

A Proteomics Approach to Study the Molecular Consequence of IgE-Mediated Cell Signaling in RBL-2H3.1 Cells and 2-D Reference Map Preparation for RBL-2H3.1 Cell Line

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Abbreviations: MALDI-TOF=Matrix assisted laser desorption /ionization-time of flight; ESI=Electrospray ionization; RBL cell=Rat basophilic leukemia cell; FcεRI=high-affinity receptor for IgE; DNP-HSA=Dinitrophenol coupled to human serum albumin

Abstract

A highly reproducible 2-D map for proteome and a pattern of protein phosphorylation of high secretory variant of RBL-2H3 cells (RBL-2H3.1) (a model cell in allergy studies) in resting and treated cells with IgE or IgE+Ag were presented in this study.

The major molecular changes were observed at the proteome of 3 hour-activated cells with IgE+Ag, especially for proteins around 17 kD, compared to control. The study proceeded by identification of 13 proteins on 11 corresponding spots as up-regulated proteins in response to IgE+Ag activation. Also, protein identification on 55 spots with MALDI-TOF and ESI mass spectrometry resulted in providing a reliable 2-D reference map and opportunity for subsequent use of 1 min-activated cells' map for a phosphoproteomics study.

Key Words: Proteomics, IgE, FcεRI, RBL-2H3.1 cells

1.Introduction

High affinity receptors for IgE (FcεRI) mediated signaling is an event followed by cellular responses such as degranulation and cytokine release in mast cells and rat basophilic leukemia cells (RBL cells). The consequences of degranulation are best known for their pathological role in allergic diseases and asthma, as well as host defense against some parasitic and bacterial infections [Echtenacher et al., 1996; Urban et al., 2000]. As a result of the pivotal importance of mast cell physiology in health and disease, molecular responses of FcεRI-activated RBL/mast cells have been investigated in several laboratories by a variety of genomic-based methods such as, Northern blotting [Burd et al., 1989], subtraction libraries [Cho et al., 1998], RT-PCR [Chang et al., 1997], and SAGE [Chen et al., 1998]. However, very little is known regarding molecular changes of their whole proteome at protein level.

This study aimed to generate a reproducible 2-D map and a pattern of protein tyrosine phosphorylation for proteome of resting and FcεRI-activated RBL-2H3.1 cell line.

2. Materials and Methods

RBL-2H3.1 cells were cultured in DMEM supplemented with FCS and antibiotics, and anti-DNP IgE-sensitized cells were stimulated at presence of DNP-HSA. Cellular proteins extracted and used for 2-D PAGE. The gels were stained with colloidal Coomassie staining [Neuhoff et al., 1988] or mass spectrometry compatible silver stain (ProteoSilver™ Plus, Sigma). ImageJ software (<http://rsbweb.nih.gov/ij/index.html>) was applied to calculate the gray stain value for protein spots.

On-gel protein digest was carried out by trypsin digestion. Mass analysis was performed using an ABI Voyager-DE STR MALDI-TOF mass spectrometer operating in a positive ion and reflector mode using α -cyano-4-hydroxy-cinnamic acid (Sigma) as matrix. In parallel, protein identification for some spots was carried out by loading the trypsin-digest on ESI-MS. Mascot database was used for protein identification.

Proteins on 2-D PAGE were translocation onto PVDF membrane. Tyrosine-phosphorylated proteins were detected using anti-phosphotyrosine antibody (clone 4G10, Millipore) as primary, and HRP-conjugated antibody as a secondary antibody.

3. Results and Discussion

Highly reproducible 2-D maps were generated for IgE+Ag activated cells [Lyse (‡)] for time courses of 1, 2, 3 and 4 h along with two sets of controls; non-activated cells [Lyse (=)] and cells sensitized with IgE [Lyse (±)], along with an IgE control for the potency of IgE alone at generation of similar cellular responses in mast cells to IgE+Ag [Kitaura et al., 2003; Kalesnikoff et al., 2001] (Figure 1).

More than 700 proteins spots were visualized on colloidal coomassie blue stained gels at the pH range of 4-7, which majority of them were located at position upper than 34 kD. Protein

identification was carried out on 55 spots with MALDI-TOF or ESI mass spectrometry (Table 1).

The comparative analysis of 2-D maps from Lyse (=) samples with that of Lyse ($\pm$) did not show any significant difference. However, the differences between 2-D map of Lyse (2h $\ddagger$), Lyse (3h $\ddagger$) and Lyse (4h $\ddagger$) compared to Lyse (=) and Lyse ($\pm$), especially on the proteins located around 17 kD was identical (the area inside the rectangles, Figure 1).

The comparative analysis of protein expression on Lyse (3h $\ddagger$) against Lyse (=) by ImageJ software led to the identification of 13 proteins on 11 spots with increased level of expression at the rate of >1.5 fold (Table 2).

Protein identification was carried out by MALDI-TOF or ESI mass spectrometry (Table 1).

Collectively, This study provides evidence for the active role of IgE-mediated cell signaling toward induction of protein synthesis by introducing an array of inducible proteins (Table 2). Earlier support for this hypothesis comes from a study carried out by Chen et al. (1998) and Jayapal et al. (2006). Although, the list of up-regulated proteins identified must be considered controversial with that of introduced in two similar studies [Chen et al., 1998, Jayapal et al., 2006] this might be explained by the different sensitivities of methods applied and the established lack of definitive correlation between gene transcription and protein expression [Cox and Mann, 2007].

Induced expression of proteins involved in metabolic pathways (e.g. *Isocitrate dehydrogenase* and *ETHE 1*), cytoskeleton dynamics (e.g. *Stathmin 1*, *WDR61*, *Armc6* and *TCPI*), cell secretion and membrane function (e.g. *Rab GDI*, *Lamin-A* and *MIPi*) and biosynthesis and turnover of nucleic acids and proteins (e.g. *eIF5A*, *Adenylosuccinate synthase*, *bifunctional purine biosynthesis protein PURH* and *proteosome*) suggests a wide range of molecular changes in response to IgE-mediated mast cell activation.

The availability of highly reproducible 2-D maps made possible providing a map for distribution of tyrosine-phosphorylated proteins (Y~P) (Figure 3). 2-D maps were provided for 1 min (IgE+Ag)-activated [Lyse (1m $\ddagger$)] and non-sensitized/non-activated cells' [Lyse (=)] proteome. About 42 spots were visualized with enhanced chemiluminescence (ECL) on Lyse (1m $\ddagger$) compared to about 25 spots on Lyse (=). The difference could be assigned to the induction of tyrosine-phosphorylation following cell activation.

Apart from the various numbers of Y~P detected in Lyse(1m $\ddagger$) and Lyse(=), relatively quantitative analysis of Y~P spots' density from activated cells and non-activated cells showed an obvious difference in phosphorylation of same proteins on activated cells sample compared to reference control (Figure 4). To identify the subject protein, Y~Ps maps were aligned to the 2-D reference map of cell proteome. This study gave rise to identification of *78kD glucose-regulated protein* and *endoplasmic* which are mainly involved in protein folding and facilitate secretory proteins translocation [Gonzalez-Gronow et al., 2009; Randow and Seed, 2001; Srivastava, 2002]. Although they are mostly reported as serine and treonine phosphorylated proteins, sequence analysis with NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>), verified their potency as a target for tyrosine phosphorylation.

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Table 1. List of proteins with significant Mascot score identified on 2-D gel using MALDI-TOF and ESI mass spectrometry. Spot numbers 1-43 were identified by MALDI-TOF and 44-53 by ESI-MS (The supportive data for protein function and subcellular location are from ExPASy proteomics server and empty columns in front of some proteins means that there was not cited information in ExPASy database at the date of data analysis. Using this database, theoretical MW and PI were calculated for each protein).

No	Protein name (AC No) Mascot score	Queries matched	Function
1	Actin, cytoplasmic I (ATRTC) 197	16	Various types of cell motility
2	60 kDa heat shock protein, mitochondrial (CAA37654) 186	17	Mitochondrial protein import, Macromolecular assembly, Protein folding
3	Protein disulfide-isomerase (AAA40620) 201	23	Rearrangement of disulfide bonds, and protein folding
4	40S ribosomal protein SA (RSSA_RAT) 146	12	Cell-cell adhesion, Cell differentiation, Translation
5	60S acidic ribosomal protein P0 (AAH62028) 137	11	Translation
6	Prohibitin (A39682) 140	11	DNA synthesis, Cell proliferation
7	Pyruvate dehydrogenase E1 subunit beta (Q6AY95_RAT) 87	7	Glycolysis
8	Phosphatidylethanolamine-binding protein 1 (PEBP1_RAT) 87	6	Various forms of biological process
9	EF-hand domain-containing protein C14orf143 homolog (Q6AXQ2_RAT) 40*	4	Ca ²⁺ -binding activity
10**	Alpha-enolase (ENOA_RAT) 77	6	Glycolysis, Plasminogen activation
	Enolase (Q5EB49_RAT) 77	6	Glycolysis,
11	Nucleoside diphosphate kinase A (A45208) 96	7	Differentiation, Endocytosis, Neurogenesis, Nucleotide metabolism
12	Superoxide dismutase [Cu-Zn] (CAA29121) 98	6	Free radical destruction
13	D-3-phosphoglycerate dehydrogenase (SERA_RAT) 114	13	Amino acid biosynthesis
14	Protein disulfide-isomerase A3 (AAH62393) 94	13	Ref to No 3
15	Thioredoxin (S04352) 78	9	Electron transport
16	Proteasome subunit alpha type-6 (JX0230) 100	12	Intracellular proteinase
17	Protein-L-isoaspartate (D-aspartate) O-methyltransferase (PMT_RAT) 55 *	5	Protein repair
18	Cytochrome-c oxidase (EC 1.9.3.1) chain Va precursor (S04592) 57	6	Electron transport
19	Malate dehydrogenase, cytoplasmic (MDHC_RAT) 91	7	Tricarboxylic acid cycle
20	Rho GDP-dissociation inhibitor 1 (Q5XI73_RAT) 117	7	Regulation of Rho proteins function
21**	Chain B, Rat Liver F1-Atpase (IMAB_B)		Electron transport

No	Protein name (AC No) Mascot score	Queries matched	Function
	356 Tubulin, beta 6 (Q4QQV0_RAT) 200 Tubulin beta-2B chain (A25113) 190		Microtubule formation Microtubule formation
	Tubulin beta-5 chain 190 Tubulin beta-2C chain (Q6P9T8_RAT) 138		Microtubule formation Microtubule formation
	ATP synthase subunit beta, mitochondrial (Q499W0_RAT) 132		ATP synthesis, Ion transport
22	T-complex protein 1 subunit epsilon (Q68FQ0_RAT) 194	18	Protein folding
23	60 kDa heat shock protein, mitochondrial (CAA37654) 207	18	Ref to No 2
24	Chloride intracellular channel 1 (Q6MG61_RAT) 126	9	Chloride ion transport
25	Chloride intracellular channel 1 (Q6MG61_RAT) 195	11	Ref to No 24
26	Translationally-controlled tumor protein (S00775)*** 76	6	Microtubule stabilization
27	Rho, GDP dissociation inhibitor (GDI) beta (Q5M860_RAT) 81	5	Ref to No 20
28**	Proteasome subunit beta type-6 (PSB6_RAT) 156 Rho, GDP dissociation inhibitor (GDI) beta (Q5M860_RAT) 74	7	Ref to No 16 Ref to No 20
29	Sorcin (BAB25652)*** 92	9	Ca ion transport
30	Mitogen-activated protein kinase scaffold protein 1 (AAH86353) 49*	4	Involved in MAPK cascade
31	Adenine phosphoribosyltransferase (AAA40757) 60	6	Purine salvage
32	Proteasome subunit alpha type-5 (JX0229) 72	6	Ref to No 16
33	78 kDa glucose-regulated protein (HHRTGB) 203	19	Protein assembly
34	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial (Q99NA5) 406	16	Tricarboxylic acid cycle
35	Eukaryotic translation initiation factor 3 subunit I (BAB22440)*** 117	13	Tricarboxylic acid cycle
36	Endoplasmic (Q66HD0_RAT) 94	16	Transport of secretory proteins
37	Transitional endoplasmic reticulum ATPase (TERA_RAT) 135	23	Golgi fragmentation, membrane transport
38	dnaK-type molecular chaperone hsp72-ps1 – rat,Heat shock cognate 71 kDa (S31716) 96	20	Chaperon activity
39	Stress-70 protein, mitochondrial (I56581) 201	18	Cell proliferation and aging
40	Proteasome subunit alpha type (Q6IE67_RAT) 119	11	Ref to No 16
41	translation initiation factor 5A (A8K9A0) 291	12	Protein biosynthesis
42	Stathmin (STMN1_RAT) 75	8	Microtubule destabilization
43	Ferritin light chain 1 (Q6P7T1_RAT) 131	9	Iron homeostasis
44	Hypoxanthine-guanine (I51842) phosphoribosyltransferase 62	8	Purine recycling
45	Proteasome subunit alpha type-1 (SNRTC2)	14	Ref to No 16

No	Protein name (AC No) Mascot score	Queries matched	Function
46	194 WD repeat-containing domain 61 (Q4V7A0) 468	15	Various biological process
47	WD repeat-containing domain 61 (Q4V7A0) 468	15	Various biological process
48**	adenylosuccinate synthase (gi 157787018) 413	12	purine nucleotide biosynthesis
	Armc6 protein (B2RYL4) 187	4	
	Rab GDP dissociation inhibitor beta (P50399) 116	5	Ref to No 20
49	T-complex protein 1 subunit gamma (Q6P502) 532	22	Ref to No 22
50**	Lamin-A (P48679) 620	29	Nuclear envelop formation
	Bifunctional purine biosynthesis protein PURH (O35567) 94	6	Purine biosynthesis
51**	Proteasome subunit alpha type-6 (P60901) 802	20	Ref to No 16
	Ethylmalonic encephalopathy 1 (B0BNJ4) 91	5	Hydrolase activity
52**	Heterogeneous nuclear ribonucleoprotein C (Q4V8K6) 603	20	mRNA and nucleotide binding activity
	SUMO-activating enzyme subunit 1 (Q6AXQ0) 102	7	Protein modification
53	Methylthioribose-1-phosphate isomerase (Q5HZE4) 593	12	Amino acid biosynthesis, Methionine biosynthesis
54	Calreticulin (P18418) 568	23	Oligomeric assembly and quality control in ER
55	Proteasome subunit alpha type-5 (Q9Z2U1) 521	18	Ref to No 16

No, Protein spot number on gel; Ref, Refer to; (*), Proteins with lower score (<56) are not mentioned, except in the case of co-ordinancy of mass spectrometry data for MW and calculated PI in database with those are estimated from the 2-D gel; (**), From several proteins identified in each spot only the proteins with significant score and matched MW and PI in MS and 2-D are included; (***), Database was searched against RAT, however, proteins from other species are included if no proteins identified in RAT and PI and MW on 2-D gel were similar to those proteins.

Table 2. List of proteins identified as over-expressed proteins on the gel for (IgE+Ag)-stimulated cells for 3 h (lyse (3h₊)) compared to non-sensitized/non-activated cells as control (Lyse (=)). From 270 spots analyzed on corresponding 2-D maps of activated and non-activated cells with image analysis software, ImageJ, for gray stain density (GSD), 11 protein spots were recognized with the increased rate of ≥ 1.5 .

No on gel (Fig 2)	Protein name (Ac No)	GSD 3h ₊ /=
34	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial (Q99NA5)	1.63
41	translation initiation factor 5A (A8K9A0)	1.65
42	Stathmin 1 (STMN1_RAT)	1.50
46	WD repeat-containing domain 61 (Q4V7A0)	2.04
47	WD repeat-containing domain 61 (Q4V7A0)	3.57
48	adenylosuccinate synthase (gi 157787018)	1.51
	Armc6 protein (B2RYL4)	
	Rab GDP dissociation inhibitor beta (P50399)	
49	T-complex protein 1 subunit gamma (Q6P502)	1.56
50	Lamin-A(P48679)	1.61
	Bifunctional purine biosynthesis protein PURH(Q35567)	
51	Proteasome subunit alpha type-6 (P60901)	2.5
	Ethylmalonic encephalopathy 1 (B0BNJ4)	
53	Methylthioribose-1-phosphate isomerase (Q5HZE4)	1.8
55	Proteasome subunit alpha type-5 (Q9Z2U1)	1.5

Figure legends

Fig. 1. 2-D gel electrophoresis of proteins from RBL-2H3.1 cell lysates in resting state (without IgE sensitization and Ag stimulation) (**A**), sensitized cells with IgE for 16h (**B**) and activated cells with IgE+Ag for 1h (**C**), 2h (**D**), 3h (**E**) and 4h (**F**). Each gel is representative of 500 µg proteins from cell lysate loaded on 13cm IPG DryStrip (pH 4-7). Protein spots are visualized with Silver staining method on 12% SDS gel. The rectangles surround the area that majority of differences are placed.

Fig. 2. 2-D map of proteins from cell lysate of 4h-activated RBL-2H3.1 cells and identified proteins numbered 1-55 including those detected as up-regulated proteins at presence of IgE+Ag. Proteins were excised from silver-stained gel and subjected to in-gel trypsin digestion and subsequent MALDI-TOF or ESI mass spectrometry.

Fig. 3. Tyrosine-phosphorylated proteins' pattern on 2-D map. Phosphoproteins were visualized on photographic film after ECL and their locations were determined on 2-D reference map by precisely aligning of signals on photographic film and 2-D gel. Each circle is representative of single spot detected on photographic film.

Fig. 4. Differential tyrosine phosphorylation of some proteins on 2-D map of 1min-activated RBL-2H3.1 cells with IgE+Ag (right column) in comparison to non-activated cells (left column).

Figure 1

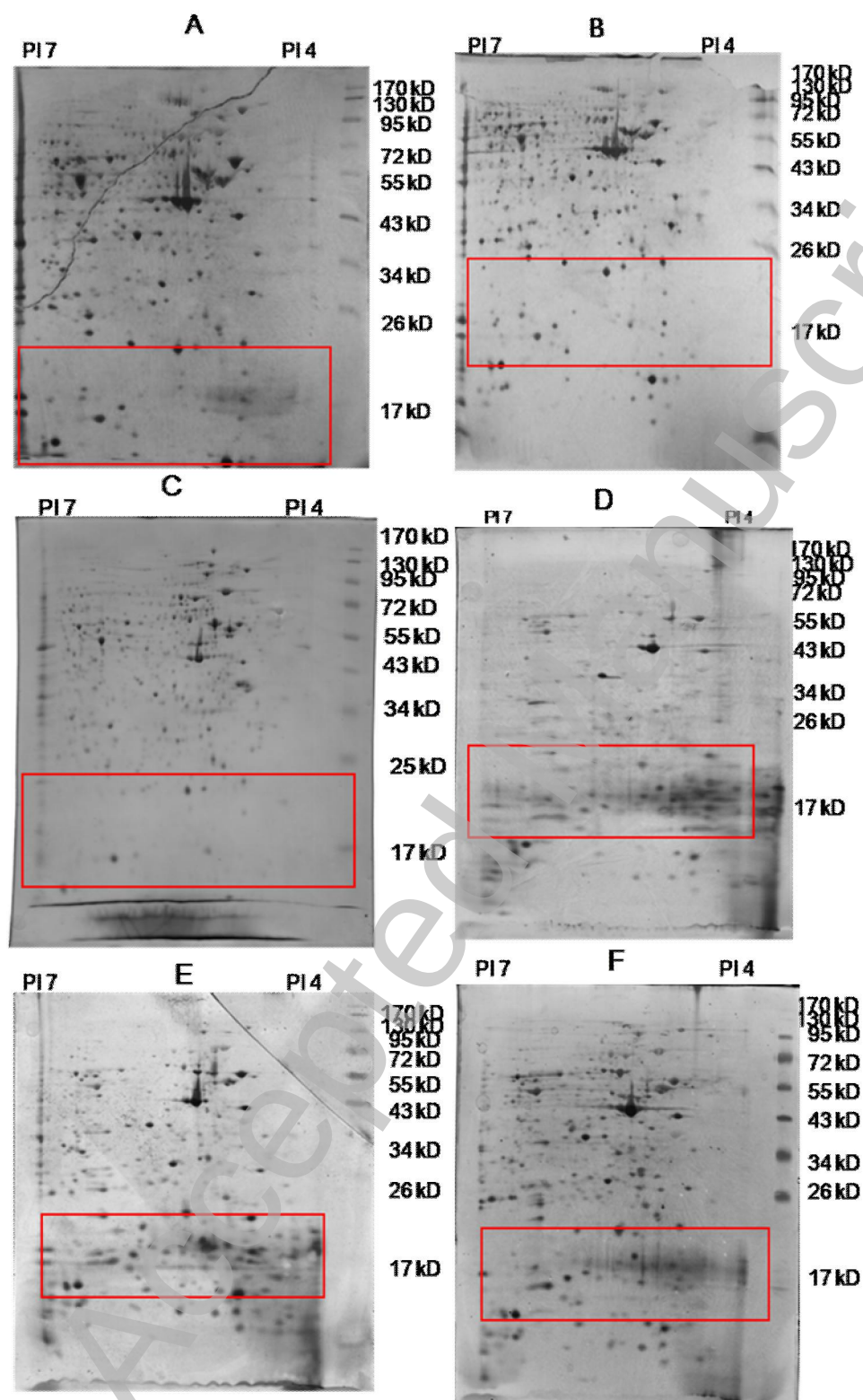


Figure 2

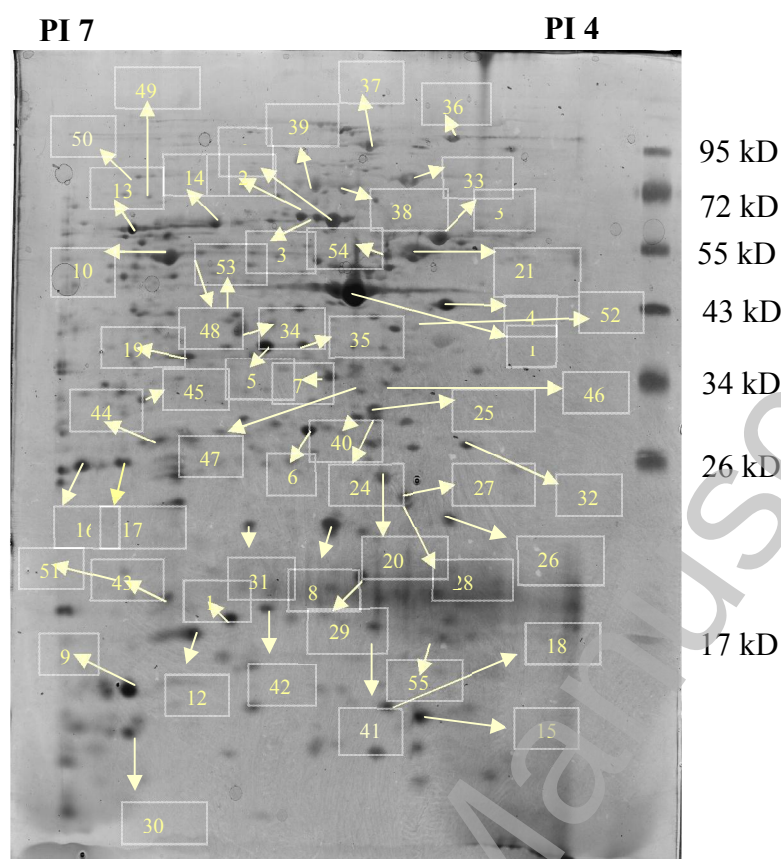


Figure 3

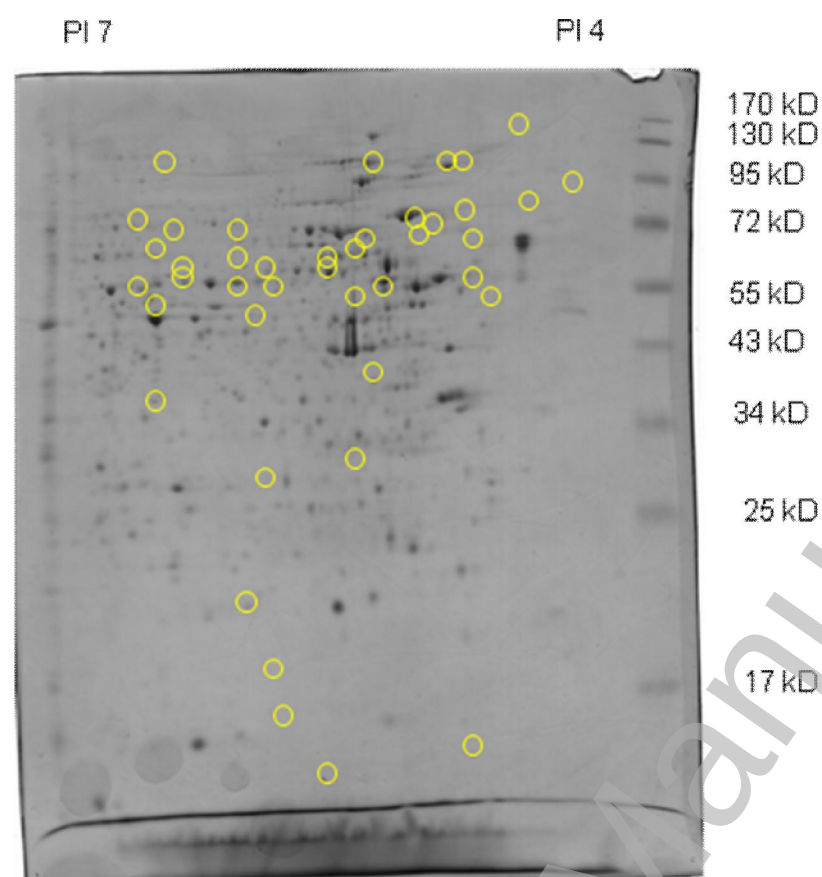


Figure 4

