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Effect of Affinity Sorbent on Proteomic Profiling of Isatin-Binding Proteins of Mouse Brain

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Abstract—Use of small molecules for isolation of particular sub-proteomes is often complicated by the need for chemical modification of a parent compound for affinity sorbent preparation. Isatin (indoledione-2,3) is an endogenous indole that exhibits a wide spectrum of biological activities. Using 5-aminocaproylisatin for proteomic profiling of fractionated rodent brain homogenates, we previously identified more than sixty individual proteins. However, proteins tested in an optical biosensor study for validation of their isatin-binding capacity demonstrated different affinity for immobilized 5-aminocaproylisatin and 5-aminoisatin. In this study, we comparatively evaluated proteomic profiles of isatin-binding proteins separated using both isatin analogs as the affinity ligands. The total number of identified proteins was higher with the shorter isatin analog (88 versus 66), and only 22 proteins were identical in the two proteomic profiles. Thus, proteomic profiling of brain isatin-binding proteins is significantly influenced by the length of the spacer between the amino group used for affinity ligand coupling to Sepharose and the isatin moiety. This suggests that the actual number of brain proteins interacting with endogenous (unmodified) isatin still remains underestimated due to different affinity of proteins for the isatin analogs used for the affinity-based proteomic profiling.

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Key words: small molecule affinity chromatography, proteomic profiling, sub-proteome isolation, brain, isatin, isatin-binding proteins

A good affinity-based sample processing for proteomic profiling of particular groups of proteins (sub-proteomes) is a key factor that determines the success of separation of particular groups of proteins and their subsequent identification [1, 2]. In the case of small molecule affinity chromatography (SMAC), coupling of a ligand to activated resins often requires chemical modification of the parent compound due to the absence of reasonable functional groups needed for its covalent attachment to the sorbent [1-4].

Isatin (indoledione-2,3) is an endogenous indole found in the mammalian brain, peripheral tissues, and body fluids [5-7]. Isatin attracts much interest due to the potential pharmacological actions of isatin itself and its many analogs [5-9]. The isatin moiety is present in a range of compounds that act as inhibitors of apoptosis, anticonvulsants and antiviral, anti-bacterial, and anti-fungal agents [7-9]. Thus, comprehensive proteomic analysis of isatin-binding proteins may help better understanding of isatin functions in the body and identification of proteins that account for numerous effects of isatin analogs reported in the literature [4-8]. Since isatin lacks functional groups suitable for covalent coupling to resins without loss of its biological activity, the use of isatin analogs as affinity ligands becomes basically an obligate step for isolation of isatin-binding proteins for subsequent proteomic analysis.

The first proteomic profiling of rat and mouse brain isatin-binding proteins was carried out using N-(6-aminocaproyl)-5-aminoisatin (Fig. 1) as the affinity ligand [10, 11]. It resulted in identification of more than 60 individual brain proteins. The isatin-binding capacity of some of the identified proteins has been validated in a surface plasmon resonance (SPR) study using a Biacore 3000 optical biosensor with 5-aminocaproylisatin or 5-aminoisatin as the affinity ligand [10]. Although K_d values obtained during the optical biosensor experiments were consistent with the range of K_d values reported for [^{3}H]isatin-binding to brain sections [11] and purified enzymes [12], the proteins tested in the biosensor study showed different affinity to these ligands [10]. In addi-

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tion, such known membrane-bound and soluble protein targets for isatin inhibition as monoamine oxidase (MAO) B, atrial natriuretic peptide (ANP)-stimulated particulate guanylate cyclase, and NO-stimulated soluble guanylate cyclase were not identified as isatin-binding proteins in either rat or mouse brains. This suggested that not all isatin-binding proteins exhibit reasonable affinity to N-(6-aminocaproyl)-5-aminoisatin required for their binding to the affinity ligand during removal of non-specifically bound proteins by extensive washing.

Since 5-aminoisatin was originally used in the SPR study that demonstrated the existence of isatin-binding protein fractions of brain and some other organs [13], and at least some purified isatin-binding proteins exhibited higher affinity to 5-aminoisatin than to N-(6-aminocaproyl)-5-aminoisatin, in this study we compared proteomic profiles of mouse brain isatin-binding proteins separated using these affinity ligands.

MATERIALS AND METHODS

Chemicals. Cyanogen bromide-activated Sepharose 4B, dithiothreitol, iodoacetamide, trypsin, Tris(hydroxymethyl)aminomethane, guanidine hydrochloride, ammonium hydrocarbonate, sodium chloride, Triton X-100, acetonitrile, and Coomassie Brilliant Blue G-250 were purchased from Sigma-Aldrich (USA). Acetic acid (sodium salt), boric acid, formic acid, sodium tetraborate, and sodium hydroxide were from Acros Organics (USA). 5-Aminoisatin and N-(6-aminocaproyl)-5-aminoisatin were synthesized as described earlier [11, 14].

Animals and preparation of brain fractions. Male C57BL/6 mice (20-25 g) obtained from the Stolbovaya nursery (Russian Academy of Medical Sciences) were used in the experiments, which were performed at least one week after their arrival from the nursery. Animals received a standard laboratory chow and water *ad libitum*, and their decapitation was performed between 11:00-13:00. The brains were immediately dissected and homogenized in 0.05 M potassium-phosphate buffer, pH 7.4, using an Ultra-Turrax T 10 homogenizer at low speed to obtain 30% homogenate (w/v). Isolation of particulate and membrane fractions of mouse brain homogenate and their subsequent treatments for affinity chromatography were performed exactly as described in [10].

Preparation of 5-aminocaproylisatin-Sepharose. N-(6-aminocaproyl)-5-aminoisatin and 5-aminoisatin were coupled to cyanogen bromide-activated Sepharose 4B according to standard protocols. The amount of immobilized isatin analog evaluated by determining the number of reactive groups of cyanogen bromide-activated Sepharose [11] was about 0.2 μmol/ml of suspension (1 : 1 v/v).

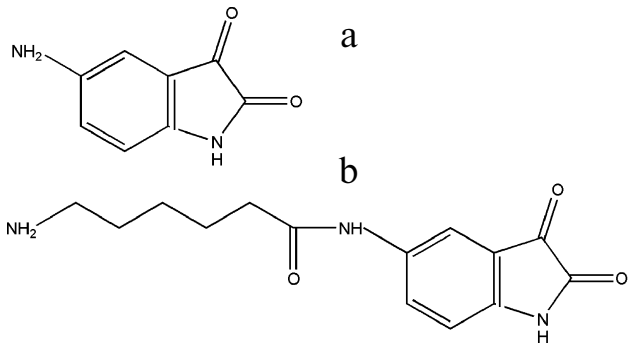

Fig. 1. Structural formulas of 5-aminoisatin (a) and 5-aminocaproylisatin (b).

For evaluation of nonspecific binding of proteins onto the affinity sorbent, we also prepared control cyanogen bromide-activated Sepharose that was run through all the same treatments as 5-aminocaproylisatin-Sepharose or 5-aminoisatin-Sepharose but without the affinity ligands.

Affinity chromatography and identification of isatin-binding proteins. The affinity chromatography separation step on 5-aminocaproylisatin- or 5-aminoisatin-Sepharose for subsequent mass spectrometric analysis was essentially the same as described in [10]. Each proteomic experiment was performed using pooled preparations obtained during affinity separation of soluble and membrane fractions of homogenates prepared using 4-6 mouse brains.

Reverse-phase nano-LCMS/MS was performed using an Agilent 1200 nano-flow HPLC-Chip cube system (Agilent Technologies Inc., USA). Peptides (1 μl, 1 μg/μl) were applied to the enrichment column (Zorbax 300 SB-C18, Agilent HPLC Chip, 40 nl trap column, 4 mm in length, particles size of 5 μm) for 3.5 min at a flow rate of 2 μl/min using isocratic mobile phase B (5% acetonitrile, 0.1% formic acid). Tryptic peptides were separated on a reverse-phase analytical column (Zorbax 300 SB-C18, Agilent HPLC Chip, 75 μm × 43 mm analytical column, 5 μm particle size) using a linear elution gradient of mobile phase A (aqueous 0.1% formic acid) and mobile phase B (80% acetonitrile, 0.1% formic acid) from 5 to 100% over 60 min at a flow rate of 300 nl/min followed by subsequent equilibration of the chromatographic system under the initial gradient conditions (A : B = 5 : 95) for 7 min.

Mass spectrometric analysis was performed using the Agilent 6340 Ion Trap (Agilent Technologies Inc.) in the positive ionization mode. Tandem spectra were scanned in the mode of automated selection of the five most intense ions from the precursor MS scans registered at m/z = 300-1800.

The following parameters were used: capillary potential of −1970 V, drying gas (nitrogen) flow of

Table 1. Proteomic identification of mouse brain isatin-binding proteins isolated using 5-aminoisatin- and 5-aminocaproylisatin-Sepharoses*

No.	Protein name	Database accession number	Molecular mass, Da**	p*I***	Number of peptides	Sequence coverage, %	Mill search score	Subcellular location**
1	2	3	4	5	6	7	8	9
	I Proteins/enzymes involved in energy generation and carbohydrate metabolism (n = 4)							
1	Creatine kinase U-type, mitochondrial (EC 2.7.3.2)	P30275	47 004.0	8.39	4	18	52.85	mitochondria
2	Dihydrolipoyl dehydrogenase mitochondrial (EC 1.8.1.4)	O08749	54 212.6	7.97	3	10	43.63	–"–
3	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	P16858	35 679.0	8.45	2	8	26.18	cytosol, nucleus
4	Alpha-enolase (EC 4.2.1.11)	P17182	47 009.9	6.36	2	7	22.75	cell membrane, cytoplasm
	II Proteins involved in cytoskeleton formation and exocytosis (n = 6)							
1	Syntaxin-binding protein 1	O08599	667 569.1	6.50	6	15	79.91	mitochondria
2	AP-2 complex subunit beta	Q9DBG3	104 583.2	5.22	5	10	69.37	cell membrane
3	AP-2 complex subunit alpha-1	P17426	107 664.7	6.63	5	6	68.88	–"–
4	Actin, cytoplasmic 2	P63260	41 793.1	5.31	4	19	58.07	cytoplasm
5	Clathrin heavy chain	Q68FD5	191 557.7	5.48	2	2	26.66	cytoplasmic vesicle membrane
6	Glial fibrillary acidic protein	P03995	49 908.3	5.36	2	5	20.70	cytoplasm
	III Proteins involved in regulation of gene expression, cell division, and differentiation (n = 10)							
1	Dihydropyrimidinase-related protein 2	O08553	62 277.9	5.95	11	35	182.87	mitochondria
2	Heterogeneous nuclear ribonucleoprotein A3	Q8BG05	39 652.2	9.09	8	22	137.85	nucleus
3	Heterogeneous nuclear ribonucleoprotein Q	Q7TMK9	69 633.0	8.68	6	13	79.54	nucleus, cytoplasm
4	Heterogeneous nuclear ribonucleoprotein K	P61979	50 976.5	5.39	5	19	75.62	–"–
5	Heterogeneous nuclear ribonucleoprotein A2/B1	O88569	37 402.8	8.97	7	18	65.93	–"–
6	Transcriptional activator protein Pur-alpha	P42669	34 883.9	6.07	3	24	55.93	nucleus
7	Heterogeneous nuclear ribonucleoprotein L	Q8R081	60 123.6	6.65	3	7	49.06	nucleus, cytoplasm
8	Heterogeneous nuclear ribonucleoproteins C1/C2	Q9Z204	34 384.9	4.91	3	11	32.14	nucleus

Table 1 (Contd.)

1	2	3	4	5	6	7	8	9
9	Heterogeneous nuclear ribonucleoprotein D0	Q60668	38 354.3	7.61	3	8	25.05	nucleus, cytoplasm
10	Heterogeneous nuclear ribonucleoprotein A/B	Q99020	30 831.4	7.69	2	10	19.39	–"–
V Antioxidant enzymes and protective proteins (n = 2)								
1	Heat shock cognate 71 kDa protein	P63017	70 871.4	5.38	12	27	193.09	cytoplasm
2	Superoxide dismutase [Mn], mitochondrial (EC 1.15.1.1)	P09671	24 603.1	8.80	2	12	28.74	mitochondria

* Minimal search scores for proteins identified during isolation of isatin-binding proteins on 5-aminoisatin- and 5-aminocaproylisatin-Sepharoses are shown. Results were corrected for proteins nonspecifically bound to control Sepharose.

** Here and in the other tables, names, molecular characteristics, and data on subcellular localization are shown as in the UniProt database used for their identification.

5 liters/min, drying gas temperature of 280°C, fragmentation amplitude of 1.15 V. Ion current control was operated at 150,000 ions with a maximum accumulation time of 70 ms and active exclusion of ions after three spectra in 36 s.

Proteins were identified using the Spectrum Mill MS Proteomics Workbench Rev A.03.03.084 SR4 software (Agilent Technologies). Proteins were identified by comparison of the experimental data with the Swiss Prot mouse subset database. The following search parameters were used: trypsin was used as the cutting enzyme, maximal allowable number of arginine and lysine of ⩽1 was allowed in tryptic peptides; mass tolerance for the monoisotopic peptide window was set to ±0.8 Da, the MS/MS tolerance window was set to ±0.3 Da, and one missed cleavage was allowed. Cysteine carbamidomethylation was chosen as a fixed modification, and oxidized methionine was chosen as a variable modification. The criteria of positive identification were set as follows: δ-forward-reverse score >2; at least two peptide identifications with a confidence score >7, and summed protein score ⩾13 [15].

Each protein listed in the tables was identified at least in three independent experiments.

Functional analysis of isatin-binding proteins. Functional analysis including enrichment analysis for the Gene Ontology (GO) categories and mapping to TRANSPATH® pathways was performed using the GeneXplain platform (www.genexplain.com) with the default settings. The algorithm of the functional analysis is intended to detect statistically significant representation of certain functional groups of genes/proteins among all genes/proteins of the input sets. Statistical significance was estimated by the p-value calculated using hypergeometrical distribution by the following formula:

$$P(+) = \sum_{i=k}^{k_{max}} \binom{D}{i}\binom{N-D}{n-i}\binom{N}{n}^{-1}$$

where $k_{max} = \min(n, D)$; N – all genes in the *Mus musculus* genome; D – number of genes in the *M. musculus* genome related to certain GO categories or pathway; n – number of genes in the input set; $i = k$ – number of genes in the input set related to certain GO categories or pathway.

Only statistically significant GO groups and pathways (p-value < 0.005) were considered for proteins isolated with 5-aminocaproylisatin- or 5-aminoisatin-Sepharose.

RESULTS

In accordance with our previous study [10], affinity separation of isatin-binding proteins of cleared Triton X-100 lysates of particulate and soluble fractions of mouse brain homogenates resulted in reliable proteomic identification of more than 60 individual proteins (Tables 1 and 2). Analysis of proteins nonspecifically bound to the control cyanogen bromide-activated Sepharose, which was run through all the same treatments as 5-aminocaproylisatin-Sepharose or 5-aminoisatin-Sepharose but without the affinity ligands, revealed the presence of type II cytoskeletal keratins (1, 1B, 6A, 6B, 7, 15, 71, 73, 75, 79) and type I cytoskeletal keratins (15, 17, 19).

The same affinity purification procedure with 5-aminoisatin-Sepharose resulted in identification of 88 proteins (Tables 1 and 3). Although most of the identified proteins could be subdivided into the same groups (Fig. 2), only 22 were identical for the two protein profiles

Table 2. Proteomic identification of mouse brain isatin-binding proteins specifically bound to 5-aminocaproylisatin-Sepharose

No.	Protein name	Database accession number	Molecular mass, Da*	p*I**	Number of peptides	Sequence coverage, %	Mill search score	Subcellular location*
1	2	3	4	5	6	7	8	9
I Proteins/enzymes involved in energy generation and carbohydrate metabolism (n = 14)								
1	Aconitate hydratase, mitochondrial (EC 4.2.1.3)	Q99KI0	85 464.0	8.08	6	11	89.15	mitochondria
2	Creatine kinase B-type (EC 2.7.3.2)	Q04447	42 713.5	5.40	6	18	86.47	–"–
3	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial precursor (EC 1.2.4.1)	Q9D051	38 937.3	6.41	4	161	68.30	–"–
4	Malate dehydrogenase, mitochondrial precursor (EC 1.1.1.37)	P08249	35 596.6	8.82	4	16	59.74	–"–
5	Gamma-enolase (EC 4.2.1.11)	P17183	47 165.7	4.99	4	14	58.05	cell membrane, cytoplasm
6	Triosephosphate isomerase (EC 5.3.1.1)	P17751	26 581.6	7.09	4	20	54.86	cytosol, nucleus
7	Fumarate hydratase, mitochondrial (EC 4.2.1.2)	P97807	54 371.1	9.11	3	10	44.68	mitochondria
8	V-type proton ATPase subunit B, brain isoform (EC 3.6.3.14)	P62814	56 551.1	5.57	2	6	37.31	mitochondrial inner membrane
9	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	P35486	43 231.9	8.49	3	11	34.73	mitochondria
10	L-lactate dehydrogenase B chain (EC 1.1.1.27)	P16125	36 441.3	5.70	3	11	34.39	cytoplasm
11	Pyruvate carboxylase, mitochondrial (EC 6.4.1.1)	Q05920	129 685.3	6.25	2	2	31.74	mitochondria
12	2-oxoglutarate dehydrogenase, mitochondrial (EC 1.2.4.2)	Q60597	116 118.3	6.52	2	3	24.54	–"–
13	Fructose-bisphosphate aldolase C (EC 4.1.2.13)	P05063	39 263.9	6.79	2	8	20.14	–"–
14	Glycogen phosphorylase, brain form (EC 2.4.1.1)	Q8CI94	96 599.3	6.29	2	3	20.12	cytoplasm
II Proteins involved in cytoskeleton formation and exocytosis (n = 7)								
1	Synaptotagmin-1	P46096	47 418.3	8.68	6	19	82.7	synaptic vesicle membrane, cytoplasm
2	Spectrin alpha chain, brain	P16546	167 554.0	5.27	3	3	49.80	cytoplasm
3	Dynamin-1 (EC 3.6.5.5)	P39053	97 803.3	7.61	3	4	41.88	–"–

Table 2 (Contd.)

1	2	3	4	5	6	7	8	9
4	Spectrin beta chain, brain 1	Q62261	274 224.5	5.40	3	1	33.06	cytoplasm
5	Rab GDP dissociation inhibitor alpha	P50396	50 521.9	4.96	2	8	32.38	–"–
6	Tubulin alpha-2 chain	P05213	50 151.9	4.94	2	7	26.93	–"–
7	AP-2 complex subunit mu-1	P84091	49 655.0	9.57	2	8	21.89	cell membrane
III Proteins involved in regulation of gene expression, cell division, and differentiation (n = 6)								
1	Transitional endoplasmic reticulum ATPase	Q01853	89 177.1	5.14	8	16	124.17	cytoplasm, nucleus
2	Heterogeneous nuclear ribonucleoprotein A1	P49312	34 065.2	9.27	5	13	70.70	nucleus, cytoplasm
3	RNA-binding protein FUS	P56959	52 673.4	9.40	2	6	32.73	nucleus
4	Cullin-associated NEDD8-dissociated protein 1	Q6ZQ38	136 332.4	5.52	2	2	25.45	–"–
5	Interleukin enhancer-binding factor 2	Q9CXY6	43 062.4	5.19	2	9	24.29	nucleus, cytoplasm
6	Polyadenilate-binding protein 1	P29341	70 643.2	9.48	2	4	22.89	–"–
IV Proteins involved in signal transduction and regulation of enzyme activity (n = 9)								
1	14-3-3 protein zeta/delta	P63101	27 771.3	4.73	10	38	152.28	cytoplasm, melanosomes
2	14-3-3 protein gamma	P61982	28 171.5	4.80	4	20	55.73	cytoplasm
3	14-3-3 protein eta	P68510	28 080.7	4.81	3	15	43.37	–"–
4	14-3-3 protein theta	P68254	27 778.4	4.69	3	20	39.23	–"–
5	Guanine nucleotide-binding-protein beta subunit 2-like 1	P68040	34 945.7	7.57	2	11	25.77	cell membrane, cytoplasm, nucleus
6	2',3'-cyclic-nucleotide 3'-phosphodiesterase (EC 3.1.4.37)	P16330	47 123.6	9.08	2	5	23.92	melanosome membranes
7	Serine/threonine-protein phosphatase 2A65 kDa regulatory subunit A, alpha isoform	Q76MZ3	65 191.8	5.00	2	5	23.48	cytoplasm
8	14-3-3 protein beta/alpha	Q9CQV8	27 955.4	4.77	2	16	23.27	cytoplasm, melanosomes
9	14-3-3 protein epsilon	P62259	29 174.1	4.63	3	13	30.28	cytoplasm
V Antioxidant enzymes and protective proteins (n = 4)								
1	Peroxiredoxin-5, mitochondrial (EC 1.11.1.15)	P99029	21 897.6	9.09	3	24	34.26	mitochondria
2	Peroxiredoxin-2 (EC 1.11.1.15)	Q61171	21 647.6	5.20	2	9	28.49	–"–
3	Glutathione S-transferase P 1 (EC 2.5.1.18)	P19157	23 478.1	8.13	2	12	28.34	cytosol, nucleus, cell membrane

Table 2 (Contd.)

1	2	3	4	5	6	7	8	9
4	Stress-70 protein, mitochondrial	P38647	73 528.7	5.91	3	6	34.92	mitochondria
VI Proteins/enzymes involved in metabolism of amino acids and other nitrogenous compounds (n = 4)								
1	Aspartate aminotransferase, mitochondrial (EC 2.6.1.1)	P05202	47 411.6	9.13	5	19	65.74	–"–
2	Pyridoxal kinase (EC 2.7.1.38)	Q8K183	35 015.4	5.88	2	7	32.80	cytoplasm
3	Ribose-phosphate pyrophospho-kinase I (EC 2.7.6.1)	Q9D7G0	34 717.3	6.57	2	10	28.87	unknown
4	4-aminobutyrate aminotrans-ferase, mitochondrial precursor (EC 1.15.1.1)	P61922	56 452.2	8.35	2	5	28.02	mitochondria

* Proteins common for both affinity ligands are listed in Table 1. Results were corrected for proteins nonspecifically bound to control Sepharose.

(Table 1). The highest number of identical proteins was found in the group of proteins involved in regulation of gene expression, cell division, and differentiation (group III; n = 10) and proteins involved in cytoskeleton formation and exocytosis/trafficking (n = 6). The groups of isatin-binding proteins involved in signal transduction and regulation of enzyme activity (group IV) and metabolism of proteins, amino acids, and related processes (group VI) that were isolated on 5-aminoisatin-Sepharose and 5-aminocaproyl-Sepharose contained completely different sets of proteins. According to the functional analysis with GO annotation, this means that they are involved in different biological processes (Fig. 3). It should be noted that isatin-binding proteins have been identified not only among highly abundant proteins (e.g. proteins involved into cytoskeleton formation, heat shock proteins, etc.), but also among less abundant cell proteins [11]. Their intracellular concentrations differing by several orders of magnitude have been quantified in some brain-derived mouse cells [16].

Proteins common for 5-aminocaproylisatin and 5-aminoisatin (Table 1) are mostly referred to the group of "cellular metabolic process" (n = 18), and most of these proteins are involved in metabolism of nitrogen compounds (n = 13). Four proteins of group II (O08599, Q9DBG3, P17426, Q68FD5) together with heat shock

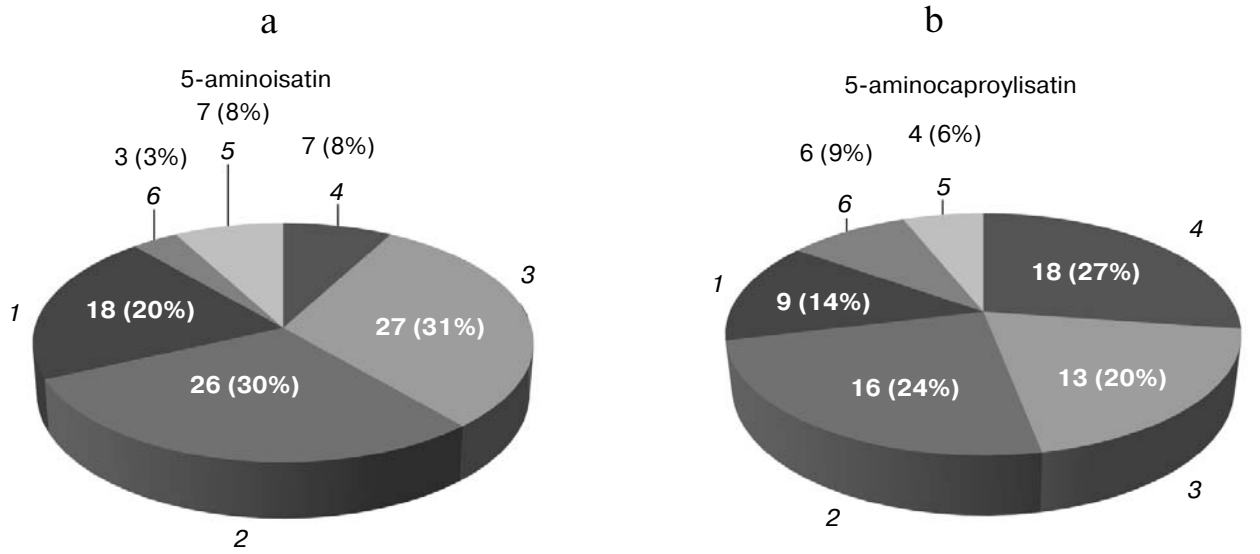

Fig. 2. Schematic subdivision of isatin-binding proteins isolated using 5-aminoisatin (n = 88) (a) and 5-aminocaproylisatin (n = 66) (b) into functional groups *1-6*: *1*) energy generation and carbohydrate metabolism; *2*) regulation of gene expression, cell division, and differentiation; *3*) cytoskeleton formation and exocytosis; *4*) signal transduction and regulation of enzyme activity; *5*) antioxidant enzymes and protective proteins; *6*) metabolism of amino acids and other nitrogenous compounds.

Table 3. Proteomic identification of mouse brain isatin-binding proteins specifically bound to 5-aminoisatin-Sepharose

No.	Protein name	Database accession number	Molecular mass, Da*	p*I**	Number of peptides	Sequence coverage, %	Mill search score	Subcellular location*
1	2	3	4	5	6	7	8	9
I Proteins/enzymes involved in energy generation and carbohydrate metabolism (n = 3)								
1	Glycogen synthase	Q9Z1E4	80 885.3	6.41	2	12	19.54	cytoplasm
2	Alcohol dehydrogenase 1	P00329	39 771.7	8.43	2	14	18.60	–"–
3	Hexokinase-1	P17710	108 303.1	6.45	2	3	15.53	outer mitochondrial membrane
II Proteins involved in cytoskeleton formation and exocytosis/transport (n = 21)								
1	Tubulin beta-2C chain	P68372	49 831.3	4.79	5	25	55.88	cytoskeleton
2	Tubulin beta-2B chain	Q9CWF2	49 953.4	4.78	5	25	55.88	–"–
3	Tubulin beta-2A chain	Q7TMM9	49 907.3	4.78	5	25	55.88	–"–
4	Tubulin beta-4 chain	Q9D6F9	49 586.1	4.78	4	13	46.88	–"–
5	Titin	A2ASS6	3 906 506.8	5.91	5	0	46.82	cytoplasm, nucleus
6	Tubulin beta-5 chain	P99024	49 671.1	4.78	4	13	44.74	cytoskeleton
7	Tubulin alpha-4A chain	P68368	49 924.7	4.95	4	13	41.61	–"–
8	Tubulin alpha-3 chain	P05214	49 959.8	4.97	3	10	36.30	–"–
9	Actin, cytoplasmic 1	P60710	41 737.0	5.29	3	10	34.30	–"–
10	Microtubule-associated protein 1A	Q9QYR6	300 141.3	4.92	3	1	33.87	–"–
11	Microtubule-associated protein 1B	P14873	270 412.1	4.76	2	1	25.55	–"–
12	Tubulin beta-6 chain	Q922F4	50 090.7	4.80	3	18	31.19	–"–
13	Tubulin beta-3 chain	Q9ERD7	50 419.0	4.82	3	10	29.49	–"–
14	Keratin, type II cytoskeletal 5	Q922U2	61 767.0	7.59	2	3	25.88	mitochondria, cytoskeleton
15	AP-1 complex subunit beta-1	O35643	103 979.2	5.02	2	6	22.54	cytoplasmic vesicles, Golgi apparatus, membranes
16	Catenin alpha	Q61301	100 106.9	5.91	2	4	21.74	cytoskeleton
17	Matrin-3	Q8K310	94 630.8	5.87	1	2	20.12	nucleus
18	Tubulin alpha-8 chain	Q9JJZ2	50 051.8	4.97	2	6	19.76	cytoskeleton
19	Coiled-coil domain-containing protein 27	Q3V036	73 651.3	5.28	2	12	18.22	unknown

Table 3 (Contd.)

1	2	3	4	5	6	7	8	9
20	Cytoplasmic dynein 1 heavy chain 1	Q9JHU4	532 028.3	6.04	2	1	17.89	cytoskeleton
21	Cytoplasmic dynein 2 heavy chain 1	Q45VK7	492 341.9	6.17	2	1	15.59	–"–
III Proteins involved in regulation of gene expression, cell division, and differentiation (n = 16)								
1	40S ribosomal protein	P62082	22 127.0	10.09	4	23	50.05	cytoplasm
2	Zinc finger transcription factor Trps 1	Q925H1	141 035.4	7.53	2	6	23.88	nucleus
3	Small nuclear ribonucleoprotein Sm D2	P62316	13 281.6	11.57	2	26	24.64	–"–
4	Zinc finger protein 280D	Q86YH2	107 836.2	6.38	2	4	21.49	–"–
5	Zinc finger protein 518 A	B2RRF6	165 604.9	9.15	2	4	21.31	–"–
6	Heterogeneous nuclear ribonucleoprotein D	Q9Z130	33 558.9	6.85	3	9	20.92	nucleus, cytoplasm
7	Huntingtin-interacting protein 1-related protein	O75146	119 486.1	6.14	2	5	19.11	cytoplasm, membranes
8	Zinc finger FYVE domain-containing protein 26	Q5DU37	282 962.5	5.82	2	3	16.29	cytoplasm
9	Sorting nexin-14 (cellular trafficking)	Q8BHY8	109 037.0	6.33	2	6	15.8	membranes
10	Elongation factor 1-alpha-2	P62631	504 54.4	9.11	2	7	15.64	nucleus
11	Zinc finger protein 608	Q56A10	162 345.5	8.94	1	2	15.02	intracellular
12	Zinc finger CCCH domain-containing protein 14	Q8BJ05	82 409.6	7.04	2	15	14.69	nucleus speckle
13	Zinc finger homeobox protein 4	Q9JJN2	39 232	6.02	1	0	14.36	nucleus
14	Synaptotagmin-4	P40749	47 659	8.83	1	7	14.23	synaptic vesicle membranes
15	Probable leucyl-tRNA ligase mitochondrial	Q8VDC0	101 480.6	8.47	1	5	14.19	mitochondria
16	Single stranded DNA-binding protein 2	P81877	37 845.8	6.16	1	14	13.91	nucleus
IV Proteins involved in signal transduction and regulation of enzyme activity (n = 18)								
1	Calcium/calmidulin-dependent protein kinase type II subunit alpha	P11798	54 115.0	6.61	4	13	41.71	cytoplasm
2	Perilipin 4	O88492	139 382.7	8.81	3	11	26.45	cell membrane, cytoplasm
3	Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing subunit alpha	Q61194	171 580.9	6.41	3	6	22.94	cell membrane, nucleus, cytoplasm

Table 3 (Contd.)

1	2	3	4	5	6	7	8	9
4	Leucine-rich repeat serine/threonine protein kinase 2	Q5S006	284 793.5	6.36	3	6	21.83	cytoplasm, mitochondrial membranes
5	Inositol 1,4,5-trisphosphate receptor type 2	Q9Z329	313 199.1	5.72	2	2	19.58	endoplasmic reticulum membranes
6	Low-density lipoprotein receptor-related protein	A2ARV4	519 211.7	4.94	2	2	19.10	membranes
7	Lymphoid enhancer-binding factor 1	P27782	44 058.8	6.90	2	22	18.76	nucleus
8	ELAV-like protein 1	P70372	36 069.1	9.23	2	12	18.1	nucleus, cytoplasm
9	G-protein coupled receptor-associated sorting protein 1	Q5U4C1	151 746.0	5.03	2	4	16.94	cytoplasm
10	Ral GTP-ase activating protein subunit alpha-2	A3KGS3	210 289.4	5.83	2	4	16.69	–"–
11	Sodium channel protein type 8 subunit alpha	Q9WTU3	223 142.6	5.93	2	3	15.88	membranes
12	Myc-box-dependent-interacting protein 1	O08539	64 470.5	4.95	2	7	14.36	nucleus, cytoplasm
13	Calcium-binding mitochondrial carrier protein ScaMC-1	Q8BMD8	52 902.3	7.02	1	6	13.98	inner mitochondrial membrane
14	1-phosphotidylinositol-4,5-bisphosphate phosphatase	Q8K4S1	255 021.7	5.81	2	2	13.63	cell membrane, cytoplasm
15	Receptor interacting serine/threonine-protein kinase 4	Q9ERK0	86 613.4	6.53	1	4	13.60	cytoplasm, membranes
16	Calcium/calmodulin-dependent protein kinase type II subunit beta	P28652	60 461.2	6.87	1	3	13.55	cytoplasm
17	Calcium/calmodulin-dependent protein kinase type II subunit gamma	Q923T9	59 607.2	7.32	1	3	13.55	membranes, sarcoplasmic reticulum
18	Calcium/calmodulin-dependent protein kinase type II subunit delta	Q6PHZ2	56 369.7	6.81	1	3	13.55	–"–
	V Antioxidant enzymes and protective proteins (n = 1)							
1	Heat shock protein HSP 90-alpha	P07901	84 788.3	4.93	6	10	65.42	cytoplasm, melanosomes
	VI Proteins/enzymes involved in metabolism of amino acids and other nitrogenous compounds (n = 7)							
1	Probable E3 ubiquitin-protein ligase MYCBP2	Q7TPH6	517 740.7	6.75	2	1	21.74	nucleus
2	Ubiquitin-carboxyl-terminal hydrolase 24	B1AY13	294 002.6	5.83	2	2	20.22	unknown

Table 3 (Contd.)

1	2	3	4	5	6	7	8	9
3	E3 ubiquitin-protein ligase MIB2	Q8R516	105 961.4	8.44	2	6	17.33	cytoplasm, endosomes
4	Alanine-glyoxylate aminotransferase 2, mitochondrial	Q3UEG6	57 115.4	8.13	2	16	16.23	mitochondria
5	E3 ubiquitin-protein ligase HUWE1	Q7TMY8	482 665.3	5.10	2	1	14.13	nucleus, cytoplasm
6	Ubiquitin-conjugating enzyme E2 variant 1	Q9CZY3	140 818.5	4.94	1	3	13.17	nucleus
7	Polyubiquitin-B	P0CG49	8564.9	6.56	1	21	12.39**	nucleus, cytoplasm

* Proteins common for both affinity ligands are listed in Table 1.

** This protein has been included in the list of identified proteins due to high coverage of its amino acid sequence and confident score significantly exceeding ($p < 0.01$) the confident score of the individual peptide, which is very close to that of the whole protein. Results were corrected for proteins nonspecifically bound to control Sepharose.

protein and dihydropyrimidinase-related protein participate in vesicle-mediated transport. All six proteins of group II and two proteins of group V and two proteins of group III (dihydropyrimidinase-related protein and heterogeneous nuclear ribonucleoprotein A/B) participate in arrangement/rearrangement of cellular components (GO:0016043). Nine proteins belonging to different groups (P16858, P17182 from group I; P63260, Q9DBG3, P17426, Q68FD5 from group II; O08553 from group III; and P63017, P09671 from group V; see Table 1) are involved in cell response to chemical stimulus.

Comparison of results of the protein annotation for the GO categories demonstrates some differences between biological processes in which proteins isolated on 5-aminocaproylisatin or 5-aminoisatin are involved. For example, 13 proteins isolated on 5-aminocaproylisatin (Q9D051, P08249, Q60597, P35486, P16125, Q8CI94, P17751 from group I; P99029, Q61171 from group V; and P61982, P68510, P68254, Q9CQV8 from group IV; see Table 2) contribute to oxidation–reduction processes that result in abstraction or addition of one or more electrons to or from corresponding substrates, with or without the concomitant abstraction or addition of proton(s) (GO:0055114). Three other proteins (14-3-3 protein zeta/delta, synaptotagmin-1 and 4-aminobutyrate aminotransferase) isolated by this affinity ligand participate in a signal release process, in which a signal is released into the extracellular medium from cellular sources.

On the other hand, using 5-aminoisatin as the affinity separation reagent, 17 proteins (mostly proteins from group II, Table 3) that are involved in the process of cellular component movement have been recognized. Calcium/calmodulin-dependent protein kinases, tubulins, ubiquitin, 90-kDa heat shock protein, cytoplasmic dynein heavy chain 1, and titin are involved in the cell cycle process ($n = 12$). Another group of proteins identified using 5-aminoisatin represents proteins involved in regulation of molecular function ($n = 14$), which is defined as any process that modulates the frequency, rate, or extent of a molecular function, an elemental biological activity occurring at the molecular level, such as catalysis or binding (GO:0065009). These include nine proteins from group IV (O08539, Q9Z329, P11798, P28652, A2ARV4, P27782, Q5S006, Q8K4S1, A3KGS3) along with proteins from other groups (P17710, A2ASS6, P07901, Q9CZY3, P0CG49) (see Table 3).

DISCUSSION

Selection of two principal isatin analogs as the affinity ligands for isolation of isatin-binding subproteome was based on several crucial observations [2]: (i) 5-amino- and 5-carboxyisatins were successfully used in ELISA-based quantitative isatin determination in urine [17]; (ii) 5-aminoisatin was a more effective antagonist of atrial natriuretic peptide (ANP)-stimulated guanylate cyclase than isatin itself [15]; (iii) 5-aminoisatin was originally used for surface plasmon resonance (SPR)-based optical biosensor detection of isatin-binding capacity of subcellular fractions of rat tissues [13]. However, monoamine oxidase inhibitory activity of 5-aminoisatin was much lower than that of isatin itself [13], while monoamine oxidase inhibitory activity of the 5-aminocaproyl analog was basically the same or even somewhat higher than that of isatin [11]. The latter was the decisive argument for the use of the 5-aminocaproyl analog in the first experiments on isolation of isatin-binding sub-proteome from rat and mouse brain homogenates [10, 11]. Although proteomic profiling of isatin-binding proteins resulted in identifica-

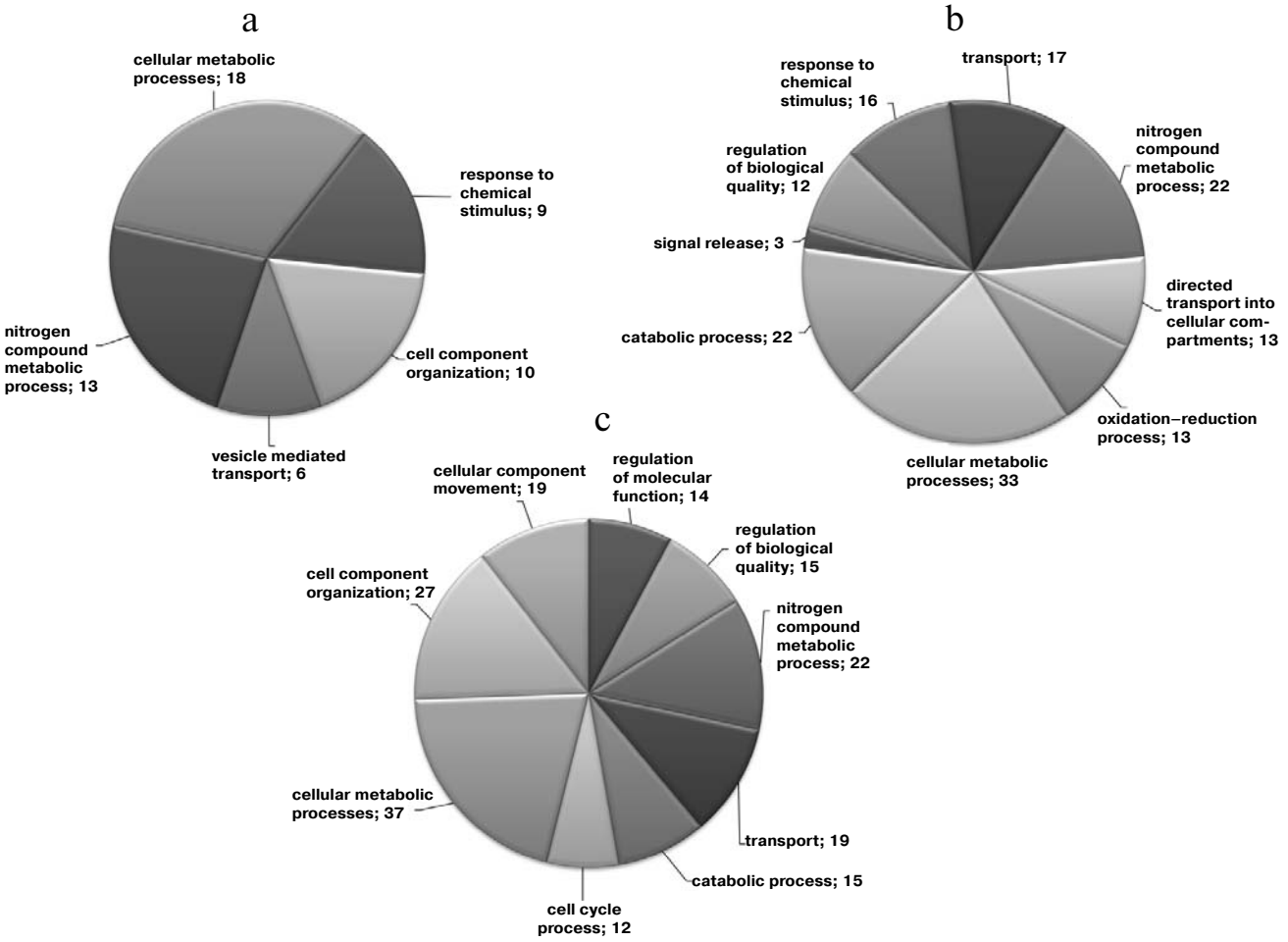

Fig. 3. GO annotation of proteins isolated on both 5-aminocaproylisatin and 5-aminoisatin affinity sorbents (a) and those specific for 5-aminoisatin (b) and 5-aminocaproylisatin (c). Color intensity reflects classification significance: the darker the coloring, the higher the statistical significance is. Names of processes are given in terms of the GO resources.

tion of more than 60 individual proteins, the known and most sensitive targets of isatin were not identified as isatin-binding proteins in either rat or mouse brains. Use of 5-aminoisatin as the affinity ligand for isolation of isatin-binding proteins significantly (by 25%) increased the number of identified proteins; however, the most sensitive targets for inhibition by isatin were not found again. Since the vast majority of the identified proteins are located in soluble subcellular fractions (e.g. cytosol, mitochondrial matrix), this might be attributed to common problems of membrane protein isolation by means of affinity chromatography [2]. However, it is also possible that the affinity of the isatin analogs used for separation of isatin-binding proteins is not sufficient for biospecific isolation of all of the relevant proteins.

The latter might also account for the modest number of common proteins identified during proteomic profiling of mouse brain preparations on the two affinity sorbents. The highest coincidence was found in the groups of proteins involved in regulation of gene expression, cell division, and differentiation and also proteins involved in cytoskeleton formation and exocytosis/trafficking. In the former group ($n = 10$) this corresponds to 38.5 and 62.5% of the total number of proteins of this group isolated on 5-aminoisatin and 5-aminocaproylisatin, respectively. The majority of this group is formed by homologous ribonucleoproteins that contain the consensus sequence-type RNA-binding domain (CS-RBD) and often differ by small peptide inserts [18], which probably determine diversity of heterogeneous nuclear ribonucleoproteins. Thus, if one member of the family of RNA-binding proteins exhibits affinity to isatin(s), it is reasonable to expect that others will behave similarly.

The group of proteins involved in cytoskeleton formation and exocytosis/trafficking contains six common proteins; this corresponds to 22.2 and 46.2% of the total number of proteins of this group isolated on 5-aminoisatin and 5-aminocaproylisatin, respectively. Some of these proteins are considered as orthologs involved in the synaptic vesicle cycle (KEGG pathway

ko04721), and thus it appears that they share not only functional but also regulatory similarities (interaction with immobilized isatins).

This is also true for common (enzyme) proteins involved in carbohydrate metabolism and energy generation: they all share their nucleotide-binding (NAD, FAD) property. In the case of enolase, certain evidence exists that in addition to its particular role in glycolysis, this enzyme is involved in targeting imported tRNA toward the mitochondria [19].

Mapping of identified proteins isolated on 5-aminocaproylisatin and 5-aminoisatin to known pathways suggest that they share some common pathways (such as glycolysis, EGF pathway) and also differential pathways. The most interesting process in which the proteins isolated by 5-aminoisatin affinity separation might participate is the parkin-associated pathway (CH000000947). Proteins that hit this pathway include tubulin α- and β-chains, ubiquitin, and heat shock cognate 71-kDa protein. The probability that hits in the pathway represent an accidental event is as low as $2.5 \cdot 10^{-5}$.

Parkin is an E3-ubiquitin-protein ligase that ubiquitinates itself and specific substrate proteins including proteins tubulin α and tubulin β and thus increases their degradation by the 26S proteasome (see for review [20, 21]). It appears that parkin enhances the ubiquitination and degradation of misfolded tubulins, and therefore it may play a significant role in protecting neurons against toxins causing Parkinson's disease [22].

On the other hand, Hsp70 and its co-chaperone CHIP are known as parkin-binding partners, and in concert with Hsp70, CHIP also enhances parkin inhibition of cell death induced by Pael-R [23]. Also, it has been recently shown that Hsp70 and Hsc70 are substrates for parkin, which mediates multiple mono-ubiquitination of Hsp70/Hsc70 consistent with a degradation-independent role for this ubiquitin modification [24].

Thus, taking into consideration the known neuroprotective role of isatin in different animal models of parkinsonism [25, 26], it is reasonable to suggest that this role may be associated with interaction of isatin with some of the isatin-binding proteins identified in this study.

Some complementarity in isatin-binding proteins separated using the two isatin analogs as the affinity ligands suggests that the actual number of brain proteins interacting with endogenous (unmodified) isatin still remains underestimated due to different affinity of proteins for the isatin analogs used for the affinity-based proteomic profiling. Since the isatin moiety is present in a range of compounds exhibiting potential pharmacological activities [8-10], it appears that identification of particular isatin-binding sub-proteomes (pharmacoproteomes) should employ particular analogs containing reactive groups for covalent attachment to sorbents. This is a much easier task.

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