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Review

Proteome analysis
I. Gene products are where the biological action is

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Abstract

Two-dimensional electrophoresis has rapidly become the method of choice for resolving complex mixtures of proteins. Since the technique was pioneered in 1975, 2-D gel methods have undergone a series of enhancements to optimize resolution and reproducibility. Recent improvements in the sensitivity of mass spectrometry have allowed the direct identification of polypeptides from 2-D gels by a procedure termed ''mass profiling''. In combination, these two techniques have made possible the characterization of the complete collection of gene products, or proteome, of an organism. Proteomes are increasingly being documented as interactive informational databases available on the World Wide Web (WWW). This availability of organismic global protein patterns will no doubt be an invaluable resource aiding the discovery of diagnostic and therapeutic disease markers.

Keywords: Review; Proteins; Proteomes

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1. Introduction

1.1. The concept of the proteome

Any biologist who started his or her research career in the 1970s and 1980s will attest to the fact that at that time, proteins had become definitely out of vogue. All of biology was focused on Genomics and Genetic Engineering, and if you couldn't clone it, no one was interested in it. Over the past decade, however, there has been a renaissance of sorts in the protein biochemistry world. This has been prompted by the phenomenal accumulation of genetic sequence data housed in easily accessible databases. As more and more gene sequence data accumulated, it became evident that it was necessary to determine what all those genes did in the cell. In addition, it was obvious that systematic sequencing of the entire genome was going to be a long, arduous process and no shortcut to the determination of disease markers. An obvious alternative approach, and perhaps a shortcut, was to look at the gene products directly and then determine the genes from them. This approach could be viewed as ''reverse genetics''. The highest resolution technique for analysis of gene products, or proteins, was first described by O'Farrell in 1975 [1]. Two-dimensional electrophoresis (2-D PAGE) combines a charge separation, isoelectric focusing (IEF) with a mass separation to produce a global map, or profile, of all (above the level of detection of the visualization technique) of the gene products present in the cell at that moment.

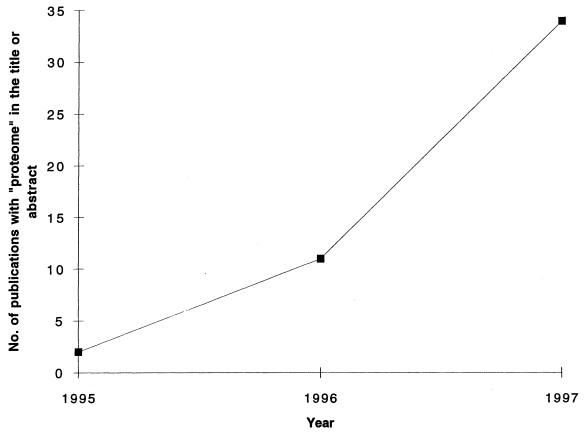

Fig. 1. Increase since 1995 in the number of publications with ''proteome'' in the title.

In 1993, the term ''Proteome'' was coined by Marc Wilkins and Keith Williams of Maquarie University in Sydney, Australia, to refer to the systematic identification of the total protein complement of the genome [2]. The subsequent increase in interest in the analysis and description of proteomes is illustrated by the rapid rise in the number of publications that include ''proteome'' in the title (Fig. 1).

2. Two-dimensional gel electrophoresis: a highly parallel analytical technique that has become central to proteome studies

Although 2-D PAGE is not a new technique [1], it has recently become the preferred method for creating protein maps of complex samples. The resolving power of 2-D PAGE is superior to that of any other protein separation techniques [3–5]. The 2-D PAGE technique separates proteins by charge (Isoelectric focusing or IEF) in the first dimension and by mass (SDS PAGE) in the second dimension. Conventional one-dimensional SDS PAGE and one-dimensional IEF will each clearly resolve (at best) approximately 100 proteins in a heterogeneous sample. Combining IEF and SDS PAGE in a two-dimensional separation results in the theoretical resolution of 10 000 individual spots, (the product of the two), since protein charge and molecular weight are orthogonal techniques. In practice, high resolution 2-D gels will resolve ca. 3000 proteins depending on the sample and sensitivity of the staining technique (although up to 10 000 spots per gel have been reported) [6].

2.1. The mechanics of 2-D gels

2.1.1. 2-D gel equipment

To simplify and enhance the reproducibility of the 2-D process, it is convenient to use a dedicated 2-D system. Dedicated high resolution 2-D gel systems are available from several electrophoresis equipment manufacturers, including Bio-Rad (Hercules, CA, USA), ESA Inc. (Chelmsford, MA, USA), and Pharmacia Biotech (Uppsala, Sweden). Most dedicated systems include the following components:

(a) A power supply; preferably one that is programmable and which can supply a consistent number of volt hours during the isoelectric focusing separations. It is also important to select a power supply which can deliver both low currents (microamps) and high voltages (3–8000 V) accurately for IEF. This feature will prevent overheating of the IEF gels as they focus.

(b) A gel casting system for first and second dimension gels, or pre-cast gels. If the researcher will be casting his or her own gels, the casting system should deliver highly reproducible IEF tube or strip gels and slab gels. Some manufacturers now provide pre-cast gels. Use different acrylamide concentrations in the slab gels for separating different molecular weight protein mixtures. High molecular weight proteins are best resolved with 7.5% acrylamide gels, lower molecular weight proteins with 15–20%. Use 10%–12.5% for most whole cell protein mixture applications. Some researchers prefer an acrylamide gradient to enhance the mass separation even further.

(c) Running systems for first and second dimension gels. The IEF running system should be able to run multiple gels simultaneously to enhance reproducibility and throughput. The capacity to run gels with analytical (25–100 μg) and preparative (1–3 mg) loads is essential since 2-D gels will probably be used for both profiling and sample preparation. For convenience and throughput, the 2-D running tank should be configured to run multiple SDS–PAGE slab gels simultaneously. The second dimension gels can be run at constant current, voltage or power. Running the gels on constant power maintains the speed of migration consistent since the resistance in the gel will change over the course of the run ($P=IV$, where P is power measured in watts, I is current measured in amperes and V is voltage).

(d) Cooling system. It is necessary to cool the second dimension gels while running since a significant amount of heat is generated during the second dimension separation. Large format gel separations require higher currents and are therefore subject to more Joule heating. Second dimension tanks are commercially available with different cooling strategies, including glycol recirculation or Peltier chips. A less desirable option is to run the second dimension tank in the cold room. The optimum temperature for running the second dimension gels is the coolest possible without precipitation of the SDS. Practically, this is between 10–20°C.

2.1.2. 2-D gel chemistry

Several strategies exist for optimization of the pI separation during isoelectric focusing. IEF separations can be run using the traditional carrier ampholyte separation or with the newer immobilized pH gradient chemistry [5,7–9]; each method has its advantages and disadvantages (for a thorough discussion, see Refs.). There are several suppliers of very good quality carrier ampholyte mixtures worldwide. Some of the larger manufacturers are Serva (Heidelberg, Germany), BDH (Poole, UK), ESA Inc. Chelmsford, MA, USA), Pharmacia Biotech (Uppsala, Sweden) and Bio-Rad (Hercules, CA, USA). Use wide range ampholyte mixtures such as pH 3–10 for complex protein mixtures. Narrow range mixtures can help to resolve close charge isoforms. Pre-cast immobilized pH gradient strip gels can be obtained from Pharmacia Biotech (Uppsala, Sweden); immobilized tube gels from ESA Inc. (Chelmsford, MA, USA). The pre-cast IPG strips are currently available in wide pH ranges; however, narrow ranges can be cast by the researcher.

When running 2-D gels it is important to use a high quality acrylamide to minimize artefacts due to acrylic acid and other high conductivity contaminants. High tensile strength acrylamide solutions are available [3–5] since large format gels can be very fragile. In fact, 2-D electrophoresis is one of the most demanding applications for reagent purity. All reagents should be the highest purity available, especially if the gels are to be visualized by silver staining. An extremely important reagent is the SDS used in the second dimension. All SDS preparations should be analyzed for consistent alkyl chain length. Using inferior quality detergents or mixed chain length detergents for the second dimension can affect polypeptide migration rate and cause doubling or streaking artefacts [10]. A variety of chemistries for the second dimension are compatible with 2-D electrophoresis separations. By far the most popular and well known is the Laemmli chemistry [11], however, other chemistries such as Tris/Tricine can be more accurate with respect to relative molecular weights [12].

Non ionic detergents used in the isoelectric focusing (IEF) gels can also have a large effect on pattern resolution. NP-40 or Triton-X 100 which have traditionally been used to solubilize proteins in IEF gels bind to proteins very tenaciously and can be difficult to exchange for SDS during equilibration to the second dimension gels. If the NP-40 is not removed, mixed micelles can result and cause large, rocket-like artefacts at the dye front (see Fig. 2). This is less a problem if the surface to volume ratio in the IEF gel is great, such as in an analytical gel, but can become serious with preparative or thicker IEF gels. In this case, a better choice of detergent for IEF gels would be octyl β-D-glucopyranoside (OBG), which is lower molecular weight and can easily be exchanged for SDS in the equilibration step. OBG is useful for solubilization of difficult proteins, such as membrane proteins (authors laboratory, unpublished results).

2.2. Sample preparation

Proper preparation of samples for IEF is the key to good 2-D results. Samples MUST be free of salts and other contaminants such as pigments, phenolic compounds and nucleic acids. Nucleic acids can be eliminated by either precipitation with protamine sulfate or digestion with DNAse/RNAse. The samples can then be cleaned up further either by precipitation with acetone or by dialysis into a diluted sample buffer. Protein concentration must be above 1 mg/ml for good recovery when using precipitation methods. Very good results for more dilute samples are obtained by dialyzing into a 1:10 dilution of a 2-D sample buffer containing urea and non-ionic detergent and then concentrating the dialysate in a speed vac [8].

2.3. Enhancing 2-D gel sensitivity: affinity enrichment techniques

Although 2-D gel PAGE is the highest resolution technique available for resolving protein mixtures, low abundance proteins can be problematic to detect in a whole cell lysate or untreated body fluids. In this case, several strategies can be proposed to enhance the sensitivity of 2-D gels. One of the most interesting has been to use chromatography (in most cases affinity chromatography) as a prefractionation step to 2-D PAGE [13–20]. The idea is not necessarily to purify individual proteins, but to enrich for classes of proteins using group specific resins. An example of

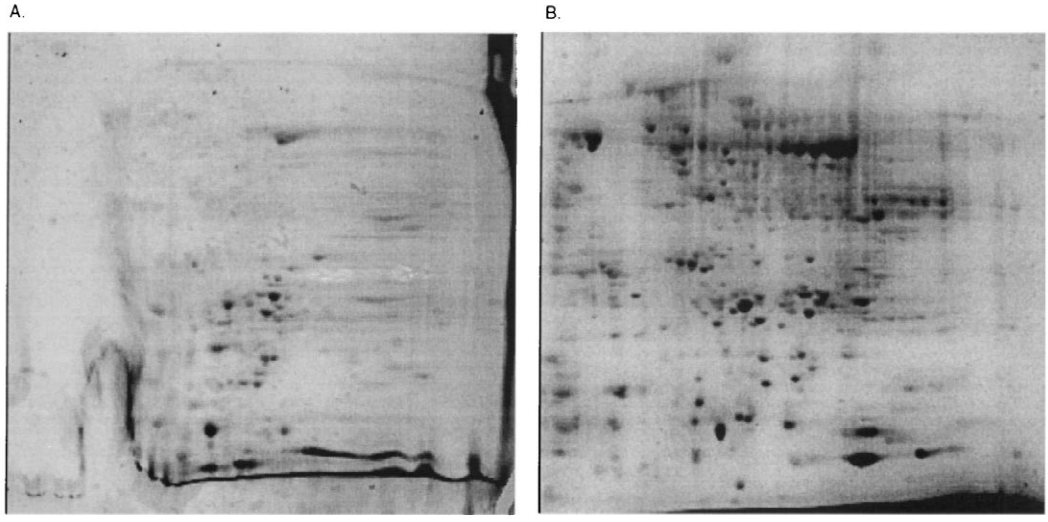

Fig. 2. Digital images of Coomassie blue stained 2-D preparative gels. (A) IEF gel solution prepared with NP-40 non-ionic detergent. (B) IEF gel solution prepared with OBG non-ionic detergent. Gels were run as described in [20]. The sample was prepared from *Brassica oleracea* (broccoli) plant tissue as described in [20]. Polypeptides range from high to low molecular weight (top to bottom) and acidic to basic (left to right). The pH range of the gel is approximately 3.8 to 8.0. Digital images were acquired and analyzed as described in [5].

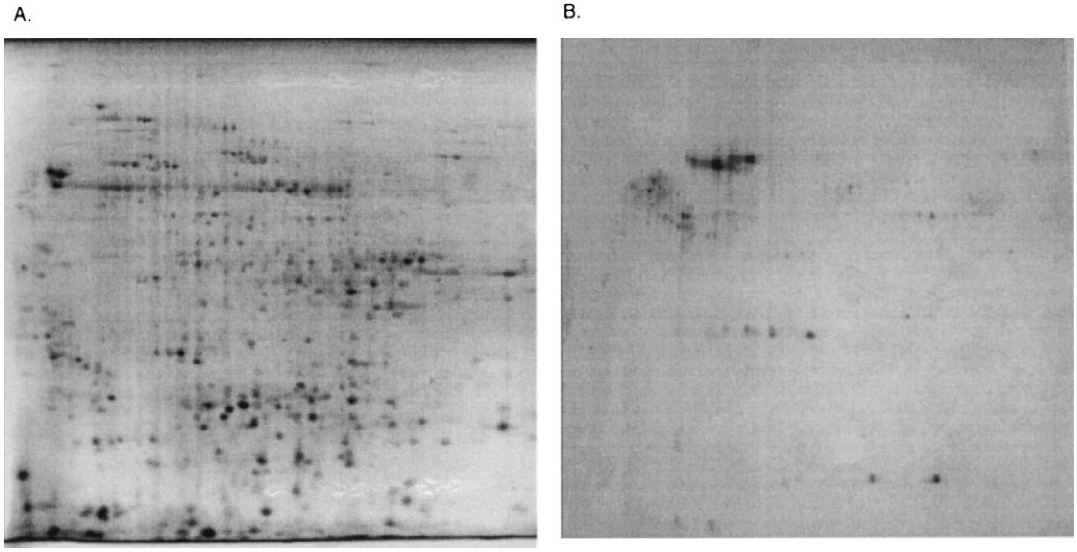

Fig. 3. Digital images of silver stained 2-D gels. One sample was treated with Concanavalin A (Con A) affinity resin prior to electrophoresis. (A) Control gel. (B) Sample treated with Con A lectin affinity resin (Con A sepharose, ≠ 17-0440-03, Pharmacia Biotech, Piscataway, NJ, USA) as described in [92]. Con A binds glycoproteins with α-D-mannose or α-D-glucose residues [93]. Gels were run as described in [5]. The sample was prepared from *Brassica oleracea* (broccoli) plant tissue as described in [20]. Polypeptides range from high to low molecular weight (top to bottom) and acidic to basic (left to right). The pH range of the gel is approximately 3.8 to 8.0. Digital images were acquired and analyzed as described in [5].

this technique is batch treatment of the sample with a lectin affinity resin to enrich for glycoproteins (see Fig. 3). Another approach is to use proteins involved in protein complexes as ligands for enrichment of all the members of the protein complex [16,17]. Still a third approach is to pre-treat the sample with an affinity resin such as Cibachron-blue agarose to reduce high abundance proteins such as albumin, which normally obscures many other plasma or serum polypeptides in the 50 kDa region [16–18], (Fig. 4). These pre-fractionation techniques will surely become more important in the near future as most of the high abundance proteins on 2-D gels are progressively identified and researchers become more interested in proteins that are now currently below the level of detection of the most sensitive staining techniques.

2.4. Enhancing 2-D gel sensitivity: in-gel activity assays

Another approach to enhancing the sensitivity of 2-D gels is to couple the 2-D protein patterns with in-gel activity assays, or zymography. Zymograms require either the inclusion of an enzyme substrate in the electrophoretic separating gel, or the overlay of a substrate containing gel onto the separating gel in a type of ''gel sandwich''. After electrophoresis, the gel/substrate sandwich is incubated and subsequently the substrate gels are stained. Digested areas appear as cleared spots thereby pinpointing the exact location of the enzyme on the electrophoretic gel. This technique has been used extensively to detect robust enzymes such as proteases and polysaccharide digestion enzymes in one dimensional SDS PAGE gels [21–29] but has recently been elegantly coupled with 2-D gels to detect esterase and protease isozymes [30,31].

3. The evolution of high throughput protein characterization from 2-D gels using mass spectrometry

In 1993, a series of papers linking the techniques of MALDI TOF MS (matrix-assisted laser desorption

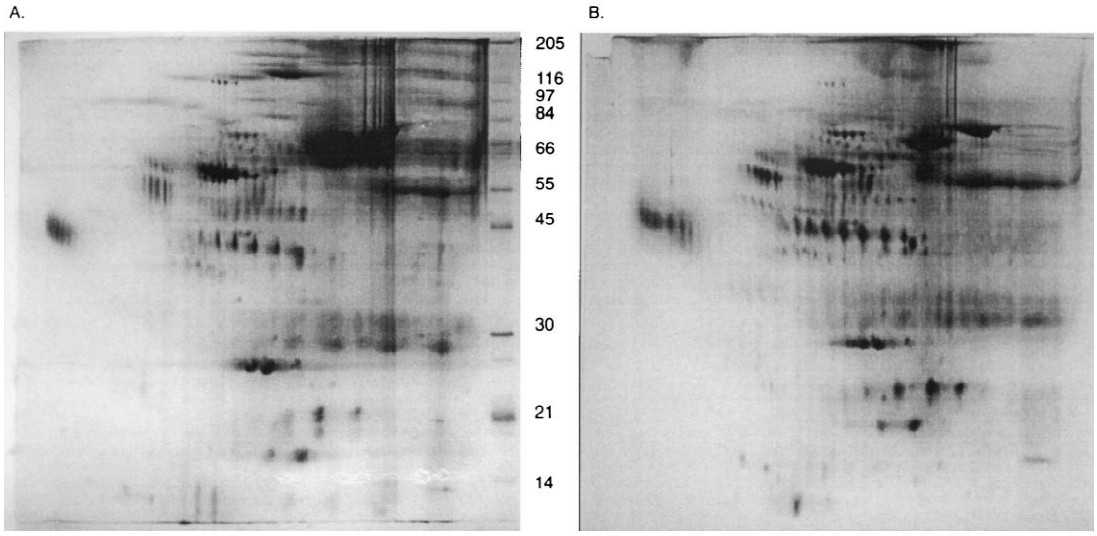

Fig. 4. Digital images of silver stained 2-D gels. One sample was treated with Cibachron Blue resin prior to electrophoresis. (A) Control gel, human plasma. (B) Plasma sample treated with Cibachron Blue affinity resin prior to electrophoresis using an Albumin Removal Kit, (#70-3701, ESA Inc., Chelmsford, MA, USA). Plasma samples were prepared as described in [94]. Polypeptides range from high to low molecular weight (top to bottom) and acidic to basic (left to right). The pH range of the gel is approximately 3.8 to 8.0. Molecular weight standards in kilodaltons are shown along the right edge of gel A. Digital images were acquired and analyzed as described in [5].

time of flight mass spectrometry) or electrospray mass spectrometry with 2-D opened the door to high throughput polypeptide characterization from 2-D gels [32–35]. One approach to protein characterization with 2-D and mass spectrometry is peptide-mass fingerprinting. This technique relies on the ability to very accurately determine the mass of several peptides obtained from a protease (usually tryptic) digest of a polypeptide spot from a 2-D gel. The peptide masses are then used to search various databases such as Genbank, PIR protein Database and the Swiss Prot Database and EMBL. Depending on the mass accuracy, an unknown polypeptide can be identified from as few as 5 peptides. Several web sites on the Internet now provide mass profiling software for database searches in real time and by email (see Table 1).

4. Amino acid sequencing from 2-D gels: is it still relevant?

In the mid to late 1980s, great improvements were made in the speed, sensitivity and ease of operation of automated protein sequencers. The automation of Edman degradation with picomole to femtomole sensitivities made possible for the first time analysis of single polypeptide spots from 2-D gels [36–39]. By the early 1990s, polypeptides from 2-D gels were routinely being analyzed by microsequencing and the first 2-D databases were initiated [40–43]. Until the development of mass profiling procedures using mass spectrometry, protein microsequencing (either n-terminal or internal after cleavage) was the method of choice for identification of spots from 2-D gels. The number of publications involving both 2-D electrophoresis and Edman sequencing (in the title or abstract) has rapidly increased since 1992 but now appears to be leveling off as mass profiling with mass spectrometry becomes the preferred method for protein identification from 2-D gels (Fig. 5). The advantages of protein sequencing are its accuracy and relatively moderate cost as compared with mass spectrometry procedures [44]. Unfortunately, the procedure is slower than mass profiling and is therefore of limited use to a high throughput Proteome analysis facility. However, until all potential genomes are sequenced and become part of sequence databases, some type of protein sequencing, either by traditional Edman degradation or by

Table 1
Internet resources for mass profiling searches

Name	Location	Profiling software
UCSF Mass spectrometry facility	http://donatello.ucsf.edu/	MS-Fit, MS-Tag, MS Edman
MOWSE	mowse@dl.ac.uk	Mowse database
EMBL Protein and Peptide Group	http://www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html	PeptideSearch
Proteometrics	http://www.proteometrics.com	PROWL
Computational Biochemistry Research group server	http://cbrg.inf.ethz.ch/section3_1.html	MassSearch
Peptide Mass Search	http://www.mdc-berlin.de/~emu/peptide_mass.html	Modified Fragfit
ProFound	http://prowl.rockefeller.edu/cgi-bin/ProFound	PROWL
MSI	http://tswww.cc.emory.edu/~kmurray/mslist.html	A collection of links to mass spectrometry internet sites

mass spectrometry methods, will be needed to accurately determine the amino acid sequences of unknown proteins.

5. 2-D gel databases: roadmaps for drug discovery

As thoroughly reviewed in [45], the protein patterns derived from 2-D PAGE analysis of human samples have been useful for certain types of clinical diagnoses and patient evaluation. Several diseases have been successfully diagnosed solely based on protein pattern modification, including Creutzfeld-Jacob Disease (CJD) [46], Bovine spongiform encephalopathy (TSE) [47], and neonatal sepsis [48]. Numerous studies have focused on cancer-related 2-D PAGE protein patterns and the characterization of tumor-specific markers [49–53]. Hochstrasser et al., evaluated the usefulness of high-resolution 2-D in a clinical environment. The resulting studies showed that ''the patient electrophoretograms revealed readily detectable modifications of the reference protein profile for some selected diseases'', such as monoclonal gammopathies, hypogammaglobulinemia, hepatic failure, chronic renal failure and hemolytic anemia [45].

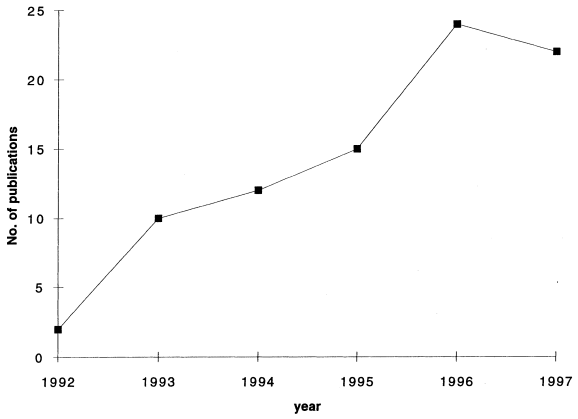

Fig. 5. Number of publications since 1992 with protein sequencing and 2-D electrophoresis in the title or abstract.

More recently, much interest has centered on the strategy of comparing 2-D PAGE patterns from various disease states to elucidate differences that may be used to develop drug targets or diagnostic markers. Practically, this leads to the creation and management of large image databases. Initially, 2-D PAGE protein databases were limited to publications in scientific journals with annotated photographs of gels. The explosion in popularity of the World Wide Web has brought with it the possibility for instant

and worldwide access to 2-D PAGE databases and linked information. The first such 2-D PAGE database, developed in 1992, is the site known as SWISS-2DPAGE [54]. This site contains archetype images of a variety of cell types, tissues, organisms and body fluids. The 2-D gel images are mouse ''clickable'' and link information from polypeptide spots to sequence databases such as SWISS PROT and to other 2-D database sites. Since the creation of SWISS-2DPAGE, numerous 2-D image and information databases have appeared on the web. A list of current sites is shown in Table 2.

6. The future of 2-D gel technology

6.1. Automation

For many laboratories, automation of the 2-D procedure would be ideal for increasing reproducibility and throughput. Several attempts have been made in the past to automate 2-D electrophoresis [72–74] and one commercial instrument was actually developed in 1991 [73,74]. Unfortunately, the TEP-1 automated 2-D instrument did not find wide acceptance, perhaps because it was limited to one gel per run. Because of the complexity of the technique, no other commercially available automated systems have as yet been developed. The future 2-D system may be a compromise between complete automation and the fully manual versions available now. Although automating the transfer from first to second dimension gels in the 2-D process itself would be convenient, in reality it is not the running of the 2-D gels, but analyzing them that is the bottleneck in most proteome projects. Nevertheless, automation of many of the intermediary steps in the complete proteome analysis process can increase throughput. For example, matching 2-D gels, picking the polypeptide spots, digestion and processing for mass spectrometry and mass profiling searches can all be fully or partially automated with robotic and software technology available or being developed now (Fig. 6).

6.2. Virtual 2-D

Any debate on the future of high throughput 2-D gel separations for proteome studies usually culminates in a discussion on ways to eliminate the gels. Theoretically, combining a high resolution charge separation in solution, such as HPLC or CE, with a mass separation in the second dimension, such as mass spectrometry, should accomplish the same thing as a traditional 2-D gel. The results would be a ''virtual'' 2-D gel or protein map. Numerous publications exist on variations of this technique [75–89]. Unfortunately, to date the resolution achieved with these methods does not approximate that of a large format, analytical 2-D gel. It is also important to note that although in-line methods such as HPLC are easier to automate, the eventual throughput using 2-D gels can actually be higher due to the parallel nature of the technique.

Recently, thin-layer IEF gels have been directly combined with mass spectrometry in an intriguing variation of the ''virtual 2-D gel'' concept [90,91]. In these experiments, the researchers obtained MALDI TOF mass spectra directly from the dried IEF gels by first soaking the gels in a matrix solution. At present there are serious limitations to this technique which include signal yield and the long time required to scan the entire gel with the laser to find the spots. However, if this technique can be optimized, it may represent a significant increase in the potential throughput for proteome analysis.

7. Conclusions

The systematic identification of the genome has been the top biological priority for many years. Now that the genomic sequences of various organisms, such as yeast and *E. coli*, have been completely defined, it has become increasingly apparent that identifying the gene products is of equal necessity. Due to post-translational modifications, the complete structures and functions of many proteins are not defined by their genetic sequence. At present, the most direct way to define the proteome of an organism is to use 2-D electrophoresis in combination with mass spectrometry. Selection of the proper components and procedures when setting up a 2-D lab for proteome analysis can enhance the speed and quality of results, since having excellent quality 2-D patterns is the first and potentially most important step in a very long process that concludes in the

Table 2
Internet resources for 2-D gel databases

Name	Location	Organism	Reference
PDD (protein disease database)	http://www-lecb.ncifcrf.gov/PDD	Human plasma, csf, urine	[55]
YPD(yeast protein database)	http://www.proteome.com/YPDhome.html	Yeast	[56–59]
Heart-2D PAGE	http://www.chemie.fu-berlin.de/user/pleiss/	Human heart	[60]
HSC-2-D PAGE	http://www.harefield.nthames.nhs.uk/nhli/protein/index.html	Human, rat and mouse heart	[61]
2DWG meta-database	http://www-lecb.ncifcrf.gov/2wgDB/	Enriched set of links to other www 2-D Databases	[62]
Biobase (Danish Center for Human Genome Research)	http://biobase.dk/cgi-bin/celis/	Human keratinocytes, tumors, serum and mouse ear, heart	[63]
Drosophila melanogaster 2D PAGE	http://tyr.cmb.ki.se/	*Drosophila melanogaster*	[64]
ECO 2DBASE	http://pcsf.brcf.med.umich.edu/eco2dbase/	*Escherichia coli*	[65]
Large Scale Biology	http://www.lsbc.com/	Rat, mouse and human liver, human plasma, corn, wheat	[66]
Yeast 2-D Gel database	http://www.ibgc.u.bordeaux2.fr/YPM	Yeast	[67]
Sub2D	http://pc13mi.biolgie.uni-griefswald.de	*Bacillus subtilis*	[68]
SWISS-2DPAGE	http://www.expasy.hcuge.ch/ch2d/ch2d-top.html	Plasma, liver, yeast, E.coli, macrophage-CL, erythroleukemia-CL, platelet, lymphoma, CSF, Hep62, RBC.	[54]
INRA Maize genome database	http://moulon.moulon.inra.fr/imgd/	Maize	NA
PPDB Phosphoprotein database	http://www-lecb.ncifcrf.gov/phosphoDB/	Phosphoproteins of neural and hematopoietic tissues	NA
Argonne PMG (Protein Mapping Group)	http://www.anl.gov/CMB/PMG/	Mouse liver, human breast cell, *Pyrococcus furiosus*	NA
Cambridge 2D PAGE	http://sunspot.bioc.cam.ac.uk	Rat neuronal	NA
Heart high performance 2-DE database	http://www.mdc-berlin.de/~emu/heart/	Human heart	[69]
2D-PAGE at Aberdeen	http://www.abdn.ac.uk/~mmb023/2dhome.htm	Virus and bacteria	[70]
JPSL 2D gel database	http://www.ludwig.edu.au/www/jpsl/jpslhome.html	Human lysosomal and carcinoma	NA
Molecular anatomy laboratory	http://iupucbio1.iupui.edu/Frankw/molan.htm	Rat kidney,liver,brain and plasma	NA
UCSF 2D PAGE	http://rafael.ucsf.edu/2DPAGEhome.html	Human melanoma	[71]

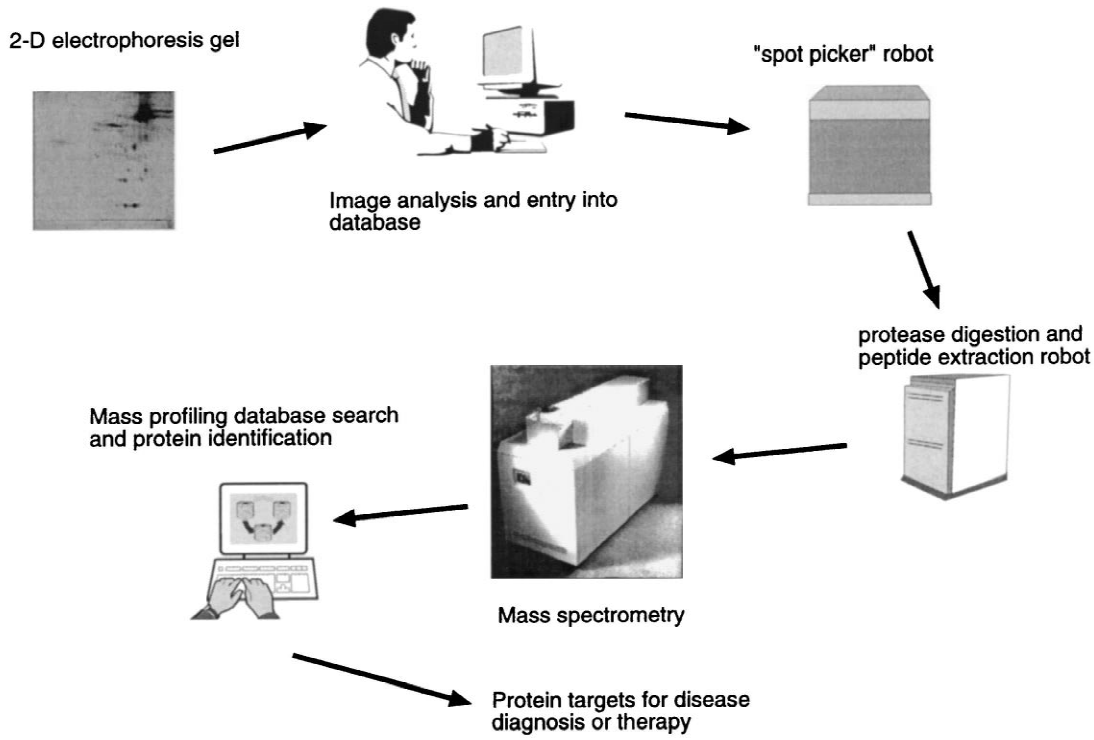

Fig. 6. Prototype core facility for proteome analysis. Many of the steps can be automated or semi-automated with robotic and software technology that exists today.

identity of a protein. Much of the information on proteomes that has been compiled over approximately the past ten years is now available on the WWW in interactive databases. This will undoubtedly have an enormous effect on the speed of searches for diagnostic and therapeutic markers for diseases.

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