

ABSTRACT

Title of Thesis: EVALUATION OF ACETONITRILE PRECIPITATION
AS A METHOD FOR SEPARATING SMALL FROM
HIGH MOLECULAR MASS PROTEINS IN CYTOSOL
FROM MCF-7 BREAST CANCER CELLS.

Ulunna K. Ofurum, Master of Science, 2004

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Acetonitrile precipitation has been carried out as an investigative method for separating small from high molecular mass proteins. The separation has been visualized on a 1-D gel electrophoresis. Acetonitrile helps drive the proteins out of solution, to precipitate them. It is expected that small proteins will remain in the supernatant while big proteins precipitate out. Visualization of the purified cytosolic extract, the precipitate and the supernatant clearly show enrichment for small molecular mass proteins in the supernatant. In addition, the high molecular mass proteins in the non-precipitated cytosolic extract are enriched in the precipitate. This has proven to be a good method for enrichment of small molecular mass proteins. This method can be combined with protein separation and or identification methods in future experiments to effectively identify proteins with high accuracy. In addition, in-gel digestion (after acetonitrile precipitation) combined with mass spectrometry can further help identify small molecular mass proteins.

EVALUATION OF ACETONITRILE PRECIPITATION AS A METHOD FOR
SEPARATING SMALL FROM HIGH MOLECULAR MASS PROTEINS IN
CYTOSOL FROM MCF-7 BREAST CANCER CELLS.

by

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Science
2004

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ACKNOWLEDGEMENTS

I would like to express my gratitude to Professor Catherine Fenselau for the opportunity to conduct this research, and for her continued support and guidance throughout this research.

I am very thankful to Dr. Yetrib Hathout for his guidance and encouragement at the initial phase of this research.

I would like to thank Dr. Marion Gehrman for providing the cells used to conduct this research and Amir Rahbar for his invaluable assistance in conducting the 1-dimensional gel electrophoresis.

Finally, I would like to thank my daughter Kayla, whose patience throughout this research made it possible.

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INTRODUCTION

Background

Breast cancer is the most common type of cancer in women, and the most important and common cause of cancer-related deaths in women [1]. It is second only to lung cancer in the number of overall cancer-related deaths [2]. Breast cancer usually becomes refractory to chemotherapy within 2 to 5 years [3]. Surgery and radiation treatment are not options for some patients, especially those with metastatic disease [3]. Some of these patients respond initially to modified chemotherapy, but, however, relapse after a short time. The development of multi-drug resistance (MDR) in breast cancer remains a major obstacle in its treatment [4, 5, 6]. MDR is defined as a tumor's resistance to an agent, and its cross-resistance to other agents structurally and functionally unrelated [6]. Cell lines (in vitro models) are important in the study of breast cancer and its resistance to chemotherapeutic agents, since experimental manipulations are more difficult to carry out on actual patients and often precluded by toxic side effects [6].

Chemotherapeutic agents inhibit cell growth by affecting different parts of interphase and mitosis of cell division, thereby inducing apoptosis. Apoptosis is defined as programmed cell death [1, 7].

Human breast cancer MCF-7 cells are one of the models used to study multidrug resistance in breast cancer. In order to learn more about the development of multi-drug resistance, investigation of cell lines at the proteomics level is necessary. Proteomics is the identification and characterization of proteins expressed by an organism [8]. One must understand which proteins are present and which of those are differentially

expressed whether upregulated or downregulated in the drug resistant cell line. Most of these proteins play important roles in the function and structure of the cell, but when either over-expressed or under-expressed can contribute to drug resistance. It is important to construct a proteomic map of proteins from the drug susceptible cell line, which can be compared to the proteins from a drug resistant cell line, in order to explore the differentially expressed proteins in the two cell lines. Therefore, comparative analysis of protein expression is an imperative aspect of the analysis of proteins contributing to diseases.

In many of the mechanisms known to contribute to MDR, cells become resilient to drug-induced apoptosis [4]. Many proteins can contribute to this. Some proteins inhibit apoptosis induced by cytotoxic drugs by preventing the release of cytochrome c into the cytosol [4]. Cytochrome c is released from the mitochondria to the cytosol in a very critical step in the activation of the apoptotic-signaling cascade [9].

Caspase-3-protein is another protein involved in drug resistance. Caspase-3-protein is a cysteine protease [10]. Caspase activation is an important step in apoptosis and cytotoxic drug-induced apoptosis is mediated by caspase-3 [1, 11]. MCF-7 cells express a truncated isoform of the caspase-3 transcript, while the drug resistant cell lines express the full-length caspase-3 transcript [12].

A major pathway involved in drug resistance is that involving growth factor receptor-mediated signal transduction [4, 13]. Although drug resistance in breast cancer has been attributed to several proteins, the pathway involving the human epidermal growth factor receptor (HER) family is a very important one. The HER family of proteins leads to the phosphorylation of a protein kinase b (Akt-a serine threonine

kinase), which in turn phosphorylates many other proteins in the phosphoinositide-3-kinase (PI-3K) pathway. Upon phosphorylation of Akt, apoptosis is suppressed [14].

Over-expression of p-glycoprotein (p-gp) leads to drug efflux and decreased intracellular drug accumulation in cells [4, 6, 15, 16]. P-gp is an ATP-dependent membrane efflux pump that maintains intracellular drug concentration below the needed cytotoxic levels [6]. Several factors, such as oncogene transfection, cell differentiation/proliferation and upregulation of heat shock proteins, can alter the cellular expression of P-gp [6]. P-gp is the most studied and best characterized drug resistance mechanism, but is highly unlikely to account for all the drug resistance seen in many tumors [15, 17]. P-gp is encoded by a group of related genes called *mdr*, of which there are two types (*mdr1* and *mdr2*) in the human genome [6]. *Mdr 1* has been shown to impart drug resistance in human tumor cells [6]. P-gp is found mainly in cells lining the luminal space in normal tissues [6]. Its localization denotes its role as an efflux pump with a protective role against cytotoxins [6]. P-gp and the level of expression of the *mdr1* gene have been suggested to be one of the prognostic factors in patients with different malignancies [6]. Topoisomerase II, which will be discussed later, has been proposed as a mechanism for non-pgp mediated drug resistance in many cell models [15]. Drugs such as verapamil, a calmodulin antagonist and calcium channel blocker, have been shown to restore the sensitivity in vitro of resistant cells to cytotoxic drugs that function by blocking the function of p-gp [6].

P53 gene is a nuclear gene involved in the normal proliferation of cells. It is expressed in very high levels in tumor derived cell lines [18]. Mutations of p53 have been identified in a number of breast tumors [19, 20].

Altered expression levels of other proteins such as type 1 topoisomerase, glutathione-related enzymes and protein kinase C (PKC) have also been observed in drug resistant cells [15, 21]. For example, tissue transglutaminase (tTGase), a protein cross-linking enzyme, which also facilitates apoptosis, has been shown to be overexpressed in the MCF-7 cells selected for resistance to doxorubicin [15].

Drug resistance is a major obstacle in the treatment of any cancers. Cellular mechanisms that contribute to MDR include increased efflux (ATP-dependent efflux pumps), decreased influx, activation of DNA repair, blocked apoptosis (example: decreased ceramide levels) and activation of detoxifying systems by the glutathione system (cytochrome p450) [6, 11, 15, 22, 23]. However, several studies have shown that acquired drug-resistant phenotypes *in vitro* express a vast variety of alterations, including protein kinase C overexpression, loss of epithelial markers, increased accumulation of p53 protein, altered expression of growth factors or changes in growth rate [11]. A study by Vickers et al showed that the overexpression of p-gp and doxorubicin resistance were accompanied by a loss of estrogen receptor and increased expression of epidermal growth factor (EGF) [24]. Loss of estrogen receptor and increased levels of EGF receptor expression have been correlated with poor prognosis in patients with breast cancer [25-29]. The recurrent alterations in growth factors suggests that they play an important role in drug resistance, although the precise mechanism is unknown [22, 30].

The MCF-7 cell line used in this experiment was originally obtained from a pleural effusion and is epithelial-like in morphology [31]. The drug-resistant cell lines used in our laboratory include doxorubicin-MCF7, etoposide-MCF7, melphalan-MCF7 and mitoxantrone-MCF7.

Doxorubicin (adriamycin) is one of the most effective therapeutic drugs available for the treatment of solid tumors including breast cancer [15]. Doxorubicin is an inhibitor of reverse transcriptase and RNA polymerase. As an RNA polymerase inhibitor, it binds to DNA and prevents transcription, thereby preventing cell growth. In addition to being an RNA polymerase and reverse transcriptase inhibitor, it is also an immunosuppressive agent, and a DNA topoisomerase II (DNA gyrase) inhibitor [2, 4, 32, 33]. Topoisomerase II is the main target of doxorubicin [33]. Doxorubicin generates free radicals that cause strand breaks in DNA [2, 33, 34]. One of the factors contributing to resistance to doxorubicin has been shown to be decreased accumulation of drug [35]. Other studies have attributed doxorubicin resistance to alterations in topoisomerase II activity, increased inactivation of radicals by increases in glutathione-dependent enzymes [2, 6], increases in drug efflux and increased p-gp expression [2, 4]. The effectiveness of doxorubicin is limited by both intrinsic drug resistance and the development of drug resistant tumor subpopulations [15]. This is the main reason why it is important to analyze proteins involved in doxorubicins' drug resistance.

Etoposide (Vp16) complexes with DNA and topoisomerase II to enhance single and double strand cleavage and reversibly inhibit relegation [2, 33, 34, 36]. Etoposide arrests the cell in both the synthesis and growth phases of the cell cycle, thereby inducing apoptosis [2, 34]. Resistance has been shown to be due to an alteration in the DNA topoisomerase II activity [36, 37].

Melphalan forms DNA cross-links that are crucial for cytotoxic effects [2, 38]. Resistance to melphalan has been shown to be mostly due to reduced drug intake [37] and increased DNA cross-linking repair [38].

Mitoxantrone is a chemotherapeutic agent similar in structure to doxorubicin [38]. Its precise mechanism of action is not fully known, however it is most likely associated with chromosomal elements, resulting in DNA damage and inhibition of nucleic acid synthesis and DNA strand breaks, which in turn result in cell death [38]. Resistance to mitoxantrone is reported to be mostly due to drug efflux [39].

To successfully analyze proteins in cells, fractionation of cellular components is preferable, if not a must. Subcellular fractionation employs differential centrifugation as its primary method. Fractionation of the cell should make it easier to analyze proteins, which contribute to drug resistance. Proteins in cells have a wide range of properties and abundance levels. This causes a great deal of complexity when analyzing whole cell extracts. Reducing this complexity by analyzing protein subsets has improved analysis of low abundance proteins and contributed information about the localization of various proteins in the cell. Finally, even proteins isolated in subcellular fractions must be further fractionated for successful global analysis.

Many high molecular mass proteins involved in MDR have been identified and studied, but only a few low molecular mass proteins have been examined. It is very likely that many more proteins than have been identified are involved in the MDR mechanism. Several methods are available for the separation and identification of large proteins, but effective methods are yet to be proven successful for small proteins. In part, this is because low molecular mass proteins are not well resolved on 1- and 2-dimensional gel electrophoresis. In order to effectively carry out a comparative analysis between two cell lines, it will be important to isolate, identify and analyze as many small proteins as possible, because these might play an integral role in the mechanism of drug

resistance. The development of new techniques is needed for the analysis of low molecular mass proteins.

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is a protein separation technique that is widely used. Proteins are separated in the first dimension according to isoelectric points (PI)/charge, and in the second dimension according to molecular weight. In-gel digestion is used to identify the proteins on the gel. Each spot on a 2D PAGE corresponds to one (or occasionally more) protein(s) due to this method's high resolution. A major advantage of this method is that crude extracts can be applied directly with high resolution. The separated proteins can be identified using mass spectrometry for microsequencing or peptide finger printing. This method, however, favors the visualization of relatively abundant proteins and requires a relatively large amount of sample. It is also difficult to separate hydrophobic proteins and proteins of extreme PI's on a 2D-gel. The limitations of 2-D electrophoresis have made it necessary to probe for more efficient methods of separating low molecular mass proteins.

One-dimensional polyacrylamide gel electrophoresis (1D PAGE) is another separation method that separates proteins according to size and molecular weight. Each band on a 1D PAGE could contain many proteins, and in-gel digestion and microsequencing are usually required to correctly identify proteins. 1D PAGE is a great visualization tool to estimate the mass range of proteins in a given mixture. Using a molecular standard as a marker, one can estimate the molecular weights of proteins in the sample.

High performance liquid chromatography is another powerful separation technique used in protein biochemistry. Biological compounds can be separated with

good resolution, convenience and sensitivity, given that the sample is soluble in the liquid phase.

Mass Spectrometry is a rapidly evolving area in proteomics. “A mass spectrometer determines the mass of a molecule by measuring its mass per charge ratio (m/z)” [40]. Ions are generated by a loss or gain of electrons or protons from a neutral species and are then directed to the analyzer where the ions are separated according to mass/charge and then detected by a detector [40]. The most common ionization sources for proteins are electrospray ionization and matrix-assisted laser desorption/ionization (MALDI). MALDI was used in this experiment due to its excellent sensitivity, suitability for peptides, relative tolerance to complex mixtures and soft ionization. Linear time-of-flight (TOF) and time-of-flight reflectron analyzers were used in this experiment due to their great compatibility with MALDI and fast scanning speed. Post source decay (PSD) is an extension of MALDI/MS that aids in the observation and identification of fragment ions from the decay of a precursor ion, taking place after leaving the ion source [41]. Mass spectrometry is usually combined with gel electrophoresis, HPLC or other separation methods. Mass spectrometry is also an excellent tool for identifying post-translational modifications [42-44].

Data from the MALDI spectra can be submitted to protein databases in an effort to identify proteins in a sample. When peptide mass or sequence information is submitted to the database, a score and probability are generated for each protein. The probability is based on the observed match between the experimental data set and each database query occurring by chance. The lowest probability results in the best match. The significance of the best match depends on the size of the database. The way that

Mascot measures the significance threshold is to see if the probability of an observed event occurring by chance is less than 1 in 20 ($p < 0.05$). Score = $-10 \log(p)$, where p is the probability of an event occurring by chance. Therefore, the best match will also have the lowest probability, which will result in the highest scores.

The objective of my research is to evaluate a new method for enrichment of low molecular mass proteins, a method that will optimize the identification of low molecular mass proteins in the cytosolic fraction of the MCF-7 cell lines. The cytosol houses the cell organelles and is the site of most cellular metabolism. It contains numerous proteins that control cellular mechanism such as signal transduction, glycolysis, intracellular receptors and transcription factors [45]. The analysis of cytosolic proteins is therefore very important in proteomics. The method used in my research, acetonitrile precipitation, was proposed in a talk by Andrew Alpert from the PolyLC Company who suggested that this might be a way of reducing the complexity of fractionation of low from high molecular mass proteins.

EXPERIMENTAL

MCF-7 Growth and Harvesting

MCF-7 drug susceptible cells were grown and harvested in our lab [46]. This cell line was a gift from K.H. Cowan (Eppley Institute, University of Nebraska Medical Center, Omaha, NE). The cells were grown on 150 cm² flasks (Corning, Inc., Corning, NY) in Minimal Essential Medium Eagle (ATCC, Manassas, VA), 5% fetal bovine serum (Sigma) and 0.1% penicillin-streptomycin antibiotic solution and 0.25% trypsin-EDTA (Sigma). The cells were maintained at 37°C with 5% carbon dioxide. Upon confluence, the cells were harvested. To harvest the cells, the cells were washed twice with 25ml 1x phosphate buffer saline solution (PBS). Then 5ml of trypsin was added to the washed cells and incubated at 37°C for 5 minutes. Next, 15 ml of media containing serum was added to inhibit the protease. The cells were centrifuged at 500g for 5 minutes. The cell pellet was washed twice with 25 ml of ice-cold 1x PBS and 25 ml of ice cold 100mM NaCl, and centrifuged at 500g in between washes.

Cytosolic Extraction

The cells were incubated with digitonin extraction buffer [.015% digitonin, 100mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 300mM sucrose, 5M NaCl, 1M MgCl₂, 100mM ethylenediaminetetraacetic acid (EDTA), and 100mM phenylmethylsulfonyl fluoride (PMSF)] with the ratio of 3.7 ml per gram of wet cells. The cells were centrifuged at 800 g for 15 minutes. The supernatant was then centrifuged at 5000 g for 20 minutes, and the supernatant obtained from this centrifuge was further

centrifuged at 90,000 g for 2 hours. The purified cytosolic extract was stored in 0.5 ml aliquots at -80°C.

Acetonitrile Precipitation

A 1:1 ratio by volume of the purified aqueous cytosolic extract and acetonitrile (HPLC grade from Fisher Scientific; Fair Lawn, NJ) was used for the precipitation studies. The cytosolic extract and acetonitrile mixture was placed on ice for 1 hour. Next, it was centrifuged (Eppendorf Centrifuge 5415 D) at 5000 revolutions per minute (rpm) for 5 minutes. The supernatant was dried in the SPD Speed Vac RVT 400 refrigerated vapor trap (Savant Instruments; Holbrook, NY). The residue was re-suspended in 0.1% Trifluoroacetic acid (TFA). The acetonitrile precipitation scheme is shown in Figure 1.

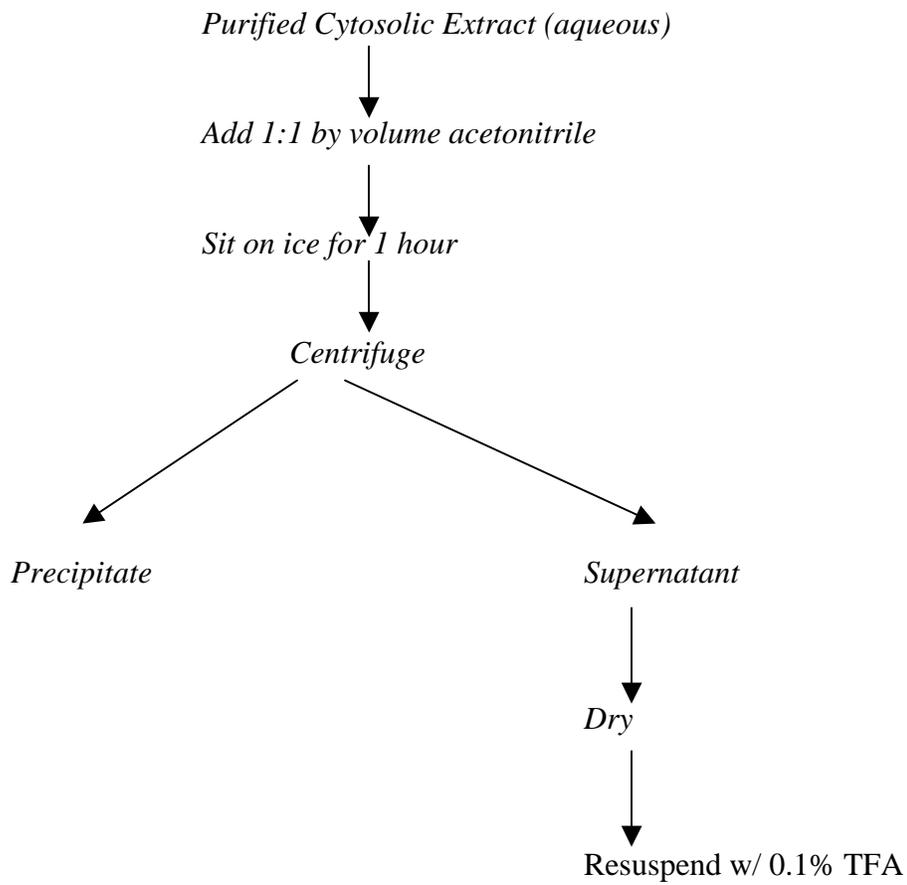


Figure 1: Acetonitrile Precipitation Scheme

Protein Assay

The protein concentration of the cytosolic extract and the supernatant were determined using the Bio-Rad Protein Assay (Hercules, CA) dye reagent following the manufacturer's instructions. The protein concentration was detected using a Beckman DU530 Life Science UV/Vis Spectrophotometer (Brea, CA). Absorbance was measured at 595nm.

1-Dimensional SDS gel electrophoresis

The precipitate was rehydrated in loading buffer (2-mercaptoethanol and Laemmli Sample Buffer) (Bio-Rad). Approximately 10 µg of the cytosolic extract, supernatant and precipitate were loaded into a well on a Bio-Rad Criterion Precast Gel (4-15%, Tris-HCl, 1.0 mm, 12 + 2 comb, 45 µL) in a Mini PROTEAN 3 (Bio-Rad) gel box with running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). The gel separation was run for approximately 45 minutes at 200 volts using the Bio-Rad Power Supply Model 1000/500. The gel was fixed in 50% methanol, 5% acetic acid and 45% water for 30 minutes and then re-hydrated with water for 15 minutes. The gel was further stained with Bio-Safe Coomassie (0.025% Coomassie blue G250, 10% acetic acid) (Bio-Rad) for 1 hour and then de-stained with water. The gel was imaged on a Bio-Rad GS-800 densitometer.

Desalting

Prior to analyzing the supernatant on the mass spectrometry, the supernatant from the acetonitrile precipitation was desalted. A Zip tip_{C4} (Millipore Corporation: Belerica, Massachusetts) was used following the manufacturer's instructions.

Mass Spectrometry

In order to further test the reliability of the acetonitrile precipitation method, the desalted supernatant sample was analyzed using the MALDI Kratos PC Axima-CFR Plus (Shimadzu Kratos, Chestnut Ridge, NY) prior to HPLC separation. 3,5-Dimethoxy-4-hydroxycinnamic acid was used as the matrix. A 1:1 ratio of matrix and sample was loaded on the sample plate and analyzed at a laser power of 65 in the linear mode.

High Performance Liquid Chromatography (HPLC)

Instrumentation and Chemicals:

A Shimadzu (Columbia, MD) HPLC system, was used with UV detection. Acetonitrile was HPLC grade from Fisher Scientific (Fair Lawn, NJ) and trifluoroacetic acid (TFA) was from Aldrich Chemicals (Milwaukee, WI).

Reversed-Phase Chromatography

Separations of the supernatant post acetonitrile precipitation were performed on a Shimadzu HRC-ODS 4.6mm x 150mm column. The mobile phase components are (A) 0.1% TFA in HPLC grade water and (B) acetonitrile in 0.1% TFA. Proteins were detected at 254 nm at a flow rate of 1 mL/min. The fractions were collected manually and dried in the SPD Speed Vac RVT 400 refrigerated vapor trap. 0.1% TFA was added to the dried sample, which was analyzed by MALDI-TOF MS in the linear mode. Fractions containing protein peaks were digested.

Tryptic digestion

Some of the HPLC fractions were selected for digestion. The fractions were dried and resuspended in 50mM ammonium bicarbonate ($\text{CH}_2\text{O}_3\cdot\text{H}_3\text{N}$). A 1:1 ratio of supernatant and trypsin (trypsin was dissolved in 50mM ammonium bicarbonate ($\text{CH}_2\text{O}_3\cdot\text{H}_3\text{N}$)). The mixture was incubated overnight at 37°C and the reaction was stopped with 0.1% TFA. The peptide mixture was then analyzed by MALDI-TOF MS in the reflectron mode using α -cyano-4-hydroxycinnamic acid as matrix. Post source decay (PSD) measurements were performed on some of the fractions.

Database searches

The MALDI spectra were analyzed using Mascot (<http://www.matrixscience.com>) as shown in figure 2. Peptide mass fingerprint was performed using the SwissProt database, allowing up to 1 missed cleavage and a peptide mass tolerance of ± 1 Dalton. The PSD analysis was performed using the Mascot sequence query and the SwissProt database, allowing up to 1 missed cleavage, a peptide tolerance of ± 2 Dalton and an MS/MS tolerance of ± 2 Dalton.

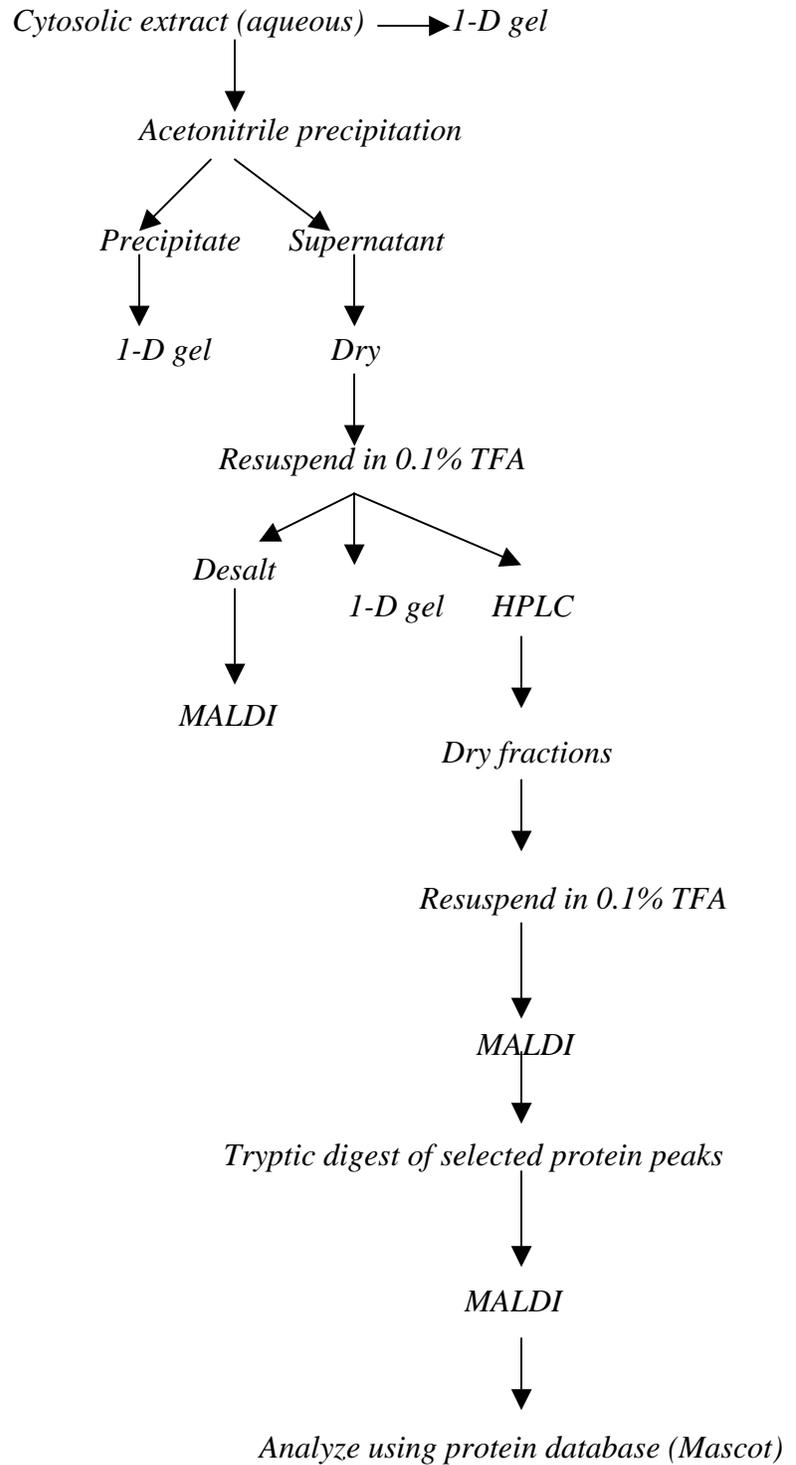


Figure 2: Overall Experimental Scheme

RESULTS

Protein Assay

The concentration of the purified cytosolic extract was determined to be 1.7 mg/ml, while that of the supernatant was determined as 0.08 mg/ml.

1-Dimensional Gel Electrophoresis

The 1-dimensional gel separations of the purified cytosolic extract, precipitate and supernatant were imaged. Gel patterns from two experiments are shown in figures 3 and 4.

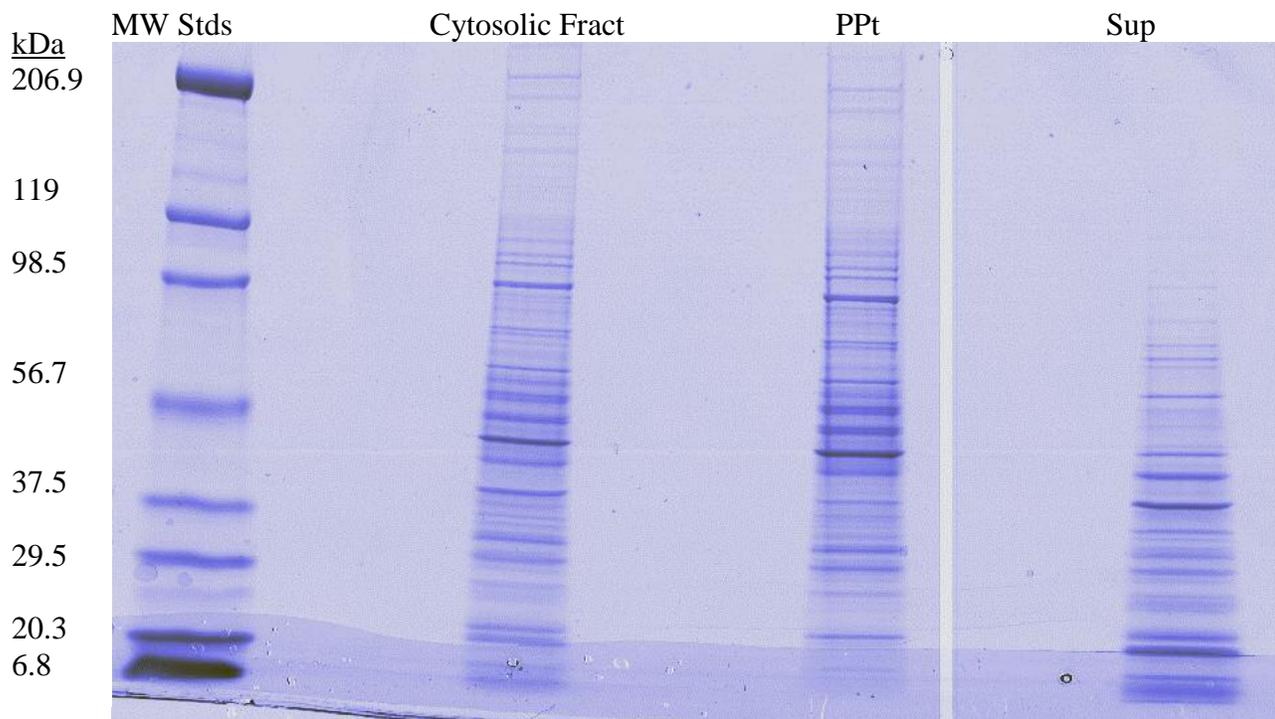


Figure 3: 1-D gel electrophoresis of the purified cytosolic extract, precipitate and supernatant respectively.

The cytosolic fraction contains all the proteins in the extract. The bands from the precipitate reveal the same large proteins as the cytosolic fraction. The supernatant can be seen to contain mainly low molecular mass proteins. A second gel separation was run using a second aliquot from the original cytosol extract and the same molecular weight standard, and is shown in figure 4. In both cases, a differential distribution of proteins can be seen between the precipitate and the supernatant, with lower molecular weight proteins being enriched in the supernatant.

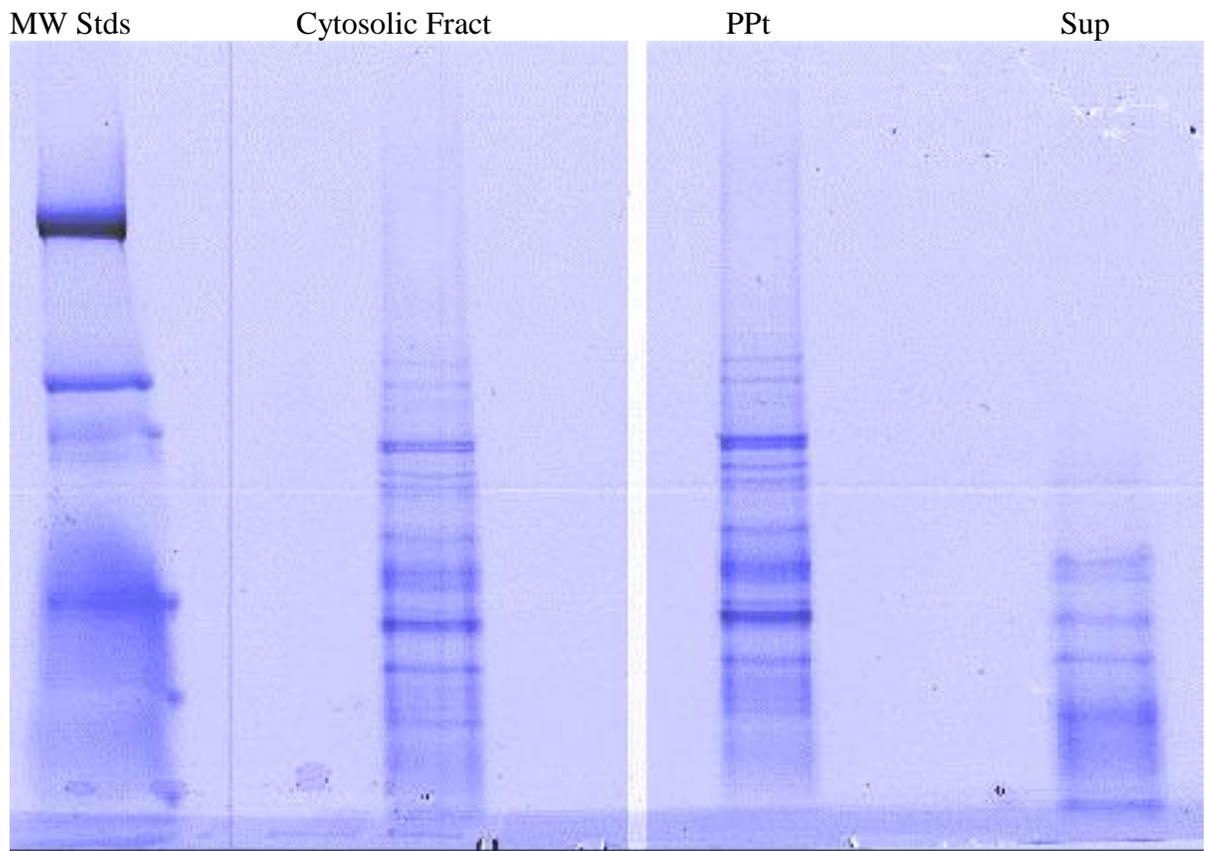


Figure 4: Duplicate 1-D gel electrophoresis of the purified cytosolic extract, precipitate and supernatant respectively.

Mass Spectrometry

The MALDI spectrum of the desalted supernatant after the acetonitrile precipitation is shown in figure 5. This shows an abundance of low mass proteins. The supernatant is enriched for low molecular weight proteins as expected. HPLC was performed on the supernatant to purify and separate the low molecular weight proteins.

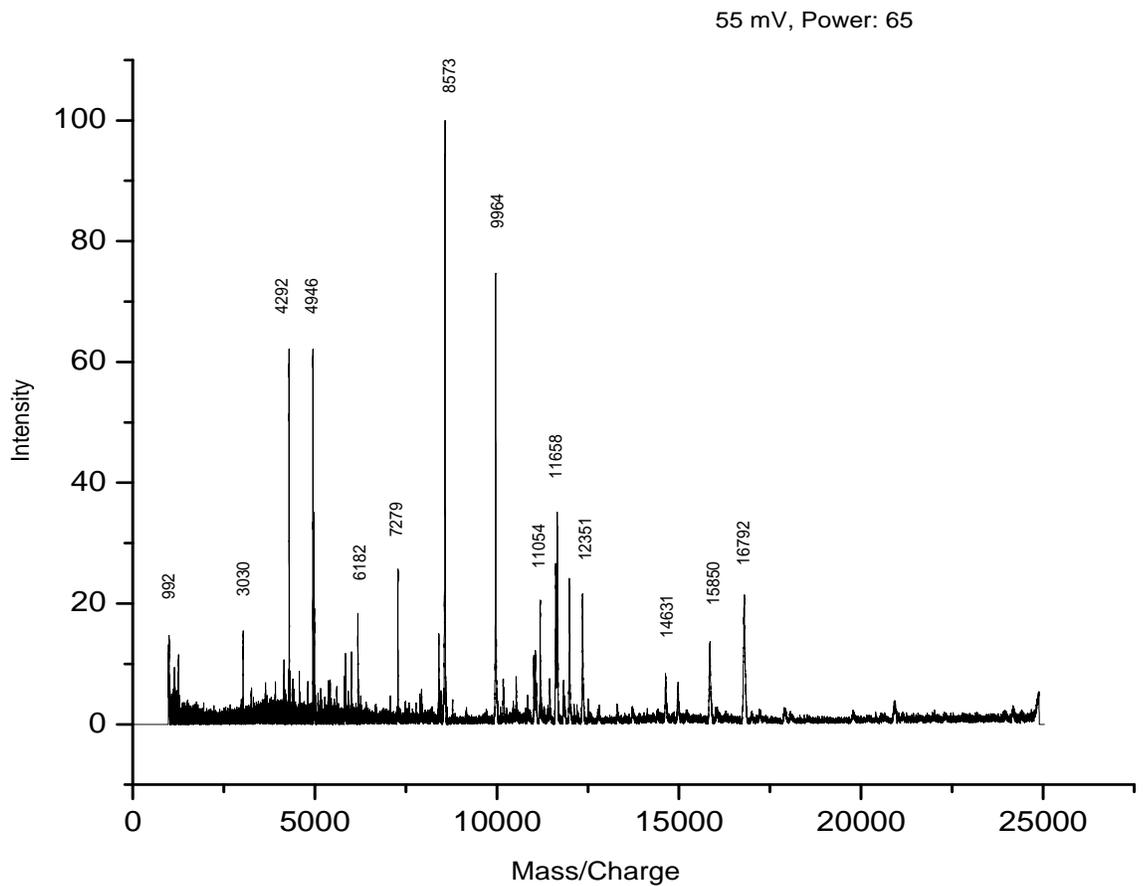


Figure 5: MALDI-TOF MS spectrum of supernatant of the acetonitrile precipitation.

The HPLC chromatogram of the supernatant is shown in figure 6. The numbered peaks were selected for tryptic digestion and further analysis by peptide mass fingerprinting and microsequencing.

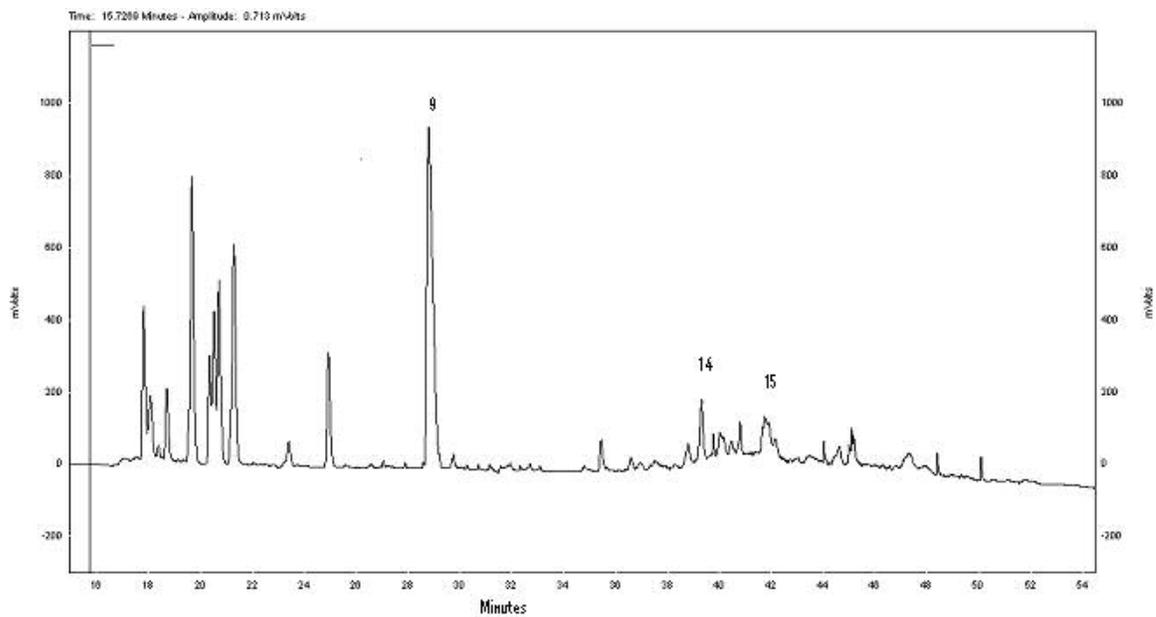


Figure 6: HPLC chromatogram of the supernatant of the acetonitrile precipitation.

A MALDI spectrum was obtained for each fraction collected (figures 7, 10 and 13). Tryptic digestion was performed for fractions 9, 14 and 15. These fractions were selected for digestion because they showed protein peaks on the MALDI spectra. The MALDI spectra of the digested peaks are shown in figures 8, 11 and 14. PSD was performed on the most abundant peptide of the tryptic digestion from each fraction. The PSD-MALDI-TOF spectra are shown in figures 9, 12 and 15.

259 mV, Power: 65

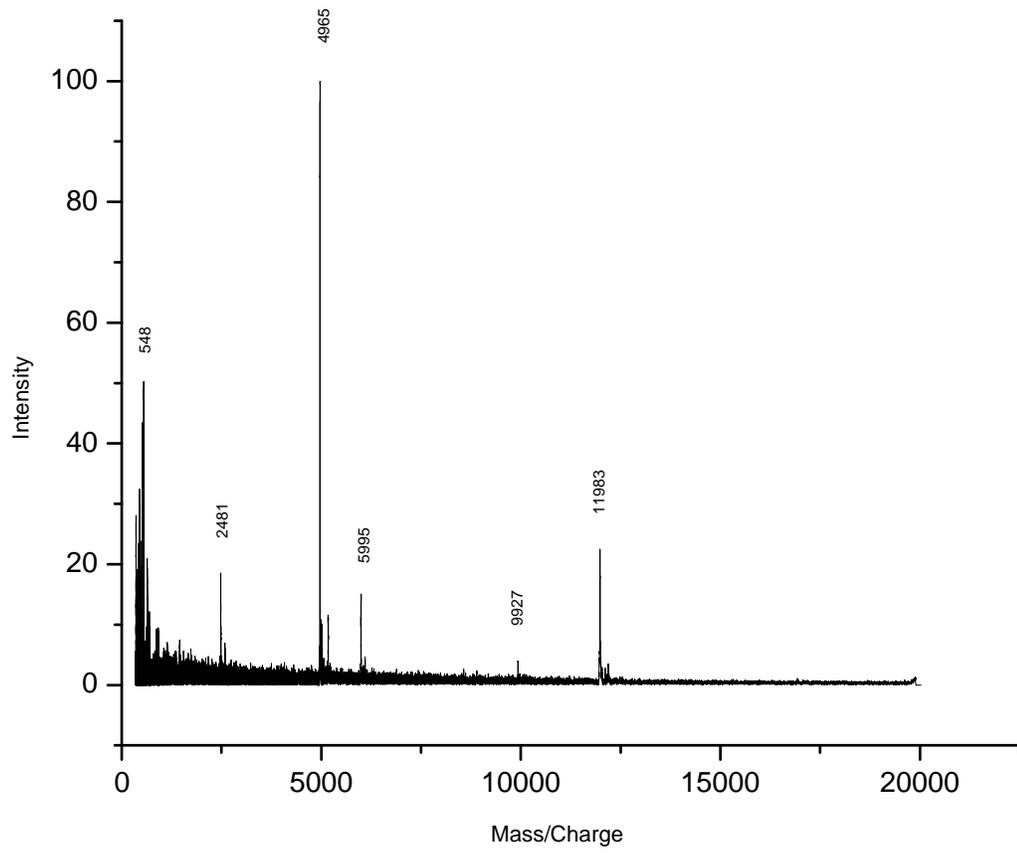


Figure 7: MALDI-TOF MS spectrum of HPLC fraction 9

273 mV, Power: 45

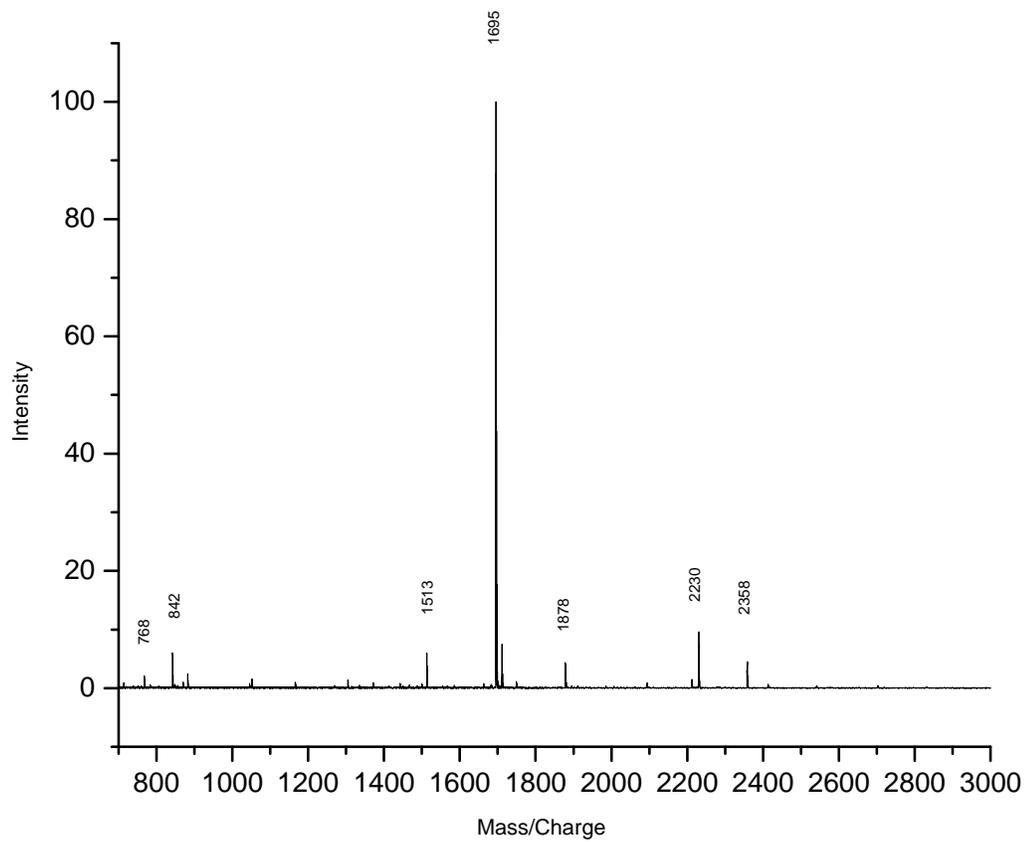


Figure 8: MALDI-TOF MS spectrum of digested HPLC fraction 9

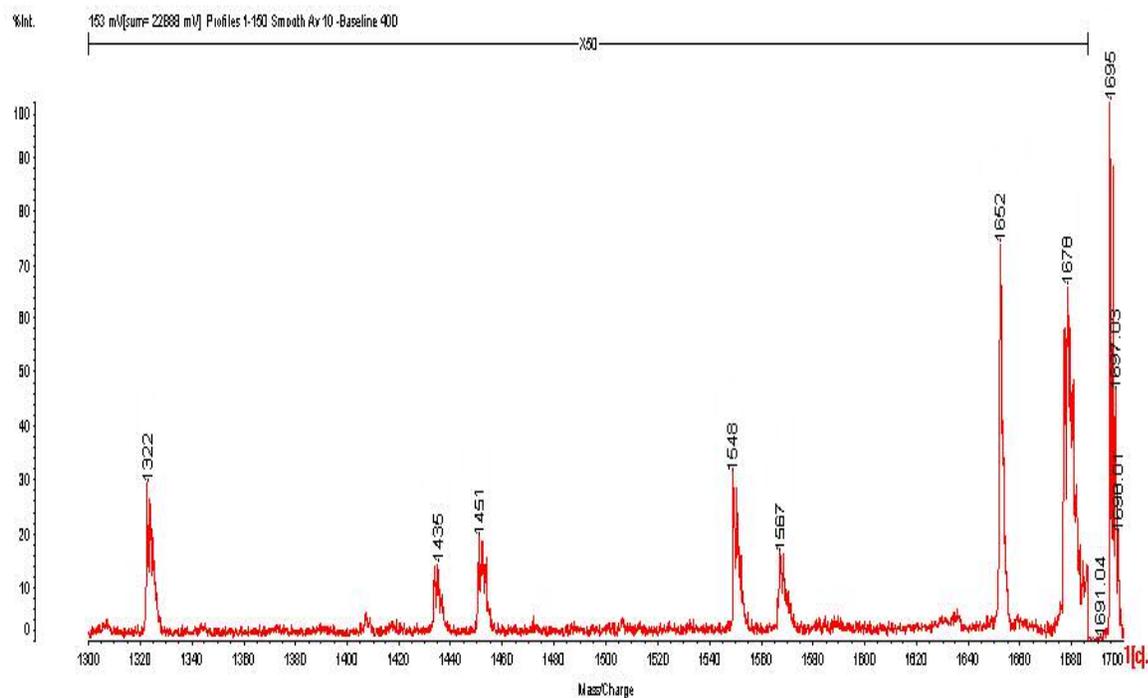


Figure 9: MALDI-TOF-PSD spectrum of HPLC fraction 9.

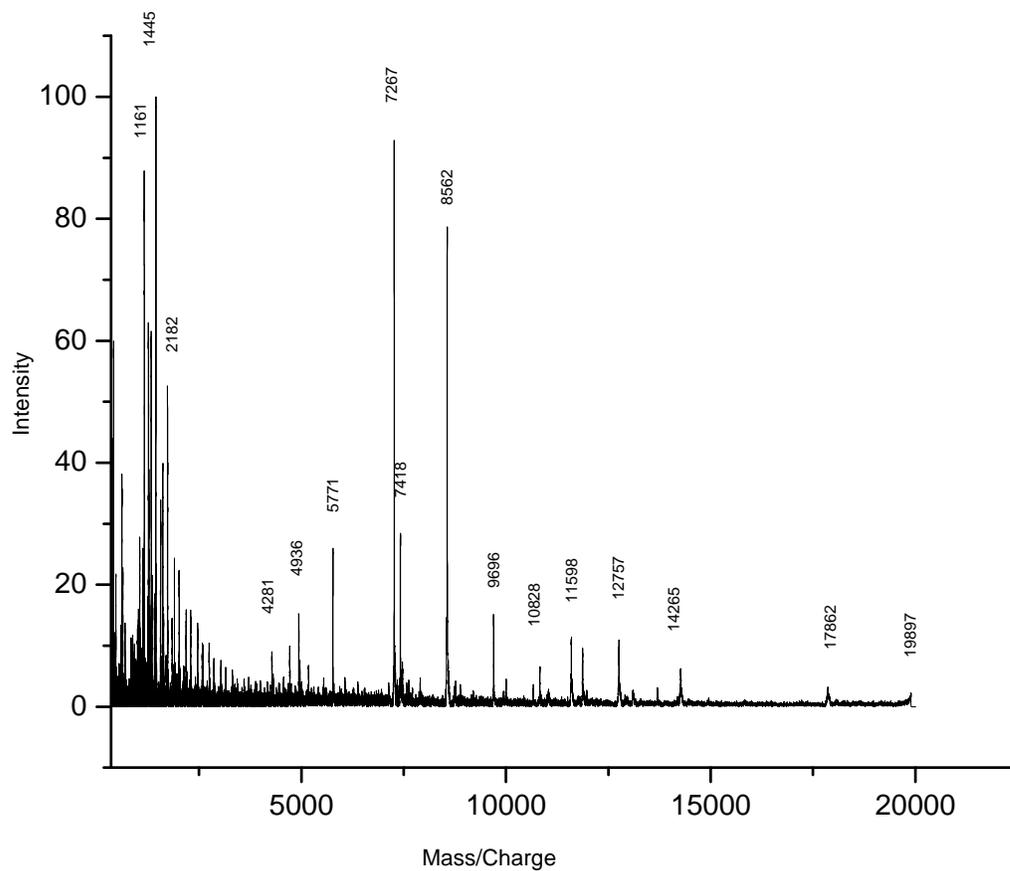


Figure 10: MALDI-TOF MS spectrum of HPLC fraction 14

130 mV, Power: 45

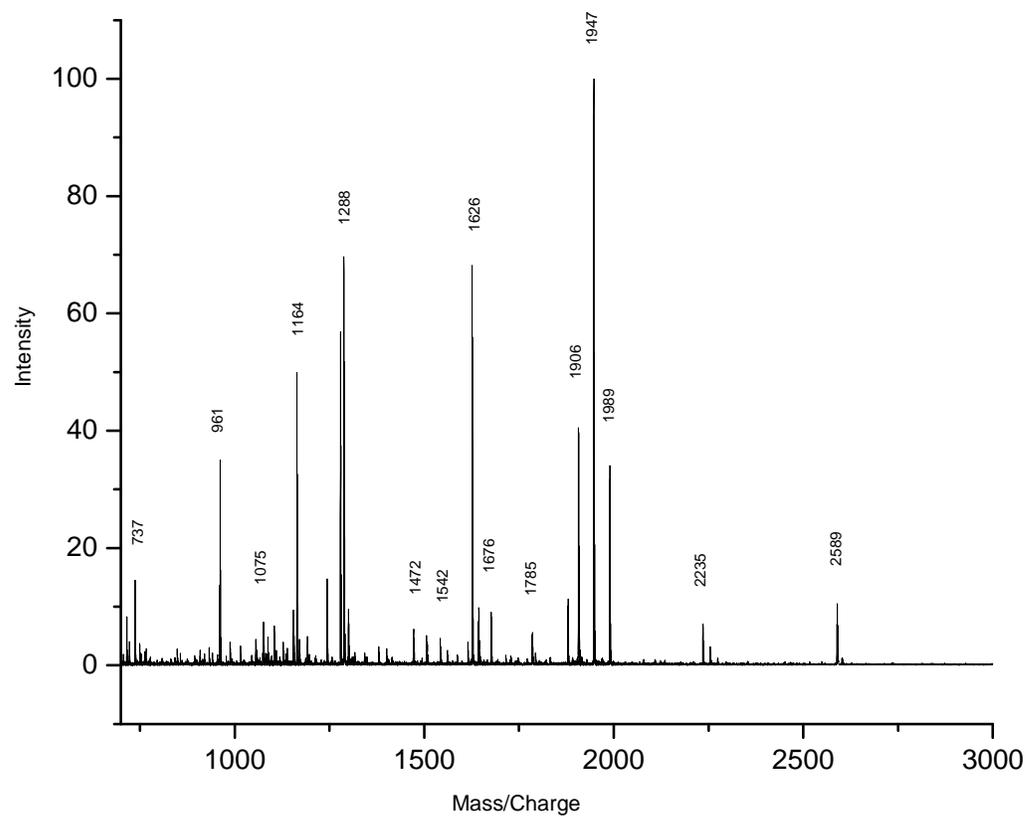


Figure 11: MALDI-TOF MS spectrum of digested HPLC fraction 14

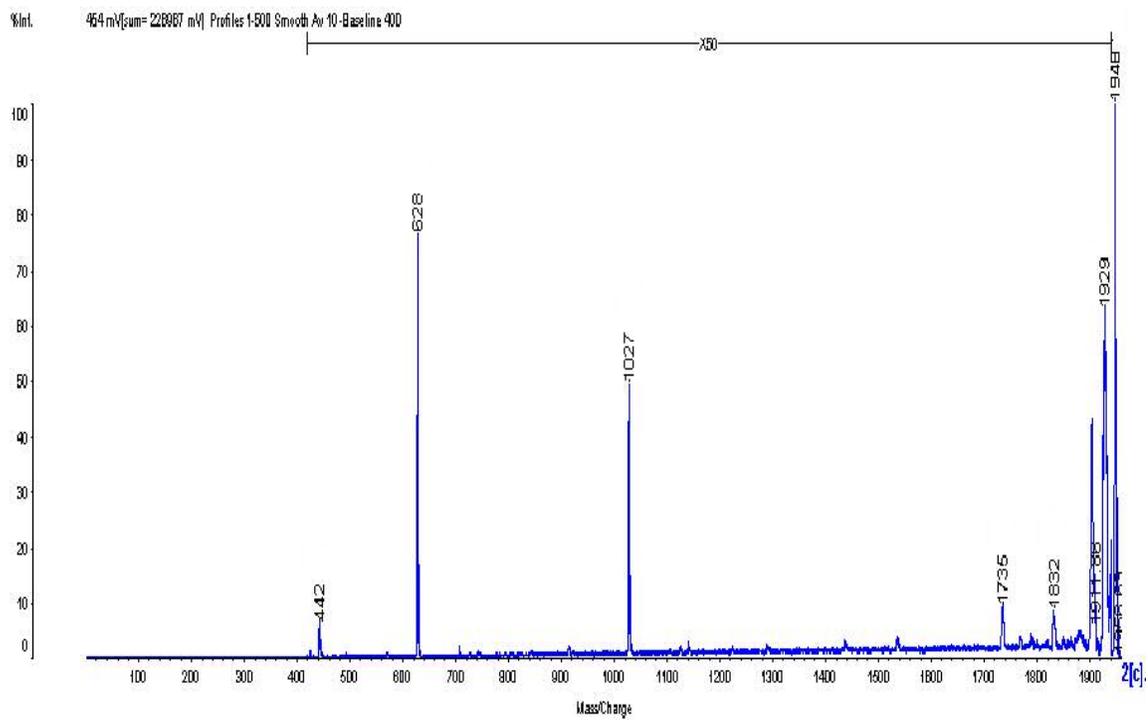


Figure 12: MALDI-TOF-PSD spectrum of HPLC fraction 14

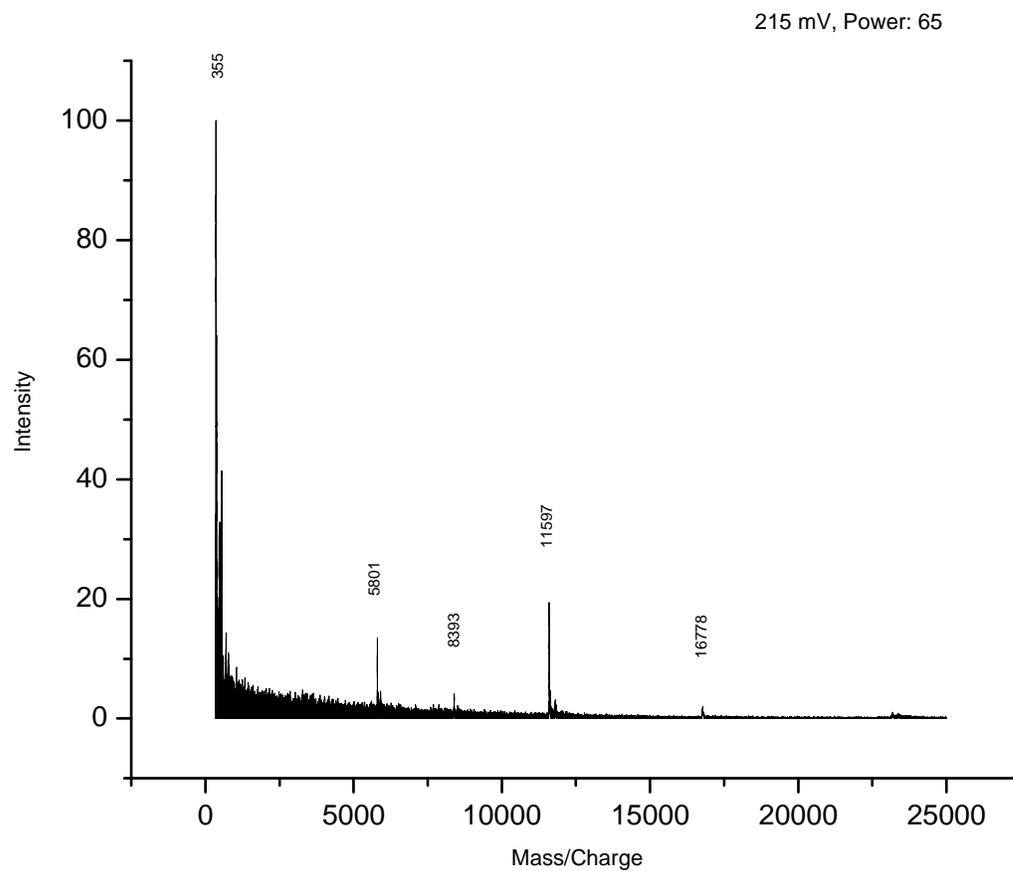


Figure 13: MALDI-TOF MS spectrum of HPLC fraction 15

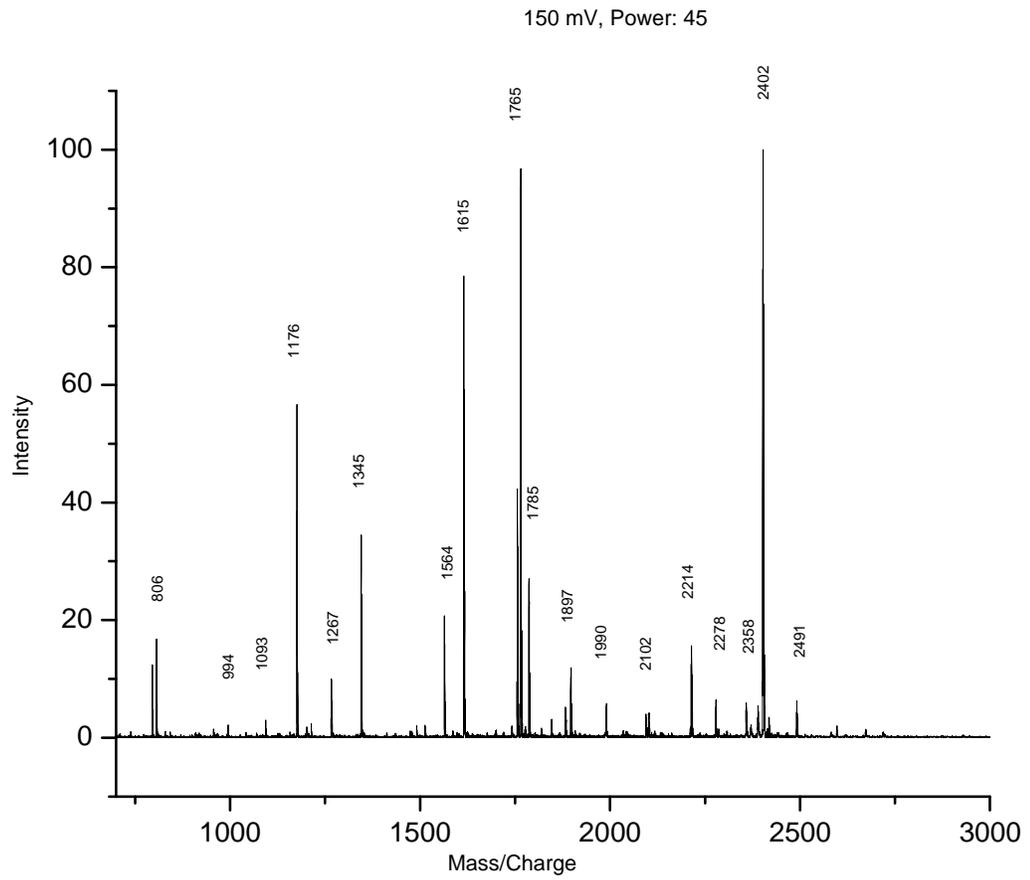


Figure 14: MALDI-TOF MS spectrum of digested HPLC fraction 15

Kratos PC Axima CTRplus V2.3.4: Mode Reflectron, Power: 55, Gate: 1620.18-1637.15, P.Exl. @ 1626 (bin 101)

%Int. 281 mV[sum= 140724 mV] Profiles 1-500 Smooth Av 10-Baseline 400

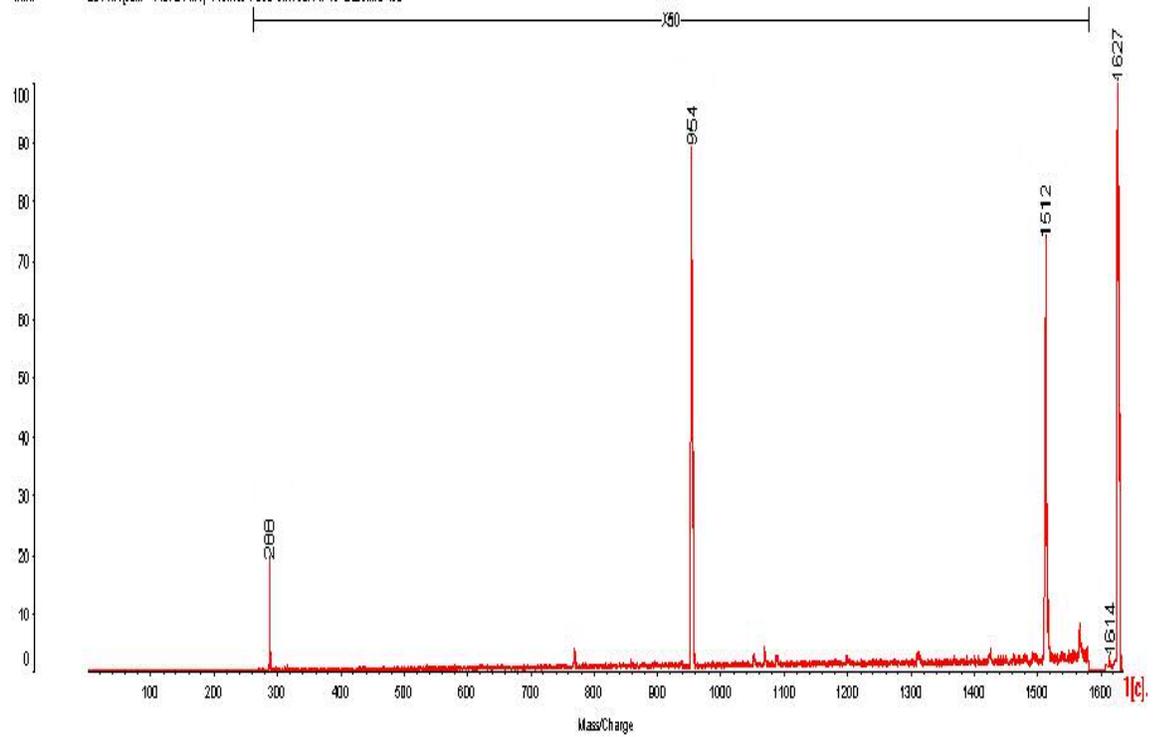


Figure 15: MALDI-TOF-PSD spectrum of HPLC fraction 15

Microsequence analysis of the PSD fragment ions was performed using Mascot and the SwissProt database. One protein each was identified for fractions 14 and 15. Table 1 shows the identified proteins, their molecular weights and scores.

Fraction Number	Protein Name	Accession Number	Mass (Daltons)	Score
14	Peptidyl-prolyl cis-trans isomerase A (PPIase)	P05092	17870	126
15	Profilin 1	P07737	14914	59

Table 1: PSD Mascot results. Scores ≥ 53 are significant.

PSD of fraction 9 resulted in proteins with no significant scores.

Sequence of PPIase

```

      10          20          30          40          50          60
      |          |          |          |          |          |
VNPTVFFDIA VDGEPLGRVS FELFADKVPK TAENFRALST GEKGFYKGS CFHRIIPGFM

      70          80          90          100         110         120
      |          |          |          |          |          |
CQGGDFTRHN GTGGKSIYGE KFEDENFILK HTGPGILSMA NAGPNTNGSQ FFICTAKTEW

      130         140         150         160
      |          |          |          |
LDGKHVVFGK VKEGMNIVEA MERFGSRNGK TSKKITIADC GQLE

```

Sequence of Profilin 1

```

      10          20          30          40          50          60
      |          |          |          |          |          |
AGWNAYIDNL MADGTCQDAA IVGYKDSPSV WAAVPGKTFV NITPAEVGVL VGKDRSSFYV

      70          80          90          100         110         120
      |          |          |          |          |          |
NGLTLGGQKC SVIRDSLLQD GEFSMDLRTK STGGAPTFNV TVTKTDKTLV LLMGKEGVHG

      130
      |
GLINKKCYEM ASHLRRSQY

```

Figure 16: Primary sequence of PPIase and Profilin 1 from the N-terminus to the C-terminus.

DISCUSSION

One dimensional gel electrophoresis is a good way of visualizing proteins in a sample, in addition to providing approximate molecular weights for these proteins. The protein mixtures loaded on the gels in figures 3 and 4 show that the proteins migrate according to molecular weight and provide an approximation for the proteins' molecular weights.

The hypothesis for this experiment is that heavier proteins come out of solution more readily than the lighter proteins, when exposed to acetonitrile and a lower temperature. The insoluble proteins are centrifuged into the precipitate. After centrifugation of the proteins, less soluble heavy proteins should be found in the precipitate, while the more soluble lighter ones remain in the supernatant. The experimental results do not show a clear separation of small from high molecular mass proteins. The proteins in the purified cytosolic extract range from approximately 6.8 – 206.9 kDa. This represents a wide range of proteins with regards to size. The proteins in the precipitated fraction are enriched for the high molecular mass proteins, and definitely contain the same high mass proteins as the protein sample prior to the acetonitrile precipitation. The 1-D gels and the MALDI mass spectrum show an abundance of low molecular mass proteins in the supernatant fraction.

Using the Mascot protein search engine and the SwissProt database, some proteins with significant scores were identified based on peptide mass maps. Most of these have masses below 30 kDa. These were discarded as potential proteins due to the complex mixture of the supernatant, and the incomplete separation by HPLC as shown in

figures 7, 10 and 13. The MALDI-TOF MS spectra in these figures indicate an abundance of several proteins, which should not be the case, had there been a complete HPLC separation and purification. Therefore it is unreliable to identify proteins in these fractions by peptide mass fingerprinting. Two proteins with significant scores were identified based on microsequencing, with masses below 20 kDa. The proteins identified are Peptidyl-prolyl cis trans isomerase A (PPIase) from fraction 14 and Profilin 1 from fraction 15. Profilin 1 has also been identified in the cytosolic extract of the drug susceptible MCF-7 cell line using 2-D gel in our laboratory. Profilin 1 is a ubiquitous protein that binds to actin and affects the structure of the cytoskeleton. It has different effects on actin at high and low concentrations. At high concentrations, it prevents the polymerization of actin, while it enhances the polymerization of actin at low concentrations [47]. PPIase is an enzyme that catalyzes the isomerization of proline residues in proteins [47].

The main goal of this experiment was to provide a means of separating small from high molecular mass proteins, so that these proteins can be analyzed to see if they play a role in drug resistance. The supernatant fraction is clearly enriched in small molecular mass proteins, which are reduced in the precipitated fraction. Although there is no cut-off for low and high molecular weight proteins, this method has proved to be successful in enriching for small molecular mass proteins. Although there is not a complete separation, there is a reproducible delineation of the small from large molecular mass proteins. Therefore this method can be used to enrich for small proteins, which can be further analyzed by HPLC, in-gel digestion, mass spectrometry and database searching.

Conclusion

Although acetonitrile precipitation did not result in complete protein separation, there is obviously enrichment for small molecular mass proteins in the supernatant. This method offers a way of preparing/separating complex mixtures of proteins for easier handling. This method can be used prior to HPLC or 1- and 2-dimensional gel electrophoresis, in an effort to effectively identify small proteins in complex mixtures such as the cytosol extract studied here.

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