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Genetic Evaluation of Proteins with High Resolution Two Dimensional (2D) Gel Electrophoresis

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ABSTRACT High resolution two dimensional gel electrophoresis with the combination of isoelectric focusing (IEF) and density gradient SDS polyacrylamide gel electrophoresis (DGSDS-PAGE) is a highly sensitive technique in population genetic, biochemical genetic and medical genetic studies. 2-D gel electrophoresis separates protein according to differences in both isoelectric point and molecular weight. The technique is so impressive and significant that one can examine accurately more than 500 proteomes (>50 proteins) in a single gel using microvolumes of the sample. Most of the laboratories dealing with protein synthesis, characterisation, protein chemistry and genetic heterogeneity have to use number of techniques to examine different known and unknown proteins. The aim of this paper is to highlight the fact that instead of going through number of complex and lengthy procedures one can make use of single high resolution two dimensional gel electrophoresis protocol for the purpose.

INTRODUCTION

All the credit goes to Arne Tiselius who discovered moving boundary electrophoresis to study serum globulin in 1937 for which he received Nobel prize in 1948. Since then number of electrophoretic procedures have increased dramatically. High resolution two dimensional gel electrophoresis (2DGE) of proteins became available for research use in 1975 mainly due to the efforts of O'Ferrell, Klose and Scheele. They used isoelectric focusing under dissociating condition (9 M urea and 20 ml of non-ionic detergent/litre) in first dimension and sodium dodecyl sulphate (SDS) gel electrophoresis in the second dimension. Then Anderson and Anderson in 1978 developed methods for running multiple parallel isoelectric focusing gels. The method though lengthy provides an overall economy of effort and record keeping relative to the multiple loci. Tracy et al. (1982) established standard maps of α_1-antitrypsin, haptoglobins, Gc globulins, α_2-HS glycoprotein and transferrins. Rosenblum et al. in 1983 proving that the method can be used to study the variants at more than 400 polypeptides of plasma proteins visible on 2DGE made it the most useful method for heterozygosity index determination. Daufeldt and Harrison (1984) evaluated the use of ISO-DALT system of 2DGE by various clinical laboratories and established standards for quality control parameters like protocol design, minimise operator dependent analytical errors and presented a map reference preparation for serum proteins (RPSP) (Fig. 1). Goldman and coworkers in 1985 carried out a linkage study in two large families. Asakawa et al. (1985) utilized the 2DGE for the study of genetic variation in 23 plasma proteins. Manabe et al. (1987) conducted a systematic analysis of serum lipoprotein and apolipoproteins using 2DGE and advocated it's use in clinical diagnostics of lipoproteinemias. Approximately more than 30 different proteins and enzymes have been studied using 2DGE so far.

In comparison to all other electrophoretic procedures apart from searching new variants, their characterisation, heterogeneity indices, this technique examines the end result of gene expression, protein post translation modifications, protein synthesis and mass polypeptide fingerprinting etc. It had been adjudged as the best procedure to investigate in vivo characteristics of plasma proteins in denatured condition (Anderson and Anderson, 1977). Recent advances in the technique allow the reproducible separation of hundreds of protein on a single gel, post separation analyses including protein sequencing, amino acid analysis, mass spectrometry and glycosylation analysis (Wilkins et al., 1996).

Purification of the Proteins

The proteins are first purified by immunoprecipitation from fresh serum with specific

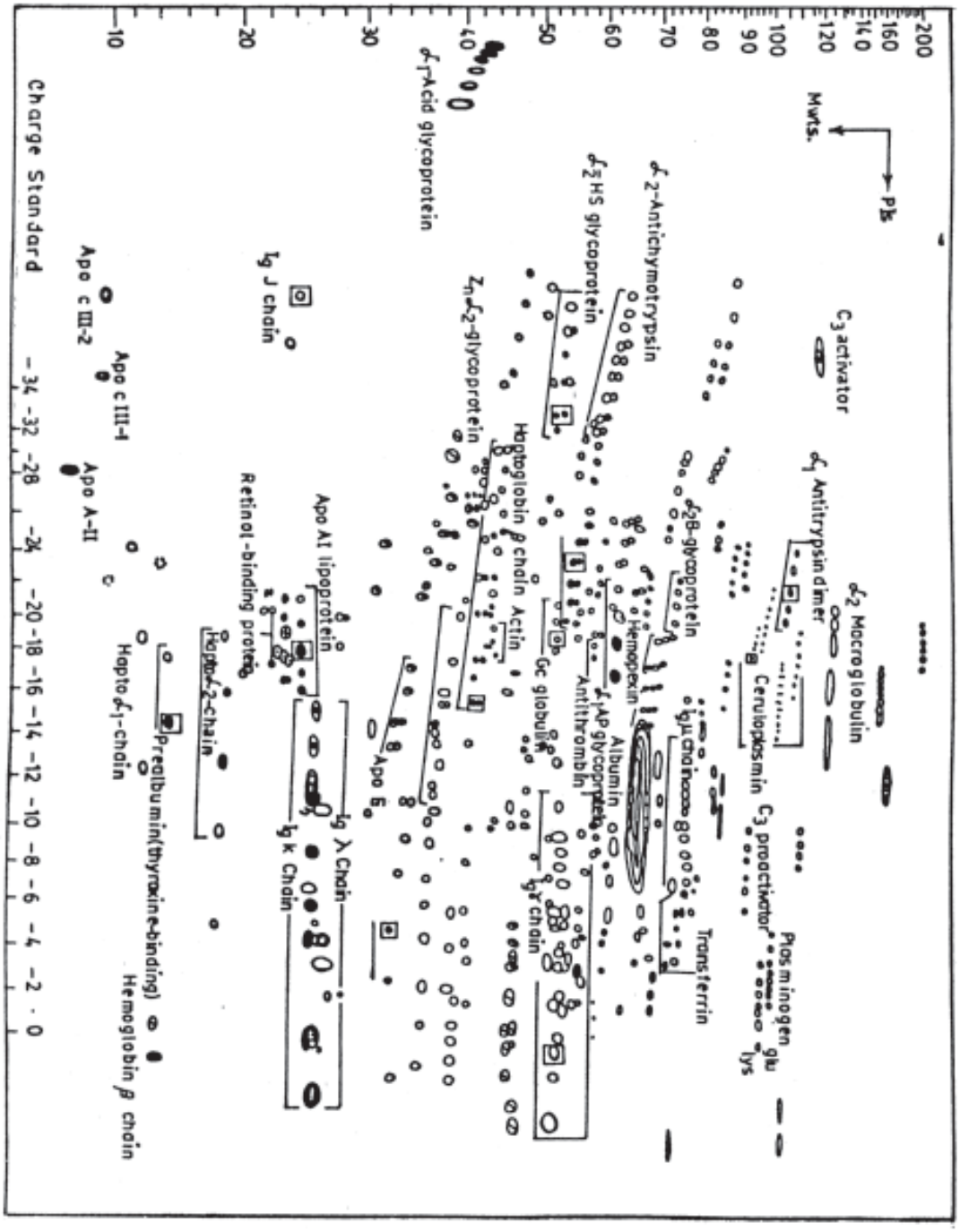


Fig. 1. Reference map of various proteins (RPSP) after 2D electrophoresis

antisera. In immunoprecipitation 100 µl of antiserum in 400 µl microfuge tube is combined in 15,30,60 µl of human serum on three successive days. The mixture is stored at -4°C and centrifuged in the microfuge for 5 minutes before each addition and before collection on fourth day. The resulting precipitate is washed twice in cold 0.1 M phosphate buffer (pH 7). Immuno precipitates made by reacting antisera with heparinised plasma generally contaminates. IgG and albumins from samples are removed by solid phase immunoadsorption. As they are oxidation sensitive the gel is rehydrated in 10m mol/L diethiothoreitol (DTT)or in 2% mercaptoethanol after polymerisation.

Purification of the Samples

Samples are first denatured in sodium dodecylsulphate ($NaDodSo_4$) to avoid any protein modifying enzyme activity and in order to separate tightly bound complexes, then sodium dodecylsulphate is removed in detergents like Urea, Nonidet P-40.

Preparation of the Samples

10 µl sample is mixed with 20 µl of 2% $NaDOdSo_4$, 5% 2- mercaptoethanol, 10% glycerol in microfuge tube and the mixture is heated for 5 minutes at 95°. After cooling this sample is applied to isoelectric focusing gel.

Iso Electric Focusing in the First Dimension (Separation w.r.t. Charge)

Isoelectric focusing is done to the proteins according to their isoelectric points. Isoelectric point is the point where the net charge of the protein is zero. The net charge of a protein is the sum of all negative and positive charges of amino acid side chains. If mixture of proteins is applied in a pH gradient, the different proteins have different net charge at this pH value. Isoelectric focusing is carried out in polyacrylamide or agarose gels but it is advantageous to use very thin gels with large pore size (Gorg et al., 1991). The pore size can be exactly controlled by the total acrylamide concentration T and degree of cross linking (bond formation).

$$T = \frac{(a+b)\times 100}{V} (\%)$$

$$C = \frac{b\times 100}{a+b} (\%)$$

Where a = mass of acrylamide in g
b = mass of methylene bis acrylamide in g
V = the volume in ml.

In IEF generally 9 molar urea is used to track hydrophobic proteins. Because of buffering capacity of urea, there is increase in the acidic pH in the gel. This leads to configurational changes in proteins and disruption of quaternary structure. So the solubility of very hydrophobic protein can be increased by addition of non-ionic detergents like Nonidet P-40, Triton X-100 or Zwitterionic detergent (CHAPS). Hoffman et al. (1989) recommended rehydrated gels for the use of IEF. Already polymerised carrier ampholyte (or immobilised pH gradient) polyacrylamide gels are commercially available (Sigma, Boerhinger manheim). The resolution capacity of isoelectric focusing was derived by Svensson et al. (1951), which can be increased accordingly.

$$pI = \frac{\sqrt{D[d(pH)/dx}}{E[-du/d(pH)]}$$

pI : resolution capacity
D : Diffusion coefficient of protein
E : Field strength (V / cm)
d(pH)/dx : pH gradient
d u/d (pH) : mobility slope at pI.

Ampholytes, Immobilines and pH Gradient

The prerequisite for highly resolved and reproducible separation, a stable and continuous pH gradient with constant conductivity and buffer capacity is required. The pH gradient can be maintained either by adding carrier ampholytes or immobilised pH gradient. Almost all the carrier ampholytes are charged, when the electric field is applied, the negatively charged carrier ampholytes migrate towards anode, the positively charged ones to the cathode and their velocity depends on the magnitude of their net negative charge. The other carrier ampholytes align themselves in between according to their pI values. The carrier ampholytes lose part of their charge so the conductivity of the gel decreases. Ampholines have low molecular weight than protein so the diffusion coefficient is small and the protein focuses in sharper zones. There

are modifications made by Brown et al. (1979), he suggested if resolution is not satisfactory it is often possible to add separators, like in separation of glycosylated HbA from neighbouring main haemoglobin bands in the pH gradient 6 to 8, addition of 0.33 mol/ l b - alanine at 15°C serves as separator. The main problem with carrier ampholytes comes when long focusing times are required, when the gradient are small and urea or non ionic detergents are used, the gradient begins to drift in both directions specially towards cathode. This leads to a conductivity gap, and gel can burn even. Some proteins migrate out of the gel. On the face of this problem alternative technique of Gorg (1991) was given using immobilised pH gradient (IPG). This gradient is built with acrylamide derivatives with immobilines by co-polymerisation with acrylamide monomers. An immobiline is a weak acid or base, is defined as its pK value. The wider the pH gradient desired, the more immobiline homologues are needed. Like ampholines, immobilines (pks 3.6, 4.6, 6.2, 7.0, 8.5, 9.3) are commercially available. pK values of immobilines and pIs of ampholines are temperature dependent so, IEF should be carried out at constant controlled temperatures (10°C). Cryo-IEF can also be used at 0°C or below this for analyses of characterisation of specific proteins, ligand bindings on enzyme substrate complexes.

Procedure for Isoelectric Focusing (IEF)

Clean the tubes with chromic acid and dry these at 60°C. Place the tubes on gel casting stand. Apply parafilm to the lower end of the tubes and seal the tubes properly. 2.5 µl acrylamide -bisacrylamide (30% acryl.+1.8% bis), 8 gm urea, 100-450 ml of triton X-100 and 8ml of deionised water is mixed. Urea is a rehydration agent, it prevents smear of the protein on the surface. Mix it properly on magnetic stirrer. Filter it and add 750 µl of ampholines (pH accrording to the pI's of the protein), 10 ml of TEMED. Deaerate the solution by applying strong vacuum for 5 minutes. Then add 20 ml of ammonium persulphate. Immediately pipette this gel solution into the gel tubes. After 10 minutes gently pour the buffer or water at the gel solution so as to prevent CO_2 diffusion into the gel which can form carbonate ions and lowers the pH of the alkaline part. Let it polymerise overnight. Put these tubes into the grommets and place these into tube cell units. Fill the lower reservoir (anolyte) with 500 ml H_3PO_4 and upper (catholyte) with NaOH. Remove the air bubbles from the gel tubes. Connect the tube cell unit to the power supply. Prefocus the gels for 1hr at 100V constant voltage. pH gradient will be formed. Disconnect it from power supply. Overlay 10 to 30 ml of test samples + lysisbuffer on the top of the gels with acupipette, To prevent the proteins from aggregating the sample is applied with dextran gel (mix 30 mg of sephadex IEF with 1 ml of solubilising mixture). Again attach the electric chords to power supply. Set the current so that the field strength in gel is low at first (40 v/cm) if the field strength is too high at the beginning some of the proteins will precipitate. Appropriate volthours should be given.

Focusing should be carried out at 10°C but at higher temperature if 10 mol/L urea is being used. The separation time depends upon the pH gradient, molecular size of the protein and power supply settings.

Maximum settings				*Time of IEF*
Prefocusing	700V	12 mA	8w	20 min
Sample entrance	520V	8mA	8w	20 min
Separation	2000V	14mA	14w	90 min
Band sharpening	2500V	14mA	18w	10 min

Current settings for separation during IEF

Procedure for Density Gradient SDS Polyacrylamide Gel Electrophoresis (DGSDS-PAGE)

Assemble the gel plates with 1.5 mm spacers with the help of agarose or cellotape. Position one feeding tube at the bottom of the plate and then apply the clamps on each side of the plates. Place the plates at casting stand. The second opening of the tube is put into first opening of peristaltic pump which is attached with gradient maker. Make adjustments so that plates are levelled and vertical. Prepare two gel solutions, one is 5% (1.28ml acrylamide- bisacrylamide {30% acrylamide + 0.4% bisacrylamide} + 5.4 ml tris HCl {pH 8.8} + 151 ml of 10% SDS, 7.0 µl TEMED and 8.28 ml distilled water) and second is 25% (6.3ml acrylamide {30% acrylamide

+ 0.8% bisacrylamide} + 5.4 ml tris HCl {pH 8.8}, 151 ml of 10% SDS, 6.4 µl TEMED and 3.1 ml distilled water). Pour both the gel solution to gradient maker. Open the mixture knob of the gradient maker to eliminate air bubbles from the solution. Place gradient maker on magnetic stirrer. Stirr gently the side containing 5% gel solution with magnet. Add 20 µl of 10% ammonium persulphate in both the gels. Start peristaltic pump (at speed key 1) and pour the gel into plates via peristaltic pump and feeding tube. After pouring 5% and 25% gels simultaneously, let the gel polymerise for 5 hrs. Overlay the gel (600 µl of 30% acrylamide, 0.4% bisacrylamide, 1.27 ml of 5M Tris HCl {pH 6.8}, 60 µl of 10% SDS, 1.25 µl TEMED, 3 ml of distilled water and 20 ml of 10% ammonium persulphate). When the overlaying gel is polymerised, remove the lower spacer and plug the empty space with 1% agarose, Thaw first dimension gel and put it on to the second dimension gel. Pipette very thin layer of 1% agarose mixed with indicator dye (Bromophenol blue) on the top of slab gel loaded with IEF gel. Mount this gel on vertical slab gel cell. Fill the upper and lower reservoir with prechilled tris-glycine buffer. Attach the cell with power pack. Give appropriate Vhrs until the tracking dye reached the end of the gel. Turnoff the power supply, remove the gels from the electrophoresis unit.After that silver staining of the gels can be done by the following method of Merril et al.(1989).Some of the oligopeptides which are not fixed by silver staining can be fixed by coomassie blue (G-250).

As this method is highly sensitive the identification of the spots can be evaluated on the light box or by photography.

Densitometric Analysis of the Gels

The high resolution two dimensional gel electrophoresis is useful in genetic investigations, where heterozygote and homozygote genotypes are not visible in simple electrophoresis. With the application of densitometry these differences can be evaluated, however the intrepretation of 2D electro-pherograms with several hundred spots is very complex process, but the analyses is very easy with the help of gelscanner and computer softwares. The patterns of the gel are recorded with densitometer and displayed as digital signals when there are huge data and data based multiple gel analyses is needed. It is also used to assess pIs, Mws. of samples assigned, even the traces are measured by densitograms.

Silver staining method by Merril et al. (1989)

Step	*Solution*	*V (ml)*	*time*
Prefixing	500 ml methanol 100 ml acetic acid with 100 ml deionised H_2O	250	> 1hr
Fixing	75 ml ethanol 25 ml acetic acid with deionised H_2O - 250 ml	250	overnight
Post fixing	150 ml ethanol 25 ml acetic acid with H_2O - 500 ml	250	two washings 10 minutes
Oxidation	0.6 g $K_2Cr_2O_7$ 600 ml H_2O deionised, 172 ml HNO_3 (65%) stirr till $K_2Cr_2O_7$ is completely dissolved	250	20 min.
Washing	with deionised H_2O 30 ml formaldehyde (37% w/v) with deionised H_2O - 300 ml	1200	4 washings for 30 seconds
Silvering	0.5g $AgNO_3$ with deionised H_2O - 300 µl	300	30 min.
Prerinse	Developer	400	2 washings (30 sec. each)
Development	53.4g Na_2Co_3 1600 ml deionised H_2O, 0.9ml formaldehyde (37% w/v)	250	15 min.
Stopping	2.5 gm glycine	250	10 min.
Washing	with distilled water	250	2 washings (10 min.)
Preserving	25 ml glycerol (87 w/v) with DH_2O - 250 ml	250 ml	15 min
Drying	Airdry (room temp.)		

In clinical chemistry where this technique is used in routine, interprets even the most close spots by enlargement on computers (the unknown variants). The conventional densitometer with white light usually does not work well with 2D and blotting methods but laser densitometer is effective for high resolution gel electrophoresis.

Evaluation of Densitograms

After running marker protein the various values (Fig. 2) can be assessed to determine isoelectric points and values of molecular weight automatically. If a mixture of protein is used, the amount of each protein applied can be calculated in mgs. If the calculations are to be done in mgs one should keep one thing in mind that every protein has a different affinity for a dye e.g. coomassie and it is complicated to calculate a constant for a protein-dye aggregate. The amount of protein is calculated by the amount of marker protein in the mixture per measured and integrated peak area (Fig. 3). The protein should be available in pure form otherwise approximate value can be assessed by comparing to albumins as marker. The absolute values lie through the identification of a protein with immuno lectin blotting and finally the values are compared with radial immunodiffusion. Correlation coefficient is applied which will indicate the substantial agreement of the results.

Scanning the Gels on Ultrascan XL Software with Computer Aid

First set the values from laser densitometer to ultrascan XL programme.

In 'Plot Menu' - no

In 'set up Menu' - ID mode

In shape of Beam - Line

To get the best results always lie the gel in same orientation on measuring tray of laser densitometer. The end of the gel is placed towards the front of light box. Enter 'Define track' menu - Enter "X-WIDTH" = 8 (6.4 mm), it indicates how often the laser beam scan the sample trail shifting of 800 mm each time. The resolution of densitometer is defined with "Y-STEP" = 1 (This is highest) the motor stops the measurement optics every 40 mm when "Y-STEP" = 1.

Enter Y-START value "20", the separation distance is 100 mm.

Enter Y-STOP value "120" —> with this all electrophoretic traces can be scanned (means range 20-120). The position of the traces can be "SAVED". Leave the 'Define Track' mode with "ESC".Choose the scan programme as 'Gel Scan XL'. Give the name of the data file.A comment can be entered in two lines.Enter the key '6' on the densitometer, the computer executes a communication test with 'Ultrascan XL'(Fig.4).

Evaluation of Molecular Weights

Pharmacia gives this software package (LMW. met.) free of charge. It can be copied in directly to c:\.

Select 'METHOD' in the main menu and choose 'Load' in submenu "file". Type in "LMW." in answer. The data of densitogram are entered in section "Edit Method". Leave the table with "ESC" and save in submenu file. In the "Evaluate" sub program, load the desired file using "File" and then "Load". A list of all the files present can be obtained by entering a question mark ("?") instead of the file name. The cursor can be moved to desired file, confirm it by pressing "Enter" key. Finally enter "LMW" under "2DGE" (or any method) and confirm with Enter. By pressing "ESC" one can turn to main menu for "Evaluate". The integration is started by choosing "Integrate" and confirm with "Enter". Once the integration is finished, the results can be printed as shown in figure 5. Choose the "Report" menu and confirm with "Enter". By choosing "Curve table" and then the results of integration and molecular weights appear. They can be saved for further analysis in sub directory.

Quantitative Analysis of Known Proteins

First pure the proteins to be tested. Apply known quantity on electrophoresis gel and is used as standard during separation, now load the method file with name "Quant.met". The peak surfaces of the gel calculated by densitometer one entered under "Edit method". Enter the separation distance which corresponds to the actual band to be tested. Then save edited method.

Quantitative Analysis of Unknown Proteins

For unknown proteins, albumin equivalents

Spot #	Area [mm²]	Volume [mm² x A]	Baseline [OD]	x-Coordinate [mm]	y-Coordinate [mm]
1	0.446	7.163	1.028	35	0
2	5.645	124.892	0.862	46	2
3	2.750	103.449	0.896	7	1
4	0.259	2.348	0.889	27	1
5	0.648	6.226	0.872	49	1
6	0.893	11.062	0.896	39	3
7	0.130	1.197	0.904	43	3
8	0.576	9			
9	5.760	1			
10	4.968	1			
11	0.850	9			
12	7.560	2			
13	4.061	1			
14	3.312	9			
15	9.288	2			
16	2.030	4			
17	0.288	2			
18	1.282	2			
19	3.514	1			
20	0.282	1			
21	2.362	2			
22	0.648	7			
23	0.691	6			

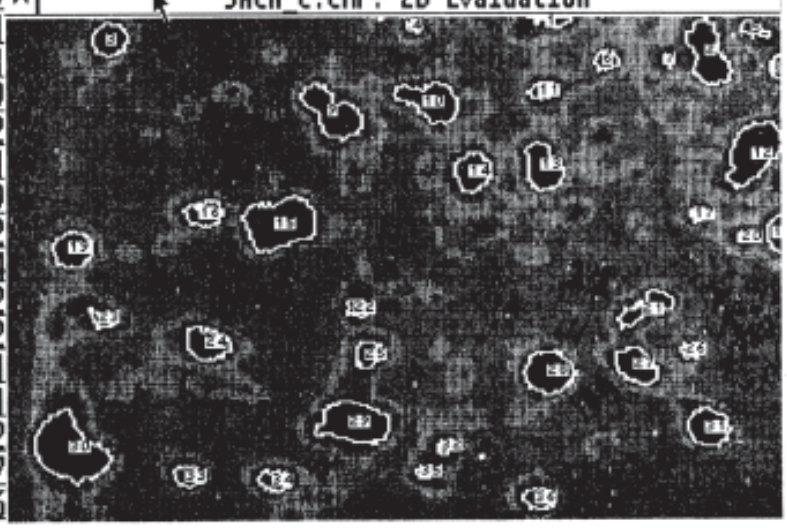


Fig. 2. Evaluation of an area (arrow) with 2D evaluation programme from laser densitogram

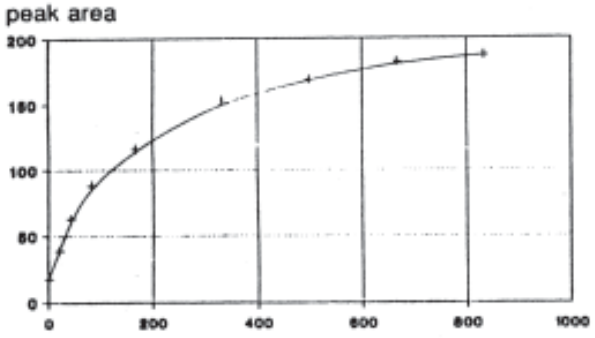


Fig. 3. Coomassie brilliant stained intensity curves of phosphorylase B, separated with SDS-PAGE

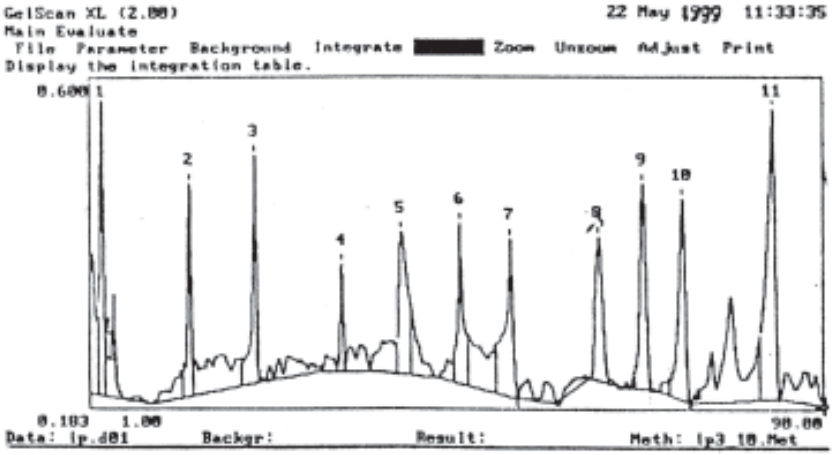
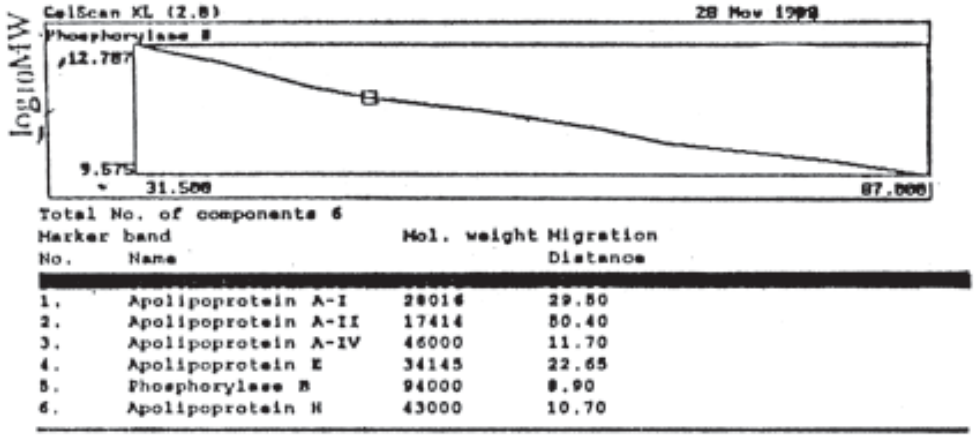


Fig. 4. Densitogram of IEF marker pI range 3 to 10



Marker band No.	Name	Mol. weight	Migration Distance
1.	Apolipoprotein A-I	28016	29.80
2.	Apolipoprotein A-II	17414	50.40
3.	Apolipoprotein A-IV	46000	11.70
4.	Apolipoprotein E	34145	22.65
5.	Phosphorylase B	94000	0.90
6.	Apolipoprotein H	43000	10.70

Fig. 5. Molecular weight calibration curve obtained by densitometric measurement of Apolipoproteins and phosphorylase B (stained with coomassie blue)

are used as external standards. Otherwise known molecular protein markers can be applied. Absolute quantification is not possible with this method. However, 'Edman's degradation' technique fecilitate the analysis with combination of peptide mass finger printing. Definite protein against which the peaks of all the other bands to be measured are compared, which gives approximate value of the amount of unknown proteins.

A home page by University hospital of Geneva and department of Medical biochemistry of Geneva University holds SWISS-2D Page databases. Data include identity, accession number, its full name as well as its reference. Currently it has 343 enteries of human S. Cerevisiae (YPD databases) and E.coli origin (ECO 2D BASE databases).

Similarly the ExPASy worldwide web (www) has SWISS 2D PAGE and SWISS PROT enteries. The web page of ExPASy server is at http://www.expasy. hcuge.ch/

Identification of Proteins through 2D Gels at Internet

AA compIdent is a program to identify

proteins from their amino acid composition by computing pIs and Mws. Similarly TaqIdent retreives protein from SWISS-PROT search engine. It enters the user name, amino acid composition of a protein and returns the answer by e-mail that are likely to be correct identification of that protein.

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