely sensitive (it can detect less than 10 fmol of peptide), it has a large mass range (up to 500 kDa), and a high accuracy (0.01-0.05 %). Another advantage of MALDI MS is that it is very tolerant to the presence of salts, buffers, and other contaminants, which makes it a well-suited instrument in biological research [22, 23].

Database Searching

There are many different software tools available on the Internet for protein identification [22, 24]. The obtained peptide mass spectrum is matched to theoretical mass spectra, which are generated by theoretically cleaving all the proteins in a database with same protease as used in the experiment (Figure 6).

A score is calculated to provide a measure of the fit between the observed and expected peptide masses. The ranking of the result is based on the number of matched peptides. If a certain score is exceeded the hit is considered significant. The approximate size and pI value of the protein, which can be obtained by the position of the protein on the gel, is often used to help exclude

false positives or confirm ‘true’ hits. However, one has to be aware that post-translational modifications can change the pI and molecular weight of a protein considerable.

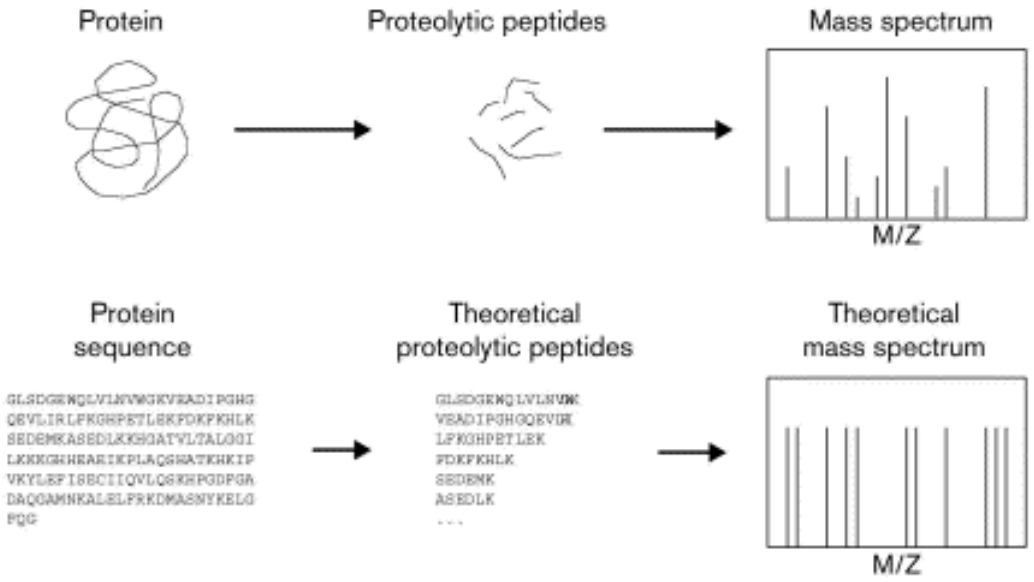

Figure 6. Protein identification using peptide mass fingerprinting [24]. The experimental mass spectrum is compared to theoretical mass spectra of a database.

Limitations

The identification of a particular protein may not however always be as straightforward. There are a few cases that can complicate the interpretation of the data: i) a high number of post-translational modifications, ii) overlapping gel spots or protein contamination (most commonly by keratin), iii) miss-cleavage by the protease, and iv) high matrix backgrounds at lower masses ($m/z < 800$). The success rate of the database search depends also, of course, on the existence of the protein in the database. Problems arise when working with proteins from organisms whose genomes are not fully sequenced, which is usually the case with mammals. Sequence homologues from other species may then be very useful in the identification. Different sequencing methods, e.g. Edman sequencing and electrospray MS-MS sequencing, can be used to give a more confident protein identification when peptide mapping does not provide enough information [22, 25].

MATERIALS AND METHODS

Chemicals

Tris, Collagen (type I), CHAPS, HEPES, hydrazine dihydrochloride (98 %), methylamine, α-cyano-4-hydroxycinnamic acid (HCCA), and iodoacetamide were purchased from Sigma-Aldrich (Vallensbæk Strand, Denmark). Insulin was obtained from Novo Nordisk (Bagsværd, Denmark), DMEM-F12 medium and foetal calf serum from Gibco BRL (Tåstrup, Denmark) and DDT from Bio-Rad (Richmond, CA, USA). Thiourea was purchased from Fluka (Vallensbæk Strand, Denmark), while dexamethasone (Decadron®) was from Merck Sharp & Dohme (Harlow, UK). BindSilane, CHAPS, Cy-dyes, IPG cover fluid, 40 % ReadySol (polyacrylamide mix), pharmalyte (pH 3-10), SDS, TEMED, and urea were all from Amersham Biosciences (Uppsala, Sweden). Glycine and ammonium persulphate were obtained from Serva (Heidelberg, Germany), trypsin was from Roche Diagnostics (Mannheim, Germany), angiotensin 1 was from PE Biosystems (Foster City, CA, USA), and SyproRuby from Molecular Probes (Eugene, OR, USA).

Animals

Non-starved, male Sprauge-Dawley rats (Charles River Laboratories, Germany) weighing between 200-300 g were used.

Cells and Culture Conditions

Hepatocytes were isolated by the two-stage collagenase perfusion technique, essentially as described by Quistorff *et al.* [26] but with the following modifications; solution 1 (0.11 M NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4 x $2H_2O$, 0.34 mM KH_2PO_4, 22 mM $C_6O_6H_{12}$, 25 mM HEPES), solution 2 (as solution 1 plus 4.9 mM $CaCl_2$ and 0.58 mg/ml collagenase), and solution 3 (as solution 1 but with 20 mM HEPES and 2 mM $CaCl_2$). Initial cell viability was determined by trypan blue exclusion and was around 85 %. Freshly prepared hepatocytes (~8 x 10^6) were plated in collagen coated 75 cm^2 flasks in DMEM-F12 medium supplemented with 4 % foetal calf serum, 2 mM L-glutamine, 48 µg/ml dexamethasone, and 1 mM insulin. After a 3 hours attachment time the old medium was replaced with serum free medium containing various

doses of HY (0 mM, 1 mM, 5 mM, and 10 mM). The cells were left to incubate for 24 hours. In order to get reliable statistics at least four flasks of each concentration were prepared.

The cells were maintained at 37 °C in a humidified atmosphere, supplemented with 5 % CO_2.

Protein Sample Preparation

After 24 hours of incubation the cells were washed with ice-cold phosphate buffered saline (PBS) to remove detached cells. The remaining cells were collected by scraping and centrifuged at 300 rpm for 5 minutes. To further remove debris the cells were washed once more with PBS and re-centrifuged. The supernatant was discarded and the cell pellet was resuspended in ~80 µl of lysis buffer (6 M urea, 2 M thiourea, 4 % CHAPS, 40 mM Tris-HCl (pH 8)). The cells were lysed using a glass homogeniser and another 50 µl of lysis buffer was used to rinse the homogeniser. Thus, a total of 130 µl of lysis buffer was added to each sample. Samples were spun down (20,000 g for 5 minutes at 4 °C). The protein concentration of the supernatant was determined using the Modified Bradford protein assay (Bio-Rad, Richmond, CA, USA). Finally, the protein samples were frozen at –80 °C.

Analytical Gels

First Dimension Separation

Rehydration

The pre-casted immobilized pH gradient gel (IPG) is rehydrated prior to IEF. 350 µl of rehydration solution (6 M urea, 2 M thiourea, 4 % CHAPS, 2 % pharmalyte) was added to each slot of a reswelling tray (Amersham Biosciences, Uppsala, Sweden). The IPG strips (18cm, pH 4-7) were placed in the trays, one in each slot, with the gel side facing down. The strips were then covered in IPG cover fluid (purified mineral oil) to prevent urea crystallization and evaporation. The strips were allowed to rehydrate for at least 10 hours.

Protein Labelling

A volume equal to 50 µg of protein was mixed with lysis buffer to make up to a total volume of 31 µl. 0.2 µl of 1 nM dye (cy2, cy3 or cy5) was added and left on ice for 15 minutes. To stop

the reaction 1µl of quencher (40 % methylamine and 0.1 M HEPES, pH 8) was added together with 0.3 µl DTT (500 mg/ml) and 0.3 µl of pharmalyte (pH 3-10). Pharmalyte is a carrier ampholyte mixture that enhances sample solubility and produces more uniform conductivity across the pH gradient without affecting the shape of the gradient. A mixture, containing equal quantities of proteins from each protein sample, was prepared as an internal standard to facilitate cross-gel quantitative analysis. This mixture was run on each gel.

Isoelectric Focusing

After the strips had been rehydrated they were loaded onto a Multiphor II unit following the instructions of the manufacturer (Amersham Biosciences, Uppsala, Sweden). The protein samples were applied using sample cups at the basic end of the IPG strips. Three differently dyed protein samples, including the internal standard, were loaded onto each strip. Thus, a total of 150 µg was added in each cup. IEF was carried out for 19.4 h (2.1 h at 200 V; 2.1 h at 500 V; 14.7 h at 3500 V; 0.5 h at 500 V). The focused strips were either frozen at –80 °C or preceded immediately to second dimension separation.

Second Dimension

Gel Casting

A gel cassette (Hoefer DALT Gel Caster, Amersham Biosciences, Uppsala, Sweden) was used, making it possible to cast up to 25 vertical gels at one time. The gels used were homogenous, 1.0 mm thick and with a polyacrylamide content of 12 % (30 % ReadySol, 0.37 M Tris-HCl (pH 8.8), 0.1 % SDS, 0.1 % ammonium persulphate, and TEMED), which gives a separation range of approximately 10-100 kDa.

Strip Equilibration

The equilibration step introduces reagents, which enhances the protein transfer between the strip and the polyacrylamide gel. The strips were placed in individual tubes containing 0.1 M Tris-HCl (pH 6.8), 4 M urea, 2 M thiourea, 30 % glycerol, 32 mM DTT, and 35 mM SDS for 10 minutes on a rocking table. The solution was discarded and displaced with a second equilibration buffer (0.1 M Tris-HCl (pH 6.8), 4 M urea, 2 M thiourea, 30 % glycerol, 0.12 M

iodoacetamide, and 35 mM SDS), also for 10 minutes. Iodoacetamide alkylates the cysteine residues, thereby preventing their re-oxidation.

SDS-PAGE

After the equilibration the strips were dipped in electrophoresis buffer (25 mM Tris, 0.19 M glycine, 35 mM SDS) and placed on top of the secondary gels. The top of the vertical gel was filled with low melting agarose (0.5 % agarose in electrophoresis buffer plus bromphenol blue) and the strip was carefully aligned against the surface of the gel using a thin plastic ruler. The agarose was left to polymerise before the gels were lowered into the buffer-filled electrophoresis tank (Hoefer DALT, Amersham Biosciences, Uppsala, Sweden). The SDS-PAGE was performed at 60-70 V and 20 °C until the tracking dye (bromphenol blue) reached the anodic end of the gels (~20 h).

Gel Scanning

The gels were scanned with the 2D 2920 Master Imager (Amersham Biosciences, Uppsala, Sweden) and the images were saved as 16-bit file format (TIFF) using 2D-Master software (V 1.95, Amersham Biosciences). Since there are three different samples on each gel (each labelled with a different dye) different scanning parameters must be set for the different images. The resolution was set to 100 µm and the excitation and emission wavelengths to 480 and 530 nm for Cy2, 540 and 590 nm for Cy3, and 620 and 680 nm for Cy5. The exposure time needed to reach the desired intensity varied a lot between the 3 dyes and between gels, but was generally 12-60 seconds per frame.

Image Analysis

Analysis of the images (spot detection, matching, editing) was carried out using DeCyder software (Version 3.5, Amersham Biosciences, Uppsala, Sweden). Proteins that were up or down regulated with more than 30 % during any of the treatments and had a significance level of 95 % or higher were marked as interesting spots.

Preparative Gels

Unlabelled proteins were separated on preparative gels for spot picking. The same procedure for first and second dimension separation was performed as described above if not otherwise stated.

2D-PAGE

The sample size was increased to 400 µg and only one protein sample was loaded per gel. Spot picking requires that the gel is attached to one of the glass plates. One of the SDS-PAGE glass plates was therefore treated with a BindSilane solution (80 % ethanol, 2 % acetic acid, 0.1 % BindSilane) over night. This step also prevents gel shrinkage upon fixation. Prior to casting the gels, reference markers were applied to the BindSilane glass plate. This is necessary for the spot-picker when localising the coordinates of the spots, which are to be picked.

Fixation, Staining and Scanning

To minimize diffusion of protein spots through the gel, the gels were fixed in 10 % methanol, 7 % acetic acid over night. The gels were then incubated in 200 ml SyproRuby dye for at least five hours. To reduce background fluorescence and increase sensitivity, the gels were washed in 10 % methanol, 7 % acetic acid for 2 hours. The gels were scanned, setting the excitation wavelength to 400 nm, the emission wavelength to 630 nm and the exposure time to 10 seconds per frame.

Spot Picking

In order to try to find the interesting spots that previously had been marked on the analytical gels, the preparative gels were visually matched to the analytical gels. In DeCyder BVA a list, containing the coordinates of the identified spots in relation to the reference markers, was established. This list was read by a robotic spot picker (Ettan Spot Picker, Amersham Biosciences, Uppsala, Sweden), which picked the protein spots of the list and transferred them into 96-well plates. The diameter of the spot picker head was 1.5 mm. A second scanning was performed after the spot picking to make sure that the right spots had been picked. A gel piece from a non-protein containing region was manually excised as a background control. Finally, the gel plugs were frozen at –20 °C.

Protein Identification by MS

Washing the Gel Pieces

The gel pieces were rinsed twice with 8 µl of double distilled water, each wash lasting ten minutes. The same procedure was repeated with 100 mM NH_4HCO_3/acetonitrile (1:1). Acetonitrile, sufficient volume to cover the gel plugs, was added. This step causes dehydration and shrinkage of the gel pieces. The gel pieces were swelled by rehydration in 100 mM NH_4HCO_3. After 5 minutes the same volume of acetonitrile was added. The liquid phase was removed after 15 minutes and the gel pieces were dried in a vacuum centrifuge.

Reduction and Alkylation

The gel pieces were swollen in a mixture of 10 mM DTT in 100 mM NH_4HCO_3 and incubated for 45 minutes at 56 °C to reduce the proteins. The samples were cooled to room temperature and the DTT solution was replaced with roughly the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3. After 30 minutes incubation at room temperature in the dark with occasional vortexing, the gel pieces were given successive washes in NH_4HCO_3 and acetonitrile as described above. The gel pieces were then completely dried in a vacuum centrifuge.

In-gel Digestion

The dried gel pieces were then incubated on ice in 8 µl freshly prepared trypsin solution (50 mM NH_4HCO_3 and 12.5 ng/µl trypsin). After 45 minutes, the remaining supernatant was removed and replaced with 3-10 µl of 50 mM NH_4HCO_3 to keep the gel pieces wet during the enzymatic cleavage. The samples were incubated for 14 hours at 37 °C.

Extraction of Peptides

The peptides were extracted from the gel by incubating the gel pieces with 8 µl of 25 mM NH_4HCO_3 for 10 minutes. The same volume of acetonitrile was added, also for 10 minutes and the supernatant was recovered. This procedure was repeated twice with 5 % formic acid and acetonitrile. All the collected extracts were pooled and dried down in a vacuum centrifuge. Finally, the peptide samples were frozen at –20 °C.

Peptide Mapping by Mass Spectrometry

The peptide extracts were resuspended in 7 µl 5% formic acid. Prior to mass spectrometric analysis, 2 µl of the digests were desalted on C18 ZipTips (Millipore, Bedford, MA, USA) following the instructions of the manufacturers except for the elution step. The peptides were eluted directly on to the MALDI target by addition of 1 µl of matrix solution (a saturated solution of HCCA in 50 % acetonitrile in 0.1 % aqueous trifluoroacetic acid). After crystallisation, mass analysis of the peptides was performed using a Voyager-DE PRO (Per Septive Biosystems, Foster City, CA, USA) MALDI-TOF mass spectrometer. Mass-to-charge ratios up to 4,000 were recorded. The obtained mass spectra were analysed using the software m/z (Proteometrics, New York, USA). The spectra were internally calibrated using two peaks from the auto-digestion (self-cleavage) of trypsin. When these peaks could not be found external calibration was performed using a peptide mixture of angiotensin 1. Sequence database searches was performed using the MASCOT program (http://www.matrixscience.com) using the following search parameters: NCBlnr database, all taxonomy, trypsin as proteolytic enzyme, 100 ppm mass error tolerance, one missed cleavage site, carboxymethylation of cysteine residues as fixed modification and methionine oxidation as variable modification.

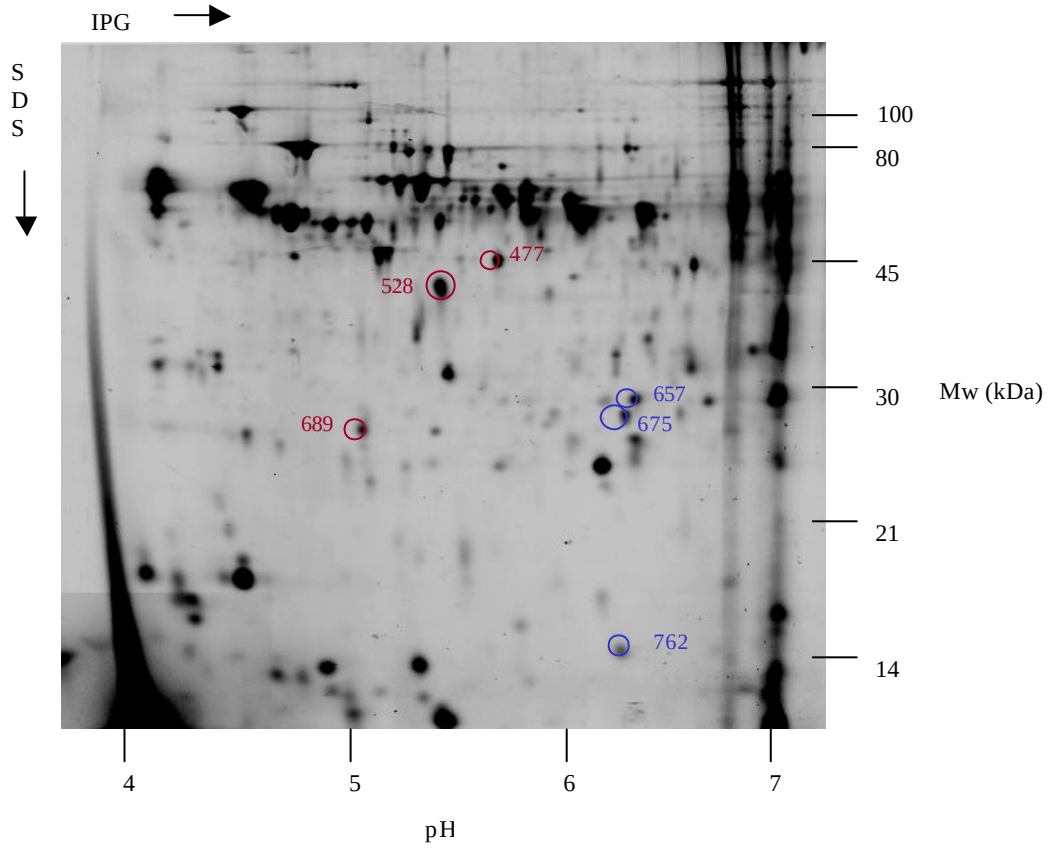

Figure 7. 2-DE gel of proteins from non-treated primary hepatocytes. The horizontal axis is the isoelectric focusing dimension, which stretches from pH 4 (left) to pH 7 (right). The vertical axis is the SDS-PAGE dimension, which stretches from approximately 10 kDa (bottom) to 100 kDa (top). Of the approximately 900 visible proteins, 62 were found to significantly change in expression following exposure to hydrazine. The encircled proteins spots are those spots that were analysed by mass spectrometry, except for spot 675, which was analysed by matching. The blue circles mark the down-regulated proteins, while the red circles mark the up-regulated ones.

2-D PAGE

Approximately 900 protein spots were visualised by Cy-staining (Figure 7). 62 of these were found to significantly change in expression following hydrazine treatment. Of these, 42 proteins were down-regulated and 20 proteins were up-regulated. To be considered significantly changed, a protein had to be up- or down-regulated with more than 30 % during any of the treatments with a significance level of 95 % or higher. The magnitude of changes ranged from a 3.4 fold up-regulation to a 41.5 fold down-regulation (Figure 8).

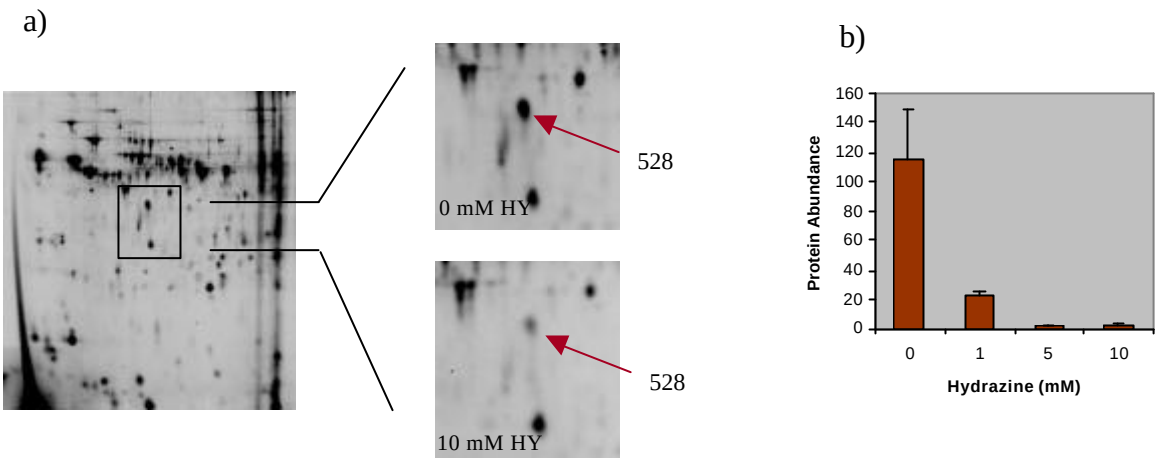

Figure 8. Concentration dependent changes of protein spot 528, which showed the greatest fold-difference of all the analysed proteins in this study. At a concentration of 10 mM hydrazine spot 528 is down regulated more than 41 times compared to non-treated cells (a). The same phenomenon is illustrated in the form of a diagram in b. The average protein abundance fold is plotted against hydrazine concentration. The y-bars represent the mean deviations from these averages.

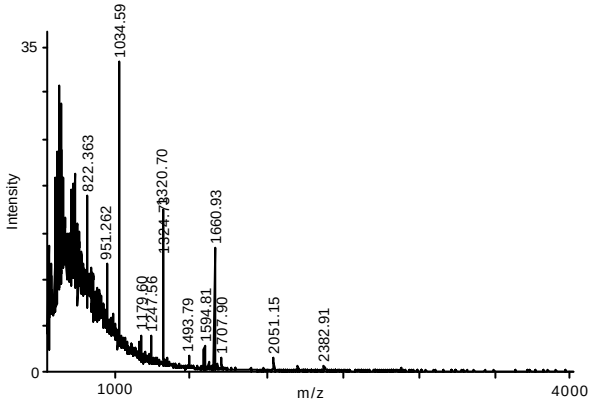

Figure 9. MALDI-TOF MS spectrum of protein spot 657. The spectrum was obtained from a tryptic in-gel digestion of the 2-DE separated protein. The data were processed using the m/z software.

Protein Identification by MS

47 of the 62 spots of interest could not be found with confidence on the SyproRuby-stained gels when preparative gels were matched against the analytical gels. Thus, only 15 spots were picked. The five most intense spots of these 15 were chosen for MS analysis. Analysis of these protein differences yielded good MALDI spectra in two cases (Figure 9), while proteins could not be identified by MALDI-TOF MS due to either low peptide levels (no results) in two cases or due to keratin contamination in one case.

Table 1. Identifications of protein spots excised from 2-DE gels

Spot no.[1]	Protein Fold Abundance Change[2]	Identified protein	Function	mw_{the} / mw_{exp}[3] (kDa)	pI_{the} / pI_{exp}[4]	Sequence Coverage[5] (%)
477	-2.5	No peptide signal	__	__	__	__
528	-41.5	No peptide signal	__	__	__	__
657	1.4	ERp29	Stress protein in the endoplasmatic reticulum	28.6 / 29.0	6.23 / 6.30	29
675[6]	1.4	Thioredoxin Peroxidase	Protection of enzymes from oxidative damage	31.0 / 28.5	6.67 / 6.20	__
689	-1.4	Keratin contamination	__	__	__	__
762	1.9	Transthyretin	Transport of thyroid hormones	13.1 / 13.5	6.04 / 6.20	42

Name and function of the identified proteins are summarised in Table 1. Sequence coverage, protein abundance fold as well as a comparison between experimental and theoretical values can also be found here.

Protein Identification by Matching

Spot 657 was identified by MS as ERp29, which also has been identified in a proteomic analysis performed by Chevalier and co-workers [27]. In their study, primary hepatocytes were used to investigate three different substances (EGF, tumour necrosis factor α, and nafenopin). The analysis was performed using the same pH-range (4-7) as in our study, which greatly facilitates comparison between gels. If a small area around spot 657 of the gel in Figure 7 is compared to the same area of Chevalier's gel, one notices that the patterns are strikingly similar (Figure 10). The similarities of the two insets indicates that spot 675 (Figure 10a) is likely to be identical to spot 46 on Chevalier's gel (Figure 10b), i.e. thioredoxin peroxidase (TP).

[1] The spot number refers to the numbers on the protein spots seen in figure 7.

[2] The column shows average fold abundance of HY treated (10 mM) verses non-treated samples, i.e. a value < -1 is a down-regulation and a value > 1 is an up-regulation

[3] Theoretical molecular weight / experimental molecular weight

[4] Theoretical pI / experimental pI

[5] A measure of the coverage of the identified protein's sequence by the obtained peptide signals

[6] Identified by matching

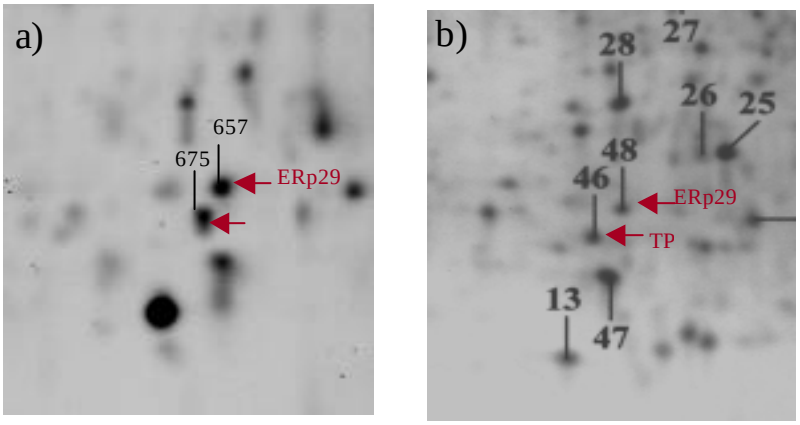

Figure 10. Both pictures represent the same area, but they originate from two different gels. In a an inset from the gel in Figure 7 is seen, while an inset from gel run by Chevalier *et al.* [27] is seen in b. Spot 657 of picture a and spot 48 of picture b are both ERp29 as identified by MS. The similarities of the two patterns indicate that spot 675 is likely to be of the same identity as spot 46, i.e. TP.

DISCUSSION

The aim of these investigations was to gain more understanding of the underlying mechanism of hydrazine induced toxicity by using proteomics technologies on rat hepatocytes. The obtained results have demonstrated a significant change of protein expression patterns following hydrazine exposure. Of the 900 analysed proteins 62 were found to be up- or down-regulated. Three of these were successfully identified by MALDI-TOF MS or by matching. These were ERp29, transthyretin and thioredoxin peroxidase.

ERp29

HY was found to cause an up-regulation of ERp29. ERp29 is a stress-inducible protein found in the endoplasmatic reticulum (ER) [28]. When an organism or a cell is exposed to environmental stress, such as heat or a chemical, it responds by increasing the expression of heat shock or stress proteins. It is believed that the damaging effect caused by different stress factors are due to the accumulation of denatured proteins in the cell. Many stress proteins are thought to protect from this type of damage by functioning as chaperones, i.e. by binding to the denatured protein thereby facilitating their refolding. ERp29 has been found to be located in the luminal compartment of the ER, which is the place where polypeptides destined for transport to the Golgi apparatus, lysosomes or cell exterior gain their three-dimensional structure. The specific function of ERp29 is not fully elucidated, but it has been observed to be strongly associated with an abundant stress protein BiP/GRP78, which is a well-studied chaperone [28].

It is known that induction of stress proteins occurs at much lower concentrations than those required to induce toxicity [4]. This is also true for the induction of ERp29 by HY. Already at 1 mM an up-regulation of 30 % is seen (data not shown), while the threshold concentration of cytotoxicity is approximately 10 mM [29].

A change in ERp29 by HY has not earlier been reported. However, previous studies have investigated the effect of HY on other heat shock proteins, HSP 25 and HSP 72/73 [4]. HY did not show to give an increase in any of these proteins at any of the used times (0-54 h) or concentrations (0-20 mM).

Transthyretin

HY causes a 90 % up-regulation of transthyretin. Transthyretin is mainly synthesised in the liver [30]. It is responsible for the transport of thyroid hormones (TH) in the blood from the thyroid glands, where TH is synthesised, to the brain, where it affects the gene regulation. Because of the hydrophobic nature of TH, it can easily enter the cells by diffusing across the plasma membrane. Once inside the cell, it binds to thyroid hormone receptors thereby stimulating transcription of specific genes. TH has been shown to increase expression of many cytosolic enzymes that catalyse the breakdown of glucose, fats and proteins [31].

Thioredoxin Peroxidase (TP)

TP was up-regulated following HY treatment. TP protects the cells from oxidative stress, i.e. damage caused by reactive oxygen species (ROS), such as free radicals and peroxides. ROS can be harmful to cells in many ways; it can cause damage of membrane lipids and degradation of proteins and nucleic acids. Most ROS are produced as by-products of normal reactions, such as energy generation. It can also be produced in detoxification reactions involving the liver cytochrome P-450 system. The cell has developed different defence mechanisms to deal with oxidative stress, one being the enzyme TP. TP reduces hydrogen peroxide to water at the expense of oxidation of thioredoxin (Figure 11).

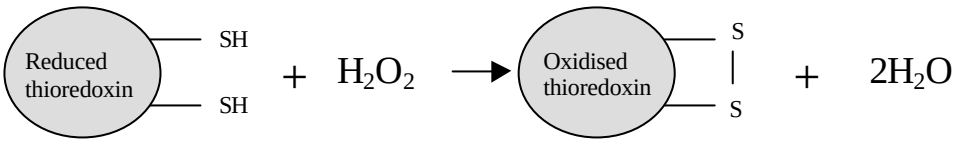

Figure 11. The mechanism of thioredoxin peroxidase.

TP was identified by matching. This type of identification is obviously not as reliable as identification by MS. Before being fully convinced that spot 675 is really TP, verification should be performed by MS.

The Role of ERp29, Transthyretin and TP in HY Toxicity

Since ERp29 is a stress protein localised in the ER, the up-regulation of ERp29 may indicate an accumulation of incorrectly folded proteins in the ER following exposure to HY. TP, which reduces hydrogen peroxide to water, was up-regulated with 40 % in the HY treated cells compared to the non-treated. This may suggest that hydrogen peroxide is produced following exposure to HY. A possible increase of H_2O_2 is consistent with a previous study, which have shown that HY is broken down by the cytochrome P-450 system. This break-down generates free radicals, which are capable of activating oxygen species (H_2O_2, $OH^{\cdot}$, etc.), thereby giving rise to oxidative stress [32].

However, the up-regulation of ERp29, transthyretin and TP cannot at this stage help explain the observed phenotype, e.g. liver fat accumulation, ATP depletion and inhibition of protein synthesis, following HY treatment. The mechanism behind HY toxicity seems to be very complex and a more complete picture, with more identified proteins, is needed before any definitive conclusions can be drawn regarding the role of these three proteins in the toxicity of HY. It is also not known whether the expression of ERp29, transthyretin and TP is regulated directly by HY as a primary response or whether their expression is regulated as a secondary response, which may follow as a result of changes in expressions of other proteins.

More experiments will be needed before the role of ERp29, transthyretin and TP in HY toxicity can be clarified.

Mass, pI and Sequence Coverage

In all three matches, the experimental molecular mass and pI were in good agreement with the theoretical mass and pI of the matched proteins.

In both cases, where the proteins were identified by MS, there was a sequence coverage of less than 50 %. Ideally, one would expect a signal for all peptides and thus be able to cover the whole sequence of the protein. There can be different reasons for not having 100 % coverage.

In some cases, the intensity of some peptides is suppressed by the presence of other peptides. A peptide can be observed if it is loaded and analysed as a single peptide, but if it is loaded at the same level in a mixture of other peptides it may fail detection. Some researchers suggest, that

this suppression can be explained by the basicity of the peptides. It has been observed that peptides containing arginine are more dominant in spectra than peptides containing lysine [21].

Another reason for the low sequence coverage may be that long stretches of sequences may not contain any trypsin cleavage site, which may give rise to large peptides whose signals appear outside the measured mass-to-charge interval (< 4,000). Problem can also arise when the cleavage sites are to close. Small peptides can be difficult to detect due to high matrix backgrounds at lower masses (m/z < 800).

In addition, peptides with modifications that are not accounted for in the database search will not give rise to a peptide hit and will therefore not contribute to the sequence coverage.

No Peptide Signal or Keratin Contamination

In half of the cases, MS analysis failed due to keratin contamination or due to no observed peptide signal (Table 1). Most peaks of the mass spectra of protein spot 689 were identified as being keratin peptides (data not shown). MS is very sensitive to contamination of keratin, since the peptide signals from the keratin can completely suppress the signals from the interesting proteins. Keratin, from hair or skin, could easily have contaminated the sample during any of the sample handling steps. No observed signal during MS can be explained by sample loss during any of the steps following excision of the protein spots from the gel.

Possible Interference of Spermidine

Only around 900 protein spots were detected on each gel image in this study. This number is very low. Normally, more than 2,000 protein spots can be visualised on one gel. The reasons for this discrepancy can only be speculated on. The low number can be partly explained by a poor isoelectric focusing. A large black band can be seen stretching over most of the size range at pH 4 in the gel of Figure 7. Interestingly, this band was not visible on 2-D gels of primary hepatocytes, which had not been cultured, i.e. the cells were frozen down straight after the separation from the liver. These gels contained almost 2,000 protein spots (Figure 12). This suggests that something happens to the cells as they are allowed to grow *in vitro*, which cannot be explained by the addition of HY since all the gels in the study, including the non-treated cells, display similar bands. This phenomenon can be explained by the possible presence of spermidine [33]. Spermidine is a polyamine (NH_3^+-$(CH_2)_3$-NH-$(CH_2)_3$-NH_3^+), which is

produced in large amounts by cultured hepatocytes [34]. The positive charges of spermidine help stabilising intercellular conformations of negatively charged nucleic acids and is required for proliferation [35]. The presences of small ionic molecules are known to cause poor focusing, but the reason for this is not fully understood. One explanation could be that the focusing of the proteins will not occur until the ions have moved to the ends of the strip, thus prolonging the time required for IEF. Another explanation could be that spermidine temporary binds to some of the proteins causing a shift in the pI, which results in the accumulation of proteins towards the cathode and thus poor focusing.

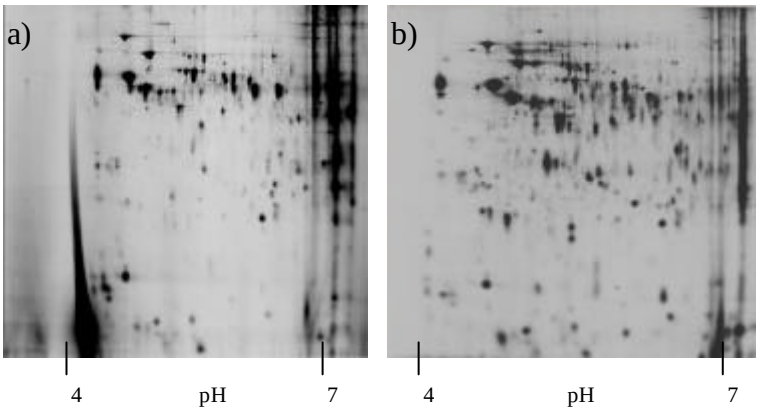

Figure 12. 2-DE gels from cultured hepatocytes (a) and non-cultured hepatocytes (b). Both gels display 50 µg of proteins from non-treated (0 mM HY) cells. A large black band can be seen stretching over most of the size range at pH 4 in a. This band is absent in b. Only about 900 protein spots are detected in a, while almost 2,000 are detected in b.

This problem can hopefully be circumvented in the future by purifying the protein sample before loading the samples on the IPG strip, e.g. by dialysis. The main problem with introducing additional steps is that the variability of the results may increase even further. One also runs the risk of sample loss or volume increase.

CONCLUSIONS AND FUTURE WORK

We have used proteomics to show changes in protein expression of primary hepatocytes following HY administration. The observed changes in protein expression pattern can be expected as a consequence of the phenotype involved in HY toxicity, e.g. liver fat accumulation, ATP depletion and inhibition of protein synthesis. Three up-regulated proteins following HY treatment were identified. They were ERp29, transthyretin, and thioredoxin peroxidase. However, the exact role of these proteins in HY hepatoxicity remains to be elucidated.

Future prospects of this project are to reproduce the results of this study and to identify the rest of the up- and down-regulated proteins. The expression of 62 proteins were found to be significantly changed after HY treatment on the analytical gels. However, 47 of these could not be found with confidence on the preparative gels. By using strips with narrow pH ranges, the resolution of the gels can be increased and hopefully more of the interesting proteins can be found.

Only about 900 proteins were visible on the gels. It has been suggested that a genome express around 6,000 proteins at any given time, excluding the post-translational modifications [14]. It would thus be desirable to increase the number of proteins included in the study. A first step could be to try to get rid of the contaminant, possible spermidine, seen on the gel in Figure 7, e.g. by dialysis. Studying more pH intervals would also increase the number of proteins in the analysis. In this report, pH-range 4 to 7 was investigated. By using strips with more basic and more acidic pH intervals the experiments would be improved even further.

It would also be very interesting to investigate HY toxicity at different time points as well as after different recovery times. The time of exposure has previously been shown to be an important factor in the toxicity of HY [29].

The response *in vitro* does not always correlate to what happens *in vivo*. It is therefore necessary to evaluate the relevance of the *in vitro* model used in these experiments. This can be performed by comparing the results of this study with results of proteomic studies that investigate the *in vivo* response of HY. Such investigations are currently ongoing at Novo Nordisk.

To summarise, by identifying more of the proteins, which have been shown to change in expression following HY treatment, together with an investigation of the relevance of the

established *in vitro* model, it will hopefully be possible to draw definitive and reliable conclusions regarding the roles of the different proteins in the cytotoxity mechanism of HY.

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