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Proteomic basis for the possible use of lymphocytes for metabolic screenings

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Summary. The advent of proteomics has provided a tool for the concomitant identification and determination of a large series of proteins using two-dimensional gel electrophoresis with subsequent mass spectrometrical analysis. We tried an approach to analyse the high abundance enzyme proteome of a lymphocytic cell line.

Immortalised lymphocytes were grown in RPMI 1640 in the presence of glutamine, harvested and the 100,000 × g supernatant of the homogenate was applied on two-dimensional gel electrophoresis with subsequent in-gel digestion of protein spots and MALDI-TOF (Matrix-associated laser desorption/ionization mass spectroscopy) analysis of resulting peptides using specific software.

A series of 57 metabolic enzymes were identified including enzymes of carbohydrate, amino acid, purine and intermediary metabolism.

We are presenting a tool for the analysis of metabolic systems including enzyme deficiencies at the protein level with the advantage of unambiguous identification of proteins and thus complementing enzyme activity determinations.

Keywords: Two-dimensional gel electrophoresis – Lymphocyte – Metabolic enzymes – Mass spectrometry

Introduction

Molecular diagnosis of metabolic disease relies on determinations of individual enzymes, mainly at the enzyme activity or protein level as well as analysis of nucleic acid sequences.

For this purpose invasive and non-invasive techniques can be used employing plasma, blood or tissues. Apart from bioptic material lymphocytes have been used that can be non-invasively obtained and are representing tissue specimen.

A series of inborn errors of metabolism (IEM) have been diagnosed in lymphocytic preparations ranging from nonketotic hyperglycinemia (Kure et al., 1992), pyruvate dehydrogenase complex of primary lactic acidemia (Kitano et al., 1990), glutaric aciduria type I (Seargeant et al., 1992), propionic acidemia (Nyhan et al., 1999), acyl-coenzyme A oxidase (Souri et al., 1994), long chain and medium chain fatty acid oxidation disorders (Brivet et al., 1995), pyruvate carboxylase deficiency (Wexler et al., 1998), biotinidase deficiency (Suormala et al., 1990), GM1 gangliosidosis (Cox et al., 1998), mitochondrial pathologies (Letellier et al., 1998), oxidative phosphorylation diseases (Artuch et al., 2000), adenosine deaminase deficiency (Apasov et al., 2001), etc., to name a few to illustrate the manifold use of lymphocytes for diagnosis of IEM.

A screening method for enzymes of IEM in lymphocytes enabling the concomitant determination and characterisation of many IEM enzyme proteins has never been reported or proposed so far and we tested the use of a classical proteomic system for the possibility of such an approach in a lymphocytic cell line.

Materials and methods

Cultivation of the lymphocytic cell line

Lymphocyte cell line 3610 is a spontaneously EBV transformed cell line from a patient with osteosarcoma and was obtained from the St. Anna Kinderspital-Forschungsinstitut (Vienna, Austria). The cell line was established from peripheral venous heparinised blood by the use of the Ficoll-Paque (Pharmacia, Uppsala, Sweden) principle and was grown in RPMI

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1640 with 10% fetal bovine serum with 70 μM gentamicin, 2 mM/L glutamine at a density of 2×10^6 cells/ml in 96 well-plates at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every other day.

Two dimensional gel electrophoresis (2-DE)

A pellet of lymphocytic cells sufficient to extract 2 mg of protein was suspended in 0.5 ml of sample buffer consisting of 40 mM Tris, 5 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO, USA), 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) (Sigma), 10 mM 1,4-dithioerythritol (Merck), 1 mM EDTA (ethylenediaminetetraacetic acid) (Merck) and protease inhibitor complete (Roche). The suspension was left at room temperature for 1 h and centrifuged at 14,000 g for 60 min. Desalting was done with Ultrafree-4 centrifugal filter unit (Millipore). The protein content in the supernatant was determined by the Coomassie blue method (Bradford, 1976).

2-DE was performed essentially as reported (Weitzdoerfer et al., 2002). Samples of 1 mg protein were applied on immobilized pH 3–10 nonlinear gradient strips in sample cups at their basic and acidic ends. Focusing started at 200 V and the voltage was gradually increased to 8000 V at 4 V/min and kept constant for a further 3 h (approximately 150,000 Vh totally). The second-dimensional separation was performed on 9–14% SDS gradient polyacrylamide gels. The gels ($180 \times 200 \times 1.5$ mm) were run at 40 mA per gel. After protein fixation for 12 h in 50% methanol, containing 10% phosphoric acid, the gels were stained with colloidal Coomassie blue (Novex, San Diego, CA, USA) for 12 h. Molecular masses were determined by running standard protein markers (Biorad), covering the range 10–250 kDa. pI values were used as given by the supplier of the immobilized pH gradient strips. Excess of dye was washed out from the gels with distilled water and the gels were scanned with ImageScanner (Amersham Pharmacia Biotech). Electronic images of the gels were recorded using Photoshop (Adobe) and PowerPoint (Microsoft) software.

Matrix-associated laser desorption ionization mass spectroscopy (MALDI-MS)

MALDI-MS analysis was performed as described elsewhere (Fountoulakis and Langen, 1997) with some modifications. The spots were excised with a spot picker and placed into 96-well microtiter plates. Each spot was destained with 100 μl of 30% acetonitrile in 50 mM ammonium bicarbonate and dried in a speedvac evaporator. Each dried gel piece was rehydrated with 4 μl of 3 mM Tris-HCl, pH 9.0, containing 50 ng trypsin (Promega, Madison, WI, USA). After 16 h at room temperature, 7 μl of distilled water was added to each gel piece and the samples were shaken in for 10 min. Four μl of 50% acetonitrile; containing 0.3% trifluoroacetic acid and the standard peptides, des-Arg-bradykinin (Sigma, 904.4681 Da) and adrenocorticotropic hormone fragment 18–39 (Sigma, 2465.1989 Da); was added to each gel piece and shaken for 10 min. Sample application was performed using SymBiot I sample processor (PE Biosystems, Framingham, MA, USA). 1.5 μl of the peptide mixture was simultaneously applied on 1 μl of matrix, consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile, containing 0.1% trifluoroacetic acid. Samples were analysed in a time-of-flight mass spectrometer (Reflex 3, Bruker Analytics, Bremen, Germany). An accelerating voltage of 20 kV was used. Peptide matching and protein searches were performed automatically. The peptide masses were compared with the theoretical peptide masses of all available proteins from all species. Monoisotopic masses were used and a mass tolerance of 0.0025% was allowed. The algorithm used for determining the probability of a false positive match with a given MS-spectrum is described elsewhere (Berndt et al., 1999).

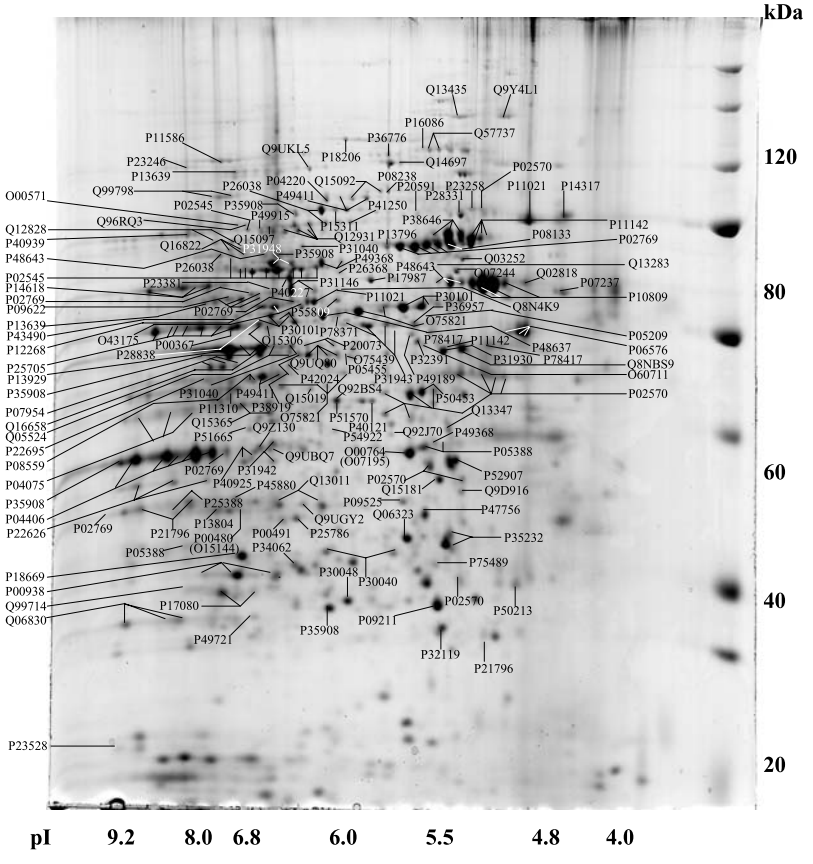

Fig. 1. Two-dimensional map of a whole cell lysate extracted from cultivated lymphocytic cell line. The proteins were extracted and separated on pH 3–10 nonlinear IPG strip, followed by 9–14% SDS-polyacrylamide gel. The gel was stained with Coomassie blue. The spots were analysed by MALDI-MS and the name of identified proteins are listed

Table 1. The list of identified proteins with accession numbers, protein names, matches and probabilities

Accession number	Name	Match	Probability
Carbohydrate handling proteins			
O43175	d-3-phosphoglycerate dehydrogenase	11	12.89
P04075	fructose-bisphosphate aldolase a	8	10.94
P09972	fructose-bisphosphate aldolase c	5	8.29
P51570	galactokinase	10	16.4
P04406	glyceraldehyde 3-phosphate dehydrogenase	5	8.25
P18669	phosphoglycerate mutase 1	5	8.37
P00938	triosephosphate isomerase	8	10.3
Amino acid handling proteins			
P09622	dihydrolipoamide dehydrogenase	4	7.21
P00367	glutamate dehydrogenase 1	5	8.23
P34897	serine hydroxymethyltransferase	7	8.28
P23381	tryptophanyl-tRNA synthetase	7	14.39
Proteins of purine metabolism			
Q9Y227	Ectonucleoside triphosphate diphospho-hydrolase 4	6	6.86
P41250	glycyl-tRNA synthetase	5	8.07
P49915	gmp synthase	8	10.71
P00491	purine nucleoside phosphorylase	5	8.39
Intermediary metabolic proteins			
Q99714	3-hydroxyacyl-CoA dehydrogenase type ii	4	8.22
Q99798	aconitate hydratase, mitochondrial precursor	10	9.67
P11310	acyl-CoA dehydrogenase, medium-chain specific	5	6.82
	alcohol dehydrogenase	7	9.81
P49189	aldehyde dehydrogenase, e3 isozyme	6	8.38
P06733	alpha enolase	8	9.09
Q05524	alpha enolase, lung specific	6	6.47
P25705	atp synthase alpha chain, mitochondrial precursor	7	11.37
P06576	atp synthase beta chain, mitochondrial precursor	10	12.15
P13929	beta enolase	5	6.79
P11586	c-1-tetrahydrofolate synthase, cytoplasmic	9	10.01
Q13011	delta3,5-delta2,4-dienoyl-CoA isomerase	5	9.08
P07954	fumarate hydratase, mitochondrial precursor	7	8.59
P09211	glutathione s-transferase p	4	8.93
P48637	glutathione synthetase	9	14.06
P78417	glutathione transferase omega 1	4	8.32
P12268	inosine-5′-monophosphate dehydrogenase 2	6	8.77
Q13907	isopentenyl-diphosphate delta-isomerase 1	5	6.8
P07195	l-lactate dehydrogenase b chain	5	8.95
P40925	malate dehydrogenase, cytoplasmic	5	8.36
Q96RQ3	methylcrotonyl-CoA carboxylase alpha chain	4	7.33
Q9HCC0	methylcrotonyl-CoA carboxylase beta chain	8	10.38
O75489	nadh-ubiquinone oxidoreductase 30 kDa subunit	8	11.49
P28331	nadh-ubiquinone oxidoreductase 75 kDa subunit	14	13.61
P22570	nadph:adrenodoxin oxidoreductase	8	8.27
Q06830	peroxiredoxin 1	4	7.93
P32119	peroxiredoxin 2	4	7.3
P49753	peroxisomal acyl-coenzyme A thioester hydrolase 2	7	8.97
Q16822	phosphoenolpyruvate carboxykinase	9	8.52
O00764	pyridoxine kinase	5	7.21
P08559	pyruvate dehydrogenase e1 component alpha subunit	4	7.49
P14618	pyruvate kinase, m1 isozyme	8	12.09
P31040	succinate dehydrogenase [ubiquinone]	12	11.69
P55809	succinyl-CoA:3-ketoacid-coenzyme A transferase	5	8.14
P30048	thioredoxin-dependent peroxide reductase	5	8.23
P40939	trifunctional enzyme alpha subunit	5	8.72
P22695	ubiquinol-cytochrome c reductase complex core protein 2	5	8.23
P31930	ubiquinol-cytochrome c reductase complex core protein I	9	10.43

(*continued*)

Table 1 (*continued*)

Accession number	Name	Match	Probability
Miscellaneous enzymes			
P28838	cytosolic aminopeptidase	10	12.42
P10768	esterase d.	5	7.21
Q15181	inorganic pyrophosphatase	5	8.4
O75439	mitochondrial processing peptidase beta subunit	8	9.54
Q9UJ70	n-acetylglucosamine kinase	9	11.23

Results

A large series of spots (SWISSPROT numbers indicated) has been unambiguously identified by MALDI-TOF and metabolic proteins were grouped into the categories: carbohydrate handling enzymes, amino acid handling enzymes, proteins of purine metabolism, enzymes of intermediary metabolism and miscellaneous.

Figure 1 presents the map of identified and assigned proteins in the lymphocytic cell line.

While most proteins were represented by a single spot, some proteins showed the presence of more than one spot (Fig. 1). The presence of several spots assigned to the identical protein may indicate posttranslational modifications or isoforms. Accession numbers, protein names, matches and probabilities are listed in Table 1.

Lymphocyte proteins were identified based upon molecular weight, pI value and peptide matches using 2-D gel electrophoresis and MALDI-MS analysis.

Peptide masses were matched with the theoretical peptide masses of all proteins of the SWISS-PROT database (data not shown). Internal standards were used to correct the measured peptide mass thus reducing the windows of mass tolerance and increasing the confidence of identification. Table 2 provides E.C. numbers, theoretical and observed pI values, and molecular weight.

Discussion

We have shown that a number of 57 metabolic enzymes can be identified on two dimensional gel electrophoresis forming the basis for the generation of metabolic screens. Another major finding is that we provide a metabolic map listing the pIs of proteins as observed rather than listing the predicted or calculated pIs which are different, a prerequisite for finding metabolic enzyme proteins on a lymphocytic map.

A general map of the lymphocyte proteome has been already published (Nyman et al., 2001; Caron et al., 2002) and our selective map specifically extends knowledge on the metabolic protein machinery. Protein maps cannot be extrapolated from one cell line or tissue to another as expressional differences are enormous. These are reflecting specific isoforms or posttranslational modifications, including phosphorylation, glycosylation, methylation, nitrosylation, etc., in the individual cell or organ systems and are mainly represented by several spots for a single protein at different pIs. In our observation several enzymes show the presence of more than one spot for an identified protein (Fig. 1).

Proteins are unambiguously identified by mass spectrometrical proteomics techniques (Lubec et al., 2003; Fountoulakis, 2001) and databases as e.g. SWISSPROT or NCBI are consulted for information on the individual structures. The information lists also pIs for the corresponding protein but this theoretical pI cannot be precisely predicted and has to be determined from the gel of the specific sample. Based upon predicted pIs from databases no position on the gel can be proposed. Our studies here enable localisation for identified proteins in lymphocytes and indeed some of the metabolic enzyme isoforms in brain show different mobility in human brain (Fountoulakis et al., 2002; Langen et al., 1999).

We here used a lymphocytic immortalised human cell line for our studies rather than primary lymphocyte culture as many different passages would be needed to grow an amount of cells sufficient to extract 2 mg of proteins, and in addition, in our view transformation per se is not relevant for the qualitative detection of metabolic enzymes.

Based upon our findings future studies can readily use the method to extend the amount of metabolic proteins found by simply modifying the conditions as e.g. pI range or gel conditions as e.g. percentage of the gels or gradients. It was the aim of this study to propose the use of the proteomic principle for screening and to provide analytical data to find the listed enzyme proteins on the gel. This method will have to be complemented by further charac-

Table 2. EC numbers, pI values and molecular weights of the individual proteins (Note that some enzymes are assigned to more than one E.C. numbers)

EC number	Name	pI value (theoretical)	pI value (our data)	Molecular weight (kDa)
Carbohydrate handling proteins				
1.1.1.95	d-3-phosphoglycerate dehydrogenase	6.29	5.8 6.2	56.65
4.1.2.13	fructose-bisphosphate aldolase a	8.39	4.6 4.8 5.0	39.29
4.1.2.13	fructose-bisphosphate aldolase c	6.46		39.32
2.7.1.6	galactokinase	6.04	6.5	42.27
1.2.1.12	glyceraldehyde 3-phosphate dehydrogenase	8.58	4.6 4.8 5.0 5.1 5.3	35.92
Amino acid handling proteins				
1.8.1.4	dihydrolipoamide dehydrogenase	7.59	5.2 5.3 5.5 5.6	54.15
1.4.1.3	glutamate dehydrogenase 1	7.66	5.5	61.4
2.1.2.1	serine hydroxymethyltransferase	8.76		55.99
6.1.1.2	tryptophanyl-tRNA synthetase	5.83	5.8	53.17
Proteins of purine metabolism				
3.6.1.6	ectonucleoside triphosphate diphosphohydrolase 4	8.55		70.26
6.1.1.14	glycyl-tRNA synthetase	6.61	6.8	83.14
6.3.5.2	GMP synthase	6.42	5.6	76.72
2.4.2.1	purine nucleoside phosphorylase	6.45	5.9	32.15
Intermediary metabolic proteins				
1.1.1.35	3-hydroxyacyl-CoA dehydrogenase type ii	7.65	4.9	26.92
4.2.1.3	aconitate hydratase, mitochondrial precursor	7.36	5.2 5.5	85.43
1.3.99.3 1.1.1.2	acyl-CoA dehydrogenase, medium-chain specific alcohol dehydrogenase	8.61	5.3	46.59
1.2.1.3	aldehyde dehydrogenase, e3 isozyme	6.01	7.3	53.53
4.2.1.11	alpha enolase	6.99		47.04
4.2.1.11	alpha enolase, lung specific	5.78	5.6 5.8	49.48
3.6.3.14	atp synthase alpha chain, mitochondrial precursor	9.16	4.5 4.7 4.8 4.9 5.0 5.2 5.4 5.5	59.75
3.6.3.14	atp synthase beta chain, mitochondrial precursor	5.26	8.0 8.3 8.4	56.56
4.2.1.11	beta enolase	7.73	5.4	46.86
1.5.1.5 3.5.4.9 6.3.4.3	c-1-tetrahydrofolate synthase, cytoplasmic	6.94	5.4	101.43
5.3.3.-	delta3,5-delta2,4-dienoyl-CoA isomerase	6.61	5.7 6.4	35.99

(*continued*)

Table 2 (*continued*)

EC number	Name	pI value (theoretical)	pI value (our data)	Molecular weight (kDa)
4.2.1.2	fumarate hydratase, mitochondrial precursor	8.85	5.0	54.64
			5.2	
			5.4	
2.5.1.18	glutathione s-transferase p	5.44	7.5	23.22
6.3.2.3	glutathione synthetase	5.67	6.4	52.38
2.5.1.18	glutathione transferase omega 1	6.24	7.8	27.57
1.1.1.205	inosine-5′-monophosphate dehydrogenase 2	6.44	5.9	55.8
5.3.3.2	isopentenyl-diphosphate delta-isomerase 1	5.93		26.32
1.1.1.27	l-lactate dehydrogenase b chain	5.72		35.51
1.1.1.37	malate dehydrogenase, cytoplasmic	6.89	5.5	36.29
			5.6	
6.4.1.4	methylcrotonyl-CoA carboxylase alpha chain	7.65	5.6	80.43
6.4.1.4	methylcrotonyl-CoA carboxylase beta chain	7.58		61.33
1.6.5.3	nadh-ubiquinone oxidoreductase 30 kDa	6.98		30.24
1.6.99.3	subunit			
1.6.5.3	nadh-ubiquinone oxidoreductase 75 kDa	5.8	7.7	79.57
1.6.99.3	subunit			
1.18.1.2	nadph:adrenodoxin oxidoreductase	8.56		53.81
1.11.1.-	peroxiredoxin 1	8.27	4.3	221.1
			4.8	
			4.9	
1.11.1.-	peroxiredoxin 2	5.66	7.6	21.89
3.1.2.2	peroxisomal acyl-coenzyme A thioester hydrolase 2	6.87		46.33
4.1.1.32	phosphoenolpyruvate carboxykinase	7.56	5.4	70.64
			5.6	
			5.7	
2.7.1.35	pyridoxine kinase	5.75	7.2	35.1
1.2.4.1	pyruvate dehydrogenase e1 component alpha subunit	8.35	5.5	43.3
			5.6	
2.7.1.40	pyruvate kinase, m1 isozyme	7.95	4.5	57.81
			4.9	
			5.1	
1.3.5.1	succinate dehydrogenase [ubiquinone]	7.06	6.2	72.69
2.8.3.5	succinyl-CoA:3-ketoacid-coenzyme A transferase	7.13	6.6	56.16
1.11.1.-	thioredoxin-dependent peroxide reductase	7.68	6.6	27.69
4.2.1.17	trifunctional enzyme alpha subunit	9.16	4.9	83
1.1.1.35				
1.10.2.2	ubiquinol-cytochrome c reductase complex core protein 2	8.74	5.1	48.44
1.10.2.2	ubiquinol-cytochrome c reductase complex core protein I	5.94	7.4	52.62
Miscellaneous proteins				
3.4.11.1	cytosolic aminopeptidase	6.29	5.8	52.64
			6.0	
3.1.1.1	esterase d	6.54		31.46
3.6.1.1	inorganic pyrophosphatase	5.54	7.4	32.66
3.4.24.64	mitochondrial processing peptidase beta subunit	6.38	6.6	54.37
2.7.1.59	n-acetylglucosamine kinase	5.82		37.38

terization of proteins including enzyme activity, detection of mutations probably by using spots for MS-MS identification as not all protein modifications maybe leading to electrophoretic shifts in a gel. This observation may challenge further work leading to the use of proteomics for large metabolic screens in a non-invasive way in human lymophocytes.

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