Original Article

An efficient method for native protein purification in the selected range from prostate cancer tissue digests

Rumana Ahmad[1,2], Carrie D. Nicora[3], Anil K. Shukla[3], Richard D. Smith[3], Wei-Jun Qian[3], Alvin Y. Liu[1,2]

[1]Department of Urology, University of Washington, Seattle, WA 98195, USA; [2]Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA 98195, USA; [3]Biological Science Division and Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA 99352, USA

Contributions: (I) Conception and design: AY Liu; (II) Administrative support: AY Liu; (III) Provision of study materials or patients: R Ahmad; (IV) Collection and assembly of data: R Ahmad; (V) Data analysis and interpretation: R Ahmad, CD Nicora, AK Shukla, RD Smith, WJ Qian; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Rumana Ahmad. Department of Biochemistry, Era's Lucknow Medical College & Hospital, Sarfarazganj, Hardoi Road, Lucknow-226003, UP, India. Email: rumana_ahmad@yahoo.co.in.

Background: Prostate cancer (CP) cells differ from their normal counterpart in gene expression. Genes encoding secreted or extracellular proteins with increased expression in CP may serve as potential biomarkers. For their detection and quantification, assays based on monoclonal antibodies are best suited for development in a clinical setting. One approach to obtain antibodies is to use recombinant proteins as immunogen. However, the synthesis of recombinant protein for each identified candidate is time-consuming and expensive. It is also not practical to generate high quality antibodies to all identified candidates individually. Furthermore, non-native forms (e.g., recombinant) of proteins may not always lead to useful antibodies. Our approach was to purify a subset of proteins from CP tissue specimens for use as immunogen.

Methods: In the present investigation, ten cancer specimens obtained from cases scored Gleason 3+3, 3+4 and 4+3 were digested by collagenase to single cells in serum-free tissue culture media. Cells were pelleted after collagenase digestion, and the cell-free supernatant from each specimen was pooled and used for isolation of proteins in the 10–30 kDa molecular weight range using a combination of sonication, dialysis and Amicon ultrafiltration. Western blotting and mass spectrometry (MS) proteomics were performed to identify the proteins in the selected size fraction.

Results: The presence of cancer-specific anterior gradient 2 (AGR2) and absence of prostate-specific antigen (PSA)/KLK3 were confirmed by Western blotting. Proteomics also detected AGR2 among many other proteins, some outside the selected molecular weight range, as well.

Conclusions: Using this approach, the potentially harmful (to the mouse host) exogenously added collagenase was removed as well as other abundant prostatic proteins like ACPP/PAP and AZGP1 to preclude the generation of antibodies against these species. The paper presents an optimized scheme for convenient and rapid isolation of native proteins in any desired size range with minor modifications.

Keywords: Prostate cancer proteins (CP proteins); purification of 10–30 kDa proteins; anterior gradient 2 (AGR2); cancer biomarkers; proteomic analysis

Submitted Nov 16, 2016. Accepted for publication Dec 02, 2016.
doi: 10.21037/cco.2016.12.03
View this article at: http://dx.doi.org/10.21037/cco.2016.12.03

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Introduction

Other than skin cancer, prostate cancer (CP) is the most common cancer in American men. According to the American Cancer Society's estimate, 180,890 new cases of CP will be diagnosed and 26,120 men will die of the disease in 2016. About 1 in 7 men will be diagnosed with CP during his lifetime (1). Nearly two-thirds of the cancer is diagnosed in men aged 65 or older. CP is the second leading cause of cancer death in American men, but most diagnosed men do not die from it. In fact, more than 2.9 million men in the United States who have been diagnosed with CP at some point are alive today. The current biomarker of elevated serum prostate-specific antigen (PSA) is a flawed test for early detection as many men with abnormal serum PSA turn out not to have cancer upon biopsy.

Researchers are working on strategies to improve molecular diagnosis. One approach is to assay for specific characteristics of PSA, or to detect PSA variants more specific to cancer (2). Another approach is to develop new tests based on other tumor markers. We used comparative analysis between the transcriptomes of isolated $CD26^+$ cancer cells and $CD26^+$ normal luminal cells to identify genes up-regulated in cancer (3,4). Genes that were found to be overexpressed by ≥8-fold, and to encode secreted or extracellular proteins were selected as biomarker candidates for assay development. Similarly, comparative analysis of isolated $CD90^+$ cancer-associated stromal cells and $CD49a^+$ benign tissue stromal cells has identified additional secreted protein candidates (5,6).

Anterior gradient 2 (AGR2; 19 kDa), breast cancer membrane protein 11/AGR3 (BCMP11; 19 kDa), cysteine-rich secretory protein-3 (CRISP3; 28 kDa), thymocyte differentiation antigen 1/CD90 (THY1; ~27 kDa) are examples of proteins secreted or released from prostate tumors. As such, these proteins can all likely be detected and measured in body fluids. Furthermore, array signals indicate that AGR2 is a moderately abundant protein while the others are less (3,7). In order to obtain antibodies that can recognize AGR2 and others, one needs to isolate these antigens preferably in their native form for mouse immunization.

The present study concerns with the development of a procedure to isolate cancer secreted/extracellular proteins such as AGR2 from media in which CP tissue was digested by collagenase (8). This media contained proteins synthesized by cell types of the tumors. Since a number of biomarker candidates are calculated to be in the 10–30 kDa size range, protein separation based on size fractionation was employed. The rationale behind the choice of 30 kDa as the upper bound was to ensure that monoclonal antibodies would not be raised against the abundant PSA (34 kDa) and other prostatic proteins such as zinc α2-glycoprotein (AZGP1, 43 kDa) and prostatic acid phosphatase (ACPP, 52 kDa), and to remove collagenase type I (68 kDa) from being present in the protein preparation. Another abundant species, β-microseminoprotein (MSMB/prostate secretory protein of 94 amino acids; 13 kDa), shows much reduced expression in tumors (3). The choice of 10 kDa as the lower bound was to remove impurities and small molecules such as peptide fragments.

The tissue digestion media used in this study was media supernatant pooled from ten tumor tissue specimens of Gleason 3+3, 3+4, and 4+3 scores (and one of 4+5), which would contain proteins synthesized by both pattern 3 and pattern 4 tumors. Proteins in the desired molecular weight range were purified by a scheme involving sonication, dialysis and ultrafiltration. Western blotting analysis was done to ascertain whether the resultant purified product was, for example, positive for AGR2 and negative for PSA. Mass spectrometry (MS)-based proteomics was also used to profile the protein composition of the purified fraction to gauge proteomic complexity.

Methods

Ethical statement

Human prostate tissue specimens were obtained from ten radical prostatectomy patients operated by surgeons in the Department of Urology with informed and written consent from all participants as per the guidelines approved by the University of Washington Institutional Review Board. Frozen sections were histologically assessed to confirm that the specimens were enriched for cancer. The pathology characteristics of the collected tumors were as follows: 05-081CP G3+3; 05-020CP G3+4; 05-036CP G3+4; 05-125CP G3+4; 05-056CP G3+4; 05-131CP G3+4; 05-081CP G3+3; 03-169CP G3+3; 05-154CP G4+5; 04-176CP G4+3 (G = Gleason score).

Tissue specimens and tissue digestion

Each CP tissue sample weighing at least 0.1 g, was rinsed with Hanks balanced salt solution (HBSS) and minced for enzymatic digestion overnight at room temperature with

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0.2% collagenase type I (Invitrogen, Carlsbad, CA, USA) in serum-free RPMI1640 media on a magnetic stirrer. The resultant cell suspension was filtered with a 70-µm Falcon cell strainer, diluted with an equal volume of HBSS, and aspirated with an 18-gauge needle. The suspension was centrifuged at 1,000 rpm for 15 min to collect the supernatant. These media preparations were screened by Western blotting analysis to show low levels of TIMP1 and high levels of CD90 compared with normal tissue (NP) or benign prostatic hyperplasia (BPH) samples digested by collagenase (5). Tumor loses TIMP1 expression (8).

Purification of proteins in the 10–30 kDa range

To 22.5 mL of pooled CP samples, the media supernatant was made to contain 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 10 µM leupeptin, 1 µM pepstatin A, 1 mM EDTA, 1% Triton-X-100 and 15 mM β-mercaptoethanol. The sample was stirred for 1 h at 4 ℃. The pH was adjusted to 5.5, and the solution was stirred for another 1 h. The sample was then sonicated three times for 2 min each, and centrifuged at 40,000 rpm for 30 min. The supernatant was then passed through a 0.22-micron filter, and fractionated using a 30-kDa MWCO Millipore filter (Millipore, Billerica, MA, USA) at 1,000 rpm for 30 min. The flow-through was then fractionated using a 10-kDa MWCO Millipore filter at 4,000 rpm for 30 min. The resultant product after these filtrations was concentrated to 1 mL. One mL MES buffer (25 mM MES, pH 5.5, 150 mM NaCl, 1 mM EDTA, 50 mM β-mercaptoethanol) was then added. The retentate was centrifuged at 4,000 rpm to about 1 mL volume. This preparation was desalted by placing in 3-kDa dialysis tubing overnight against 1 mM MES, pH 5.5.

SDS-PAGE and Western blot analysis

Protein concentration of the purified CP sample was measured by Bradford Assay (BioRad, Hercules, CA, USA). For gel analysis, loading buffer containing 0.1 M DTT was added to the amount of purified CP sample containing ~60 µg protein, heated to 70 ℃ for 10 min, electrophoresed on 4–20% gradient SDS-polyacrylamide gel (BioRad), and electrotransferred to PVDF membrane (Hybond-P, Amersham). Blotting was ascertained by visualization of protein bands on the membrane by Ponçeau S (Sigma, St. Louis, MO, USA). Afterwards, the stain was completely removed by repeated washing with triple distilled water. The membrane was immersed in 5% nonfat dry milk-PBS-Tween for 30 min, and probed with AGR2 antibody (1:2,000; clone 1C3, Abnova, Taiwan), or PSA antibody (1:1,000; clone A67-B/E3, Santa Cruz Biotechnology, Dallas, TX, USA) for 60 min, followed by horseradish peroxidase conjugated anti-mouse IgG (1:5,000; Vector, Burlingame, CA, USA). After washing, the membrane was incubated with Luminol (Santa Cruz Biotechnology), and the immunoreactive bands were visualized using Biomax MR light film (Kodak, Rochester, NY, USA).

Proteomic analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The purified protein sample was further concentrated using Amicon Ultra-15 centrifugal filter MWCO3000 (Millipore). Protein concentration was determined by BCA Protein Assay (Thermo Scientific, Rockford, IL, USA). An aliquot of the concentrated preparation containing 10 µg protein was denatured in 50% trifluoroethanol at 60 ℃ for 2 h with gentle shaking (9), and then reduced with 2 mM DTT at 37 ℃ for 1 h. The sample was diluted 5-fold with 50 mM NH_4HCO_3 before digestion with trypsin (trypsin:protein 1:50 w/w) at 37 ℃ for 3 h. After speed-vac, the resultant tryptic peptides were reconstituted in 50 mM NH_4HCO_3 for LC-MS analysis.

LC-MS/MS was performed as previously described (10). Briefly, ~1 µg peptides were loaded onto a 65-cm-long, 75-µm-i.d. reversed-phase capillary column packed in house with 3 µm Jupiter C18 particles (Phenomenex, Torrance, CA, USA). The mobile phases consisted of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in acetonitrile). An exponential gradient starting with 100% of mobile phase A to 60% of mobile phase B over the course of 100 min was employed with a flow-rate of ~500 NL/min. Eluted peptides were ionized via a nanoelectrospray ionization interface manufactured in house and analyzed on an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The instrument was operated in data-dependent mode with m/z ranging from 400–2,000, in which a full MS scan was followed by ten MS/MS scans.

MS/MS data was analyzed by SEQUEST-based database searching against the Human International Protein Index (IPI) database (version 3.54). Searching parameters were: 3 Da tolerance for precursor ion masses and 1 Da for fragment ion masses with no enzyme restraint and a maximum of three missed tryptic cleavages. Filtering criteria (11) were applied for peptide identifications

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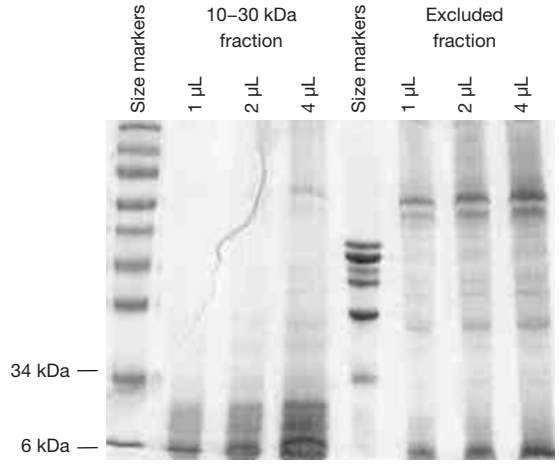

Figure 1 SDS PAGE-Coomassie analysis of purified 10–30 kDa fraction from prostate cancer (CP) tissue digest on a 4–20% gradient gel. For comparison, the excluded fraction was also analyzed.

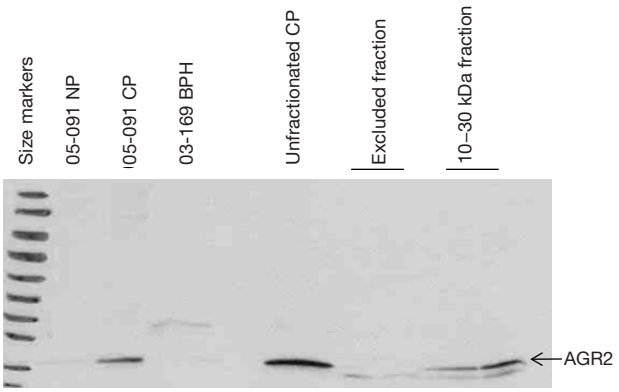

Figure 2 Western blot analysis of 10–30 kDa fraction from prostate cancer (CP) tissue digests. The membrane was probed with anti-anterior gradient 2 (AGR2) and the fraction was found to be AGR2 positive.

to achieve a <5% false discovery rate at the unique peptide level based on a reversed-database searching methodology (12).

Results

Purification of the 10–30 kDa fraction of CP tissue digests

Protein concentration of the purified CP sample was found to be at 3.2 mg/mL. *Figure 1* shows the SDS-PAGE/Coomassie profile of the purified 10–30 kDa fraction. There

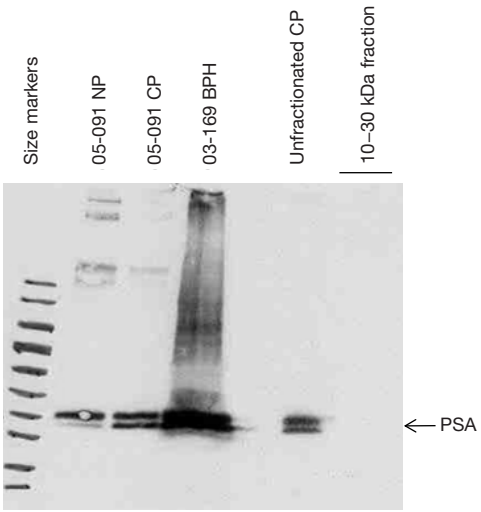

Figure 3 Western blot analysis of 10–30 kDa fraction from prostate cancer (CP) tissue digests. The membrane was probed with anti-prostate-specific antigen (PSA) and the fraction was found to be PSA negative.

was a good removal of proteins having molecular weights above 30 kDa by the purification scheme. At the same time, there were little 10–30 kDa proteins in the excluded fraction. The banding pattern also indicated minimal protein degradation.

Detection of AGR2 in the 10–30 kDa fraction

Figure 2 shows the presence of AGR2 (19 kDa) in the purified 10–30 kDa CP preparation and not in the excluded fraction. The Western blot also shows that this protein was not expressed by non-cancer (e.g., samples 05-091NP and 03-169BPH). There was an estimated loss of about 50% in the amount of AGR2 after purification (compare lanes labeled unfractionated CP and 10–30 kDa fraction, *Figure 2*). A fainter band lower than AGR2 may represent AGR3 (166 aa), which has 65% sequence homology with AGR2 (175 aa).

Absence of PSA in the 10–30 kDa fraction

The purified 10–30 kDa fraction was found to be negative for PSA as the unfractionated preparation was positive (*Figure 3*). On the blot, PSA was detected in matched 05-091CP and 05-091NP, as well as in 03-169BPH. Thus, the purification scheme was effective in eliminating an abundant protein species just 4 kDa larger than the upper size cutoff point.

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Table 1 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of the purified 10–30 kDa fraction. Anterior gradient 2 (AGR2) was identified by seven different tryptic peptides

Peptide sequence	Protein name	Charge state	Xcorr of max	DelCN20f max	PPM of min	Cleavage type	Peptide sequence of count
K.LAEQFVLLNLVYETTDK.H	IPI:IPI00007427.2	2	5.3495	0.4284	–9.38347	2	4
L.YAYEPADTALLLDNMK.K	IPI:IPI00007427.2	2	2.7295	0.1342	–3.50132	1	1
R.GWGDQLIWTQTYEEALYK.S	IPI:IPI00007427.2	2	4.1530	0.3242	–4.62505	2	2
R.IMFVDPSLTVR.A	IPI:IPI00007427.2	2	3.0653	0.1520	–6.01084	2	2
R.LYAYEPADTALLLDNMK.K	IPI:IPI00007427.2	2	4.7862	0.4556	–5.99701	2	3
Y.AYEPADTALLLDNMK.K	IPI:IPI00007427.2	2	3.6434	0.3572	–6.41511	1	2
T.VQEESEEEEVDETGVEVK.D	IPI:IPI00007471.2	2	6.8026	0.3457	–2.34021	1	2

Table 2 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) identification of the 12 most abundant proteins based on spectral counts

Protein name	Peptide count	Total count	Description	Locus	MW
IPI:IPI00302592.2	56	148	Isoform 2 of filamin-A	FLNA	280018
IPI:IPI00022434.4	19	81	Putative uncharacterized protein ALB	ALB	71704
IPI:IPI00216138.6	15	79	Transgelin	TAGLN	22611
IPI:IPI00025252.1	38	72	Protein disulfide-isomerase A3	PDIA3	56782
IPI:IPI00021812.2	48	67	Neuroblast differentiation-associated protein AHNAK	AHNAK	629101
IPI:IPI00022200.3	33	58	Isoform 1 of collagen alpha-3(VI) chain	COL6A3	343665
IPI:IPI00027230.3	25	51	Endoplasmin	HSP90B1	92469
IPI:IPI00289334.1	28	47	Isoform 1 of filamin-B	FLNB	278195
IPI:IPI00179330.6	5	42	Ubiquitin and ribosomal protein S27A precursor	UBC	17965
IPI:IPI00020501.1	20	41	Myosin-11	MYH11	227339
IPI:IPI00010796.1	22	36	Protein disulfide-isomerase	P4HB	57116
IPI:IPI00291175.7	16	32	Isoform 1 of vinculin	VCL	116722

MS analysis of purified CP fraction

The purified 10–30 kDa was analyzed using LC-MS/MS, and the proteins and peptides identified are included in *Table S1*. AGR2 was confidently identified by seven different peptides (*Table 1*). Previously, using unfractionated CP tissue digest media LC-MS/MS was unable to confidently detect AGR2 (unpublished) most likely due to its relatively low abundance or physical properties. On the other hand, the exogenously added (abundant) collagenase was not seen among the 252 proteins identified with two or more unique peptides. Although PSA was undetected by Western blotting, the protein was still identified by MS with seven spectral counts, but as evidence of a relatively low-abundance protein in the fraction. *Table 2* lists the top dozen abundant proteins identified by LC-MS/MS using spectral counts as a measure of abundance (13). Among them are several large cytoskeletal components FLNA, COL6A3, FLNB, MYH11, VCL, etc. In fact, only 51% (290/574) of the proteins had calculated molecular weights in the 10–30 kDa range (and 1.6% below 10 kDa). Many of the proteins belonged to the cytoplasmic proteome and were not necessarily secreted.

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Discussion

With protein biomarker discovery, reagents such as high quality antibodies are needed for clinical assays to measure these markers. Purified antigens are the optimal immunogens for monoclonal antibody production. However, the purification of multiple proteins from biosamples is extremely challenging and not cost-effective. The purification of 10–30 kDa protein species from pooled CP samples represents our attempt to obtain suitable materials for subsequent antibody production. Simple modifications can be done to the protocol to obtain any size fractions containing other informative biomarkers. Our results showed that this procedure is effective with exclusion of proteins outside the size range, as probed by Western blotting, while retaining the targeted proteins with acceptable losses. Importantly, the proteins are present in their native configuration with proper post-translational modifications intact. Antibodies resulting after mouse immunization would likely recognize specifically these protein analytes in human biospecimens. The laborious part would involve extensive screening of the hybridomas for the desired clones against particular antigens as the number of protein species in the 10–30 kDa CP is unknown (at least 574 based on the MS data). Removal of the abundant prostatic proteins ensures against generation of many unwanted antibodies. One drawback of this approach is that proteins in lower abundance would have fewer resultant clones, and screening for them would be hard. The first screen would compare immunoreactivity on frozen tissue sections of CP *vs.* NP. Those that show no reactivity on NP would be used for immunoprecipitation of CP digestion media or media of CP cell lines (7), and the protein(s) captured would be identified by MS. Most likely, these antibodies will not work well on Western blotting.

Using the more sensitive MS analysis, the 10–30 kDa fraction was shown to still contain nearly 50% protein species with molecular weights outside the selected range. These could result from degradation during the overnight tissue digestion. Frequently, some tumor tissue samples may contain necrotic areas not visible grossly. The cytoplasmic proteins then, most likely, resulted from breakdown of cells. This creates a potentially large obstacle for our approach as cytoskeletal proteins are some of the most immunogenic molecules.

An alternative to using CP tissue media is media supernatant of cultured cancer cell lines (and cancer-associated stromal cells) as well as that of xenograft tissue digest. Perhaps then, the problem of cell breakdown could be minimized. While no single cell line produces all the proteins found in CP (3), a panel of available cell lines and xenografts could produce most of these proteins. For example, AGR2 is made by CL1, CRISP3 by LuCaP 35, CEACAM5, BCMP11 by LuCaP 49 [results from dataset queries (4)]. In this way, we are not limited by the availability of ample material for immunization.

Our protein purification scheme has been optimized by including relatively fewer and simpler steps to obtain a good yield. It is convenient and efficient. The more common approach of using recombinant proteins as immunogens suffers from the lack of proper configuration when made in a bacterial or yeast host system, or low yield and high cost when made in mammalian cultured cells. The recombinantly produced proteins would still require purification. Nevertheless, useful monoclonal antibodies against AGR2 have been obtained by us using bacterially made recombinant AGR2. The selected antibody clones were able to recognize the recombinant protein and native AGR2 in CP (and not NP) digestion media, media supernatant of cultured CL1 cells, and in urine of patients (7). This could be due to the relatively small size of AGR2 and its lack of extensive glycosylation (small molecular weight difference between calculated size from amino acid composition and size estimated in SDS-PAGE).

The presence of stromal cell-derived proteins in the CP fraction shows that these could also be used for tumor detection. CD90 was previously detected by global proteomics in unfractionated CP tissue media (6,8). CD90 expression is increased in the tumor stroma compared with NP stroma, and can be measured in urine of patients (6). In addition, a number of other secreted CP stromal proteins have been identified. Thus, a CP tissue sample would contain proteins derived from both the cancer epithelial component and the tumor stromal component.

Conclusions

This work reports a method to selectively isolate native proteins in a particular molecular weight range using tissue digestion media. This process essentially lowers the proteomic complexity of the biosample that could be used to identify biomarker candidates, and for mouse immunization.

Acknowledgements

The authors thank Charles Luo and Michael Regnier in the

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Department of Bioengineering, Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, USA, for their expertise in developing the purification protocol. Proteomics experiments were performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the DoE and located at Pacific Northwest National Laboratory, which is operated by Battelle Memorial Institute for the DoE under Contract DE-AC05-76RL0 1830.
Funding: This work was supported by NCI-EDRN grant CA111244 and NIH P41GM103493.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by the University of Washington Institutional Review Board and written informed consent was obtained from all patients.

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Cite this article as: Ahmad R, Nicora CD, Shukla AK, Smith RD, Qian WJ, Liu AY. An efficient method for native protein purification in the selected range from prostate cancer tissue digests. Chin Clin Oncol 2016;5(6):78. doi: 10.21037/cco.2016.12.03

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Supplementary

Table S1 List of identified proteins. Spectral count indicates the number of MS/MS spectra identifying a given protein

Protein name	Unique peptides	Spectral count	Protein description	Gene symbol	MW
IPI:IPI00302592.2	56	148	Isoform 2 of filamin-A	FLNA	280018
IPI:IPI00022434.4	19	81	Putative uncharacterized protein ALB	ALB	71704
IPI:IPI00216138.6	15	79	Transgelin	TAGLN	22611
IPI:IPI00025252.1	38	72	Protein disulfide-isomerase A3.	PDIA3	56782
IPI:IPI00021812.2	48	67	Neuroblast differentiation-associated protein AHNAK	AHNAK	629101
IPI:IPI00022200.3	33	58	Isoform 1 of collagen alpha-3(VI) chain	COL6A3	343665
IPI:IPI00027230.3	25	51	Endoplasmin	HSP90B1	92469
IPI:IPI00289334.1	28	47	Isoform 1 of filamin-B	FLNB	278195
IPI:IPI00179330.6	5	42	Ubiquitin and ribosomal protein S27A precursor	UBC	17965
IPI:IPI00020501.1	20	41	Myosin-11	MYH11	227339
IPI:IPI00010796.1	22	36	Protein disulfide-isomerase	P4HB	57116
IPI:IPI00291175.7	16	32	Isoform 1 of vinculin	VCL	116722
IPI:IPI00002459.4	16	30	Annexin VI isoform 2	ANXA6	75277
IPI:IPI00220327.3	16	29	Keratin, type II cytoskeletal 1	KRT1	66018
IPI:IPI00329801.12	13	29	Annexin A5	ANXA5	35937
IPI:IPI00020984.2	16	28	CDNA FLJ55574, highly similar to calnexin	CANX	71503
IPI:IPI00024284.5	16	28	Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	468798
IPI:IPI00218918.5	12	27	Annexin A1	ANXA1	38714
IPI:IPI00076042.2	17	25	Short heat shock protein 60 HSP60S2	–	27096
IPI:IPI00784154.1	14	25	60 kDa heat shock protein, mitochondrial	HSPD1	61055
IPI:IPI00003362.2	15	24	HSPA5 protein	HSPA5	72422
IPI:IPI00178352.5	15	23	Isoform 1 of filamin-C	FLNC	290959
IPI:IPI00019359.3	14	22	Keratin, type I cytoskeletal 9	KRT9	62129
IPI:IPI00000230.6	10	20	Tropomyosin 1 alpha chain isoform 2	TPM1	32678
IPI:IPI00003865.1	12	19	Isoform 1 of heat shock cognate 71 kDa protein	HSPA8	70898
IPI:IPI00014516.1	9	19	Isoform 1 of caldesmon	CALD1	93250
IPI:IPI00025465.2	11	18	CDNA FLJ59205, highly similar to mimecan	OGN	40553
IPI:IPI00180240.2	9	18	Thymosin beta-4-like protein 3	TMSL3	5063
IPI:IPI00216691.5	4	18	Profilin-1	PFN1	15054
IPI:IPI00026314.1	14	17	Isoform 1 of gelsolin	GSN	85698
IPI:IPI00019502.3	12	17	Isoform 1 of myosin-9	MYH9	226532
IPI:IPI00220740.1	11	17	Isoform 2 of nucleophosmin	NPM1	29465
IPI:IPI00382470.3	11	17	Heat shock protein 90 kDa alpha (cytosolic), class A member 1 isoform 1	HSP90AA1	98161
IPI:IPI00453473.6	10	17	Histone H4	HIST4H4	11367

Table S1 (*continued*)

Table S1 (*continued*)

Protein name	Unique peptides	Spectral count	Protein description	Gene symbol	MW
IPI:IPI00465248.5	10	17	Isoform alpha-enolase of alpha-enolase	ENO1	47169
IPI:IPI00299571.5	10	17	Isoform 2 of protein disulfide-isomerase A6	PDIA6	53901
IPI:IPI00219301.7	9	17	Myristoylated alanine-rich c-kinase substrate	MARCKS	31555
IPI:IPI00028888.1	9	17	Isoform 1 of heterogeneous nuclear ribonucleoprotein D0	HNRNPD	38434
IPI:IPI00030929.4	7	17	Myosin regulatory light chain 9 isoform B	MYL9	13866
IPI:IPI00020599.1	10	16	Calreticulin	CALR	48142
IPI:IPI00219446.5	7	16	Phosphatidylethanolamine-binding protein 1	PEBP1	21057
IPI:IPI00075248.11	6	16	Calmodulin	CALM3	16838
IPI:IPI00419585.9	6	16	Peptidyl-prolyl CIS-trans isomerase A	PPIA	18012
IPI:IPI00220766.5	10	15	Lactoylglutathione lyase	GLO1	20778
IPI:IPI00013508.5	10	15	Alpha-actinin-1	ACTN1	103058
IPI:IPI00010182.4	7	14	Isoform a 1 of acyl-coa-binding protein	DBI	10044
IPI:IPI00009865.2	7	14	Keratin, type I cytoskeletal 10	KRT10	59511
IPI:IPI00007427.2	6	14	AGR2	AGR2	22238
IPI:IPI00005102.3	9	13	Isoform 1 of spermine synthase	SMS	41268
IPI:IPI00176903.2	8	13	Isoform 1 of polymerase i and transcript release factor	PTRF	43476
IPI:IPI00328587.4	7	13	42 kDa protein	–	42342
IPI:IPI00335168.9	6	13	Isoform non-muscle of myosin light polypeptide 6	MYL6B	16930
IPI:IPI00021405.3	6	13	Isoform a of lamin-A/C	LMNA	74139
IPI:IPI00299024.9	8	12	Brain acid soluble protein 1	BASP1	22693
IPI:IPI00024095.3	7	12	Annexin A3	ANXA3	36375
IPI:IPI00334627.3	7	12	Putative annexin A2-like protein	ANXA2P2	38659
IPI:IPI00013991.1	6	12	Isoform 1 of tropomyosin beta chain	TPM2	32851
IPI:IPI00000877.1	8	11	Hypoxia up-regulated protein 1	HYOU1	111335
IPI:IPI00026154.3	7	11	CDNA FLJ59211, highly similar to glucosidase 2 subunit beta	PRKCSH	60134
IPI:IPI00021828.1	5	11	Cystatin-B	CSTB	11140
IPI:IPI00221255.1	5	11	Isoform 2 of myosin light chain kinase, smooth muscle	MYLK	203128
IPI:IPI00295542.5	8	10	Nucleobindin-1	NUCB1	53879
IPI:IPI00216057.5	7	10	Sorbitol dehydrogenase	SORD	38297
IPI:IPI00003269.1	5	10	Beta-actin-like protein 2	ACTBL2	42003
IPI:IPI00216298.6	4	10	Thioredoxin	TXN	11737
IPI:IPI00410714.5	4	10	Hemoglobin subunit alpha	HBA2	15258
IPI:IPI00550363.3	4	10	Transgelin-2	TAGLN2	22391

Table S1 (*continued*)

Table S1 (*continued*)

Protein name	Unique peptides	Spectral count	Protein description	Gene symbol	MW
IPI:IPI00008603.1	4	10	Actin, aortic smooth muscle	ACTA2	42009
IPI:IPI00220362.5	7	9	10 kDa heat shock protein, mitochondrial	HSPE1	10932
IPI:IPI00073454.2	7	9	ISoform 2C2A' of collagen alpha-2(VI) chain	COL6A2	87280
IPI:IPI00026272.2	6	9	Histone H2A type 1-B/E	HIST1H2AE	14135
IPI:IPI00022418.1	6	9	Isoform 1 of fibronectin	FN1	262607
IPI:IPI00554788.5	5	9	Keratin, type I cytoskeletal 18	KRT18	48058
IPI:IPI00176193.6	5	9	Isoform 1 of collagen alpha-1(XIV) chain	COL14A1	193515
IPI:IPI00026964.2	6	8	Cytochrome b-c1 complex subunit Rieske, mitochondrial	UQCRFS1	29668
IPI:IPI00009123.1	6	8	Nucleobindin-2	NUCB2	50223
IPI:IPI00003935.6	5	8	Histone H2B type 2-E	HIST2H2BE	13920
IPI:IPI00654755.3	5	8	Hemoglobin subunit beta	HBB	15998
IPI:IPI00171411.4	5	8	Golgi membrane protein 1	GOLM1	46273
IPI:IPI00215948.4	5	8	Isoform 1 of catenin alpha-1	CTNNA1	100071
IPI:IPI00021785.2	4	8	Cytochrome c oxidase subunit 5B, mitochondrial	COX5B	13696
IPI:IPI00021263.3	4	8	14-3-3 protein zeta/delta	YWHAZ	27745
IPI:IPI00014230.1	4	8	Complement component 1 Q subcomponent-binding protein, mitochondrial	C1QBP	31362
IPI:IPI00216592.2	4	8	Isoform C1 of heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	32338
IPI:IPI00177728.3	4	8	Cytosolic non-specific dipeptidase	CNDP2	52878
IPI:IPI00000875.7	4	8	CDNA FLJ56389, highly similar to elongation factor 1-gamma	EEF1G	56150
IPI:IPI00015842.1	6	7	Reticulocalbin-1	RCN1	38890
IPI:IPI00440493.2	6	7	ATP synthase subunit alpha, mitochondrial	ATP5A1	59751
IPI:IPI00006482.1	5	7	Isoform long of sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	112896
IPI:IPI00646304.4	4	7	Peptidyl-prolyl cis-trans isomerase B	PPIB	23743
IPI:IPI00010858.1	4	7	Prostate-specific antigen	KLK3	28741
IPI:IPI00643920.3	4	7	CDNA FLJ54957, highly similar to transketolase	TKT	68742
IPI:IPI00218803.3	4	7	Isoform b of fibulin-1	FBLN1	77139
IPI:IPI00013895.1	3	7	Protein S100-A11	S100A11	11740
IPI:IPI00008219.1	3	7	UV excision repair protein RAD23 homolog A	RAD23A	39609
IPI:IPI00294578.1	2	7	Isoform 1 of protein-glutamine gamma-glutamyltransferase 2	TGM2	77329
IPI:IPI00025849.1	5	6	Acidic leucine-rich nuclear phosphoprotein 32 family member A	ANP32A	28585
IPI:IPI00166768.3	5	6	TUBA1C protein	TUBA1C	36743

Table S1 (*continued*)

Table S1 (*continued*)

Protein name	Unique peptides	Spectral count	Protein description	Gene symbol	MW
IPI:IPI00025318.1	4	6	SH3 domain-binding glutamic acid-rich-like protein	SH3BGRL	12774
IPI:IPI00000816.1	4	6	14-3-3 protein epsilon	YWHAE	29174
IPI:IPI00008223.3	4	6	UV excision repair protein RAD23 homolog B	RAD23B	43171
IPI:IPI00305383.1	4	6	Cytochrome b-c1 complex subunit 2, mitochondrial	UQCRC2	48443
IPI:IPI00012074.3	4	6	Heterogeneous nuclear ribonucleoprotein R	HNRNPR	70943
IPI:IPI00295414.7	4	6	Collagen alpha-1(XV) chain	COL15A1	141720
IPI:IPI00031008.1	4	6	Isoform 1 of tenascin	TNC	240866
IPI:IPI00298994.6	4	6	Talin-1	TLN1	269767
IPI:IPI00014898.3	4	6	Isoform 1 of plectin-1	PLEC1	531791
IPI:IPI00017704.3	3	6	Coactosin-like protein	COTL1	15945
IPI:IPI00442073.5	3	6	Cysteine and glycine-rich protein 1	CSRP1	20567
IPI:IPI00023048.4	3	6	Elongation factor 1-delta	EEF1D	31122
IPI:IPI00479877.4	3	6	Aldehyde dehydrogenase 9A1	ALDH9A1	56292
IPI:IPI00219330.2	3	6	Isoform 5 of interleukin enhancer-binding factor 3	ILF3	74607
IPI:IPI00026781.2	3	6	Fatty acid synthase	FASN	273400
IPI:IPI00301058.5	2	6	Vasodilator-stimulated phosphoprotein	VASP	39830
IPI:IPI00028635.4	2	6	Ribophorin II	RPN2	71217
IPI:IPI00165579.6	5	5	CNDP dipeptidase 2 (metallopeptidase M20 family), isoform CRA_B	CNDP2	43833
IPI:IPI00007765.5	5	5	Stress-70 protein, mitochondrial	HSPA9	73680
IPI:IPI00171438.2	4	5	Thioredoxin domain-containing protein 5	TXNDC5	47629
IPI:IPI00183526.6	4	5	NCL protein	NCL	50951
IPI:IPI00003021.1	4	5	Sodium/potassium-transporting atpase subunit alpha-2	ATP1A2	112265
IPI:IPI00005614.6	4	5	Isoform long of spectrin beta chain, brain 1	SPTBN1	274609
IPI:IPI00012011.6	3	5	Cofilin-1	CFL1	18502
IPI:IPI00003815.3	3	5	RHO GDP-dissociation inhibitor 1	ARHGDIA	23207
IPI:IPI00028055.4	3	5	Transmembrane EMP24 domain-containing protein 10	TMED10	24976
IPI:IPI00383071.1	3	5	Triosephosphate isomerase (fragment)	RCTPI1	26943
IPI:IPI00793199.1	3	5	Annexin IV	ANXA4	36085
IPI:IPI00289983.3	3	5	Isoform 2 of prostatic acid phosphatase	ACPP	48336
IPI:IPI00017870.1	3	5	Keratin-8-like protein 1	–	55152
IPI:IPI00291136.4	3	5	Collagen alpha-1(VI) chain	COL6A1	108529
IPI:IPI00619951.3	2	5	Tumor protein D52 isoform 2	TPD52	22477
IPI:IPI00306543.4	2	5	Growth/differentiation factor 15	GDF15	34154
IPI:IPI00216171.3	2	5	Gamma-enolase	ENO2	47269

Table S1 (*continued*)

Table S1 (*continued*)

Protein name	Unique peptides	Spectral count	Protein description	Gene symbol	MW
IPI:IPI00027165.3	2	5	Isoform R-type of pyruvate kinase isozymes R/L	PKLR	61830
IPI:IPI00018140.3	2	5	Isoform 1 of heterogeneous nuclear ribonucleoprotein Q	SYNCRIP	69603
IPI:IPI00027463.1	4	4	Protein S100-A6	S100A6	10180
IPI:IPI00220487.4	4	4	Isoform 1 of ATP synthase subunit D, mitochondrial	ATP5H	18491
IPI:IPI00220278.5	4	4	Myosin regulatory light polypeptide 9	MYL9	19827
IPI:IPI00020332.1	4	4	Arginase-2, mitochondrial	ARG2	38578
IPI:IPI00413451.1	4	4	Putative uncharacterized protein DKFZP686I04222	SERPINB6	46370
IPI:IPI00007752.1	4	4	Tubulin beta-2C chain	TUBB2C	49831
IPI:IPI00303476.1	4	4	ATP synthase subunit beta, mitochondrial	ATP5B	56560
IPI:IPI00218848.5	3	4	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit E	ATP5I	8308
IPI:IPI00020008.1	3	4	NEDD8	NEDD8	9072
IPI:IPI00216461.4	3	4	Acylphosphatase-2	ACYP2	11140
IPI:IPI00217236.4	3	4	Tubulin-specific chaperone A	TBCA	12855
IPI:IPI00385149.1	3	4	Putative uncharacterized protein	PTMA	14577
IPI:IPI00219684.3	3	4	Fatty acid-binding protein, heart	FABP3	14858
IPI:IPI00022145.6	3	4	Isoform 1 of nuclear ubiquitous casein and cyclin-dependent kinases substrate	NUCKS1	27296
IPI:IPI00291006.2	3	4	Malate dehydrogenase, mitochondrial	MDH2	35503
IPI:IPI00031812.3	3	4	Nuclease-sensitive element-binding protein 1	YBX1	35924
IPI:IPI00217966.7	3	4	Isoform 1 of L-lactate dehydrogenase A chain	LDHA	36689
IPI:IPI00059366.4	3	4	H2A histone family, member Y isoform 2	H2AFY	39489
IPI:IPI00009867.3	3	4	Keratin, type II cytoskeletal 5	KRT5	62378
IPI:IPI00141318.2	3	4	Isoform 1 of cytoskeleton-associated protein 4	CKAP4	66022
IPI:IPI00018931.6	3	4	Vacuolar protein sorting-associated protein 35	VPS35	91707
IPI:IPI00295857.7	3	4	Isoform 1 of coatomer subunit alpha	COPA	138346
IPI:IPI00448752.1	2	4	Isoform 5 of heterochromatin protein 1-binding protein 3	HP1BP3	14594
IPI:IPI00033494.3	2	4	Myosin regulatory light chain MRLC2	MRLC2	19779
IPI:IPI00306280.3	2	4	Density-regulated protein	DENR	22285
IPI:IPI00024572.3	2	4	Aspartate beta-hydroxylase isoform E	ASPH	25560
IPI:IPI00219217.3	2	4	L-lactate dehydrogenase B chain	LDHB	36638
IPI:IPI00021439.1	2	4	Actin, cytoplasmic 1	ACTB	41737
IPI:IPI00021888.5	2	4	Zinc transporter SLC39A7	SLC39A7	50118
IPI:IPI00012442.1	2	4	Ras GTPase-activating protein-binding protein 1	G3BP1	52164
IPI:IPI00010471.5	2	4	Plastin-2	LCP1	70289

Table S1 (*continued*)

Table S1 (*continued*)

Protein name	Unique peptides	Spectral count	Protein description	Gene symbol	MW
IPI:IPI00414676.6	2	4	Heat shock protein HSP 90-beta	HSP90AB1	83264
IPI:IPI00166487.3	2	4	Isoform 3 of protein transport protein SEC16A	SEC16A	231252
IPI:IPI00465315.6	3	3	Cytochrome C	CYCS	11749
IPI:IPI00015029.1	3	3	Prostaglandin E synthase 3	PTGES3	18697
IPI:IPI00296259.4	3	3	Isoform 1 of transmembrane EMP24 domain-containing protein 4	TMED4	25943
IPI:IPI00219018.7	3	3	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	36053
IPI:IPI00027107.5	3	3	Tu translation elongation factor, mitochondrial precursor	TUFM	49875
IPI:IPI00297084.7	3	3	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase precursor	DDOST	50801
IPI:IPI00002406.2	3	3	Lutheran blood group glycoprotein	BCAM	67405
IPI:IPI00644712.4	3	3	ATP-dependent DNA helicase 2 subunit 1	XRCC6	69843
IPI:IPI00304925.5	3	3	Heat shock 70 kDa protein 1	HSPA1B	70052
IPI:IPI00031522.2	3	3	Trifunctional enzyme subunit alpha, mitochondrial	HADHA	83000
IPI:IPI00007960.4	3	3	Isoform 1 of periostin	POSTN	93314
IPI:IPI00020301.1	2	3	Isoform 2 of gamma-glutamylcyclotransferase	GGCT	12943
IPI:IPI00412545.4	2	3	Putative uncharacterized protein DKFZP781K1356	NDUFA5	13563
IPI:IPI00413778.7	2	3	Peptidyl-prolyl CIS-trans isomerase	FKBP1A	15689
IPI:IPI00032293.1	2	3	Cystatin-C	CST3	15799
IPI:IPI00025086.4	2	3	Cytochrome c oxidase subunit 5A, mitochondrial	COX5A	16762
IPI:IPI00298547.3	2	3	Protein DJ-1	PARK7	19891
IPI:IPI00016608.1	2	3	Transmembrane EMP24 domain-containing protein 2	TMED2	22761
IPI:IPI00419424.3	2	3	IGKV1-5 protein	IGKV1-5	26234
IPI:IPI00328748.4	2	3	Protein ARMET	ARMET	26903
IPI:IPI00451401.3	2	3	Isoform 2 of triosephosphate isomerase	TPI1	27126
IPI:IPI00170692.4	2	3	Vesicle-associated membrane protein-associated protein A	VAPA	27893
IPI:IPI00010779.4	2	3	Isoform 1 of tropomyosin alpha-4 chain	TPM4	28522
IPI:IPI00384016.1	2	3	Full-length CDNA 5-prime end of clone CS0DJ009YL13 of T cells (Jurkat cell line) of homo sapiens (fragment)	DLST	29625
IPI:IPI00305692.5	2	3	Thioredoxin-like protein 1	TXNL1	32251
IPI:IPI00001754.1	2	3	Junctional adhesion molecule A	F11R	32583
IPI:IPI00005792.2	2	3	Isoform 1 of polyadenylate-binding protein 2	PABPN1	32749
IPI:IPI00291005.8	2	3	Malate dehydrogenase, cytoplasmic	MDH1	36426
IPI:IPI00021728.3	2	3	Eukaryotic translation initiation factor 2 subunit 2	EIF2S2	38388

Table S1 (*continued*)

Table S1 (*continued*)

Protein name	Unique peptides	Spectral count	Protein description	Gene symbol	MW
IPI:IPI00418169.3	2	3	Annexin A2 isoform 1	ANXA2	40411
IPI:IPI00032826.1	2	3	HSC70-interacting protein	ST13	41332
IPI:IPI00063234.1	2	3	Protein kinase, camp-dependent, regulatory, type II, alpha, isoform CRA_B	PRKAR2A	43067
IPI:IPI00328319.8	2	3	Histone-binding protein RBBP4	RBBP4	47656
IPI:IPI00465054.2	2	3	Putative uncharacterized protein DKFZP686C1054	THUMPD1	48549
IPI:IPI00337494.7	2	3	Isoform 1 of calcium-binding mitochondrial carrier protein SCAMC-1	SLC25A24	53354
IPI:IPI00554648.3	2	3	Keratin, type II cytoskeletal 8	KRT8	53704
IPI:IPI00014852.2	2	3	Isoform 1 of phosphoglucomutase-like protein 5	PGM5	62225
IPI:IPI00005859.4	2	3	CDNA FLJ60809, highly similar to homo sapiens cytokeratin type II (K6HF), MRNA	KRT75	65361
IPI:IPI00021304.1	2	3	Keratin, typE II cytoskeletal 2 epidermal	KRT2	65865
IPI:IPI00220857.3	2	3	Isoform 2 of calpastatin	CAST	75304
IPI:IPI00000856.7	2	3	Isoform 1 of fermitin family homolog 2	FERMT2	77861
IPI:IPI00002606.5	2	3	Isoform 1 of adseverin	SCIN	80489
IPI:IPI00604664.5	2	3	CDNA FLJ55978, highly similar to NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	NDUFS1	80997
IPI:IPI00017855.1	2	3	Aconitate hydratase, mitochondrial	ACO2	85425
IPI:IPI00002491.3	2	3	Isoform 9 of sorbin and SH3 domain-containing protein 1	SORBS1	87183
IPI:IPI00002966.2	2	3	Heat shock 70 kDa protein 4	HSPA4	94331
IPI:IPI00299608.3	2	3	Isoform 1 of 26s proteasome non-ATPase regulatory subunit 1	PSMD1	105836
IPI:IPI00011454.1	2	3	Isoform 2 of neutral alpha-glucosidase AB	GANAB	109438
IPI:IPI00221384.2	2	3	Isoform 2 of collagen alpha-1(XII) chain	COL12A1	205491
IPI:IPI00744706.2	2	3	CDNA FLJ61399, highly similar to spectrin alpha chain, brain	SPTAN1	282147
IPI:IPI00328113.2	2	3	Fibrillin-1	FBN1	312312
IPI:IPI00299244.3	2	2	G antigen family c member 1	PAGE4	11153
IPI:IPI00221222.7	2	2	Activated rna polymerase II transcriptional coactivator P15	SUB1	14395
IPI:IPI00007797.3	2	2	Fatty acid-binding protein, epidermal	FABP5L7	15164
IPI:IPI00455479.1	2	2	Similar to insulinoma protein	LOC440733	17074
IPI:IPI00056357.3	2	2	Upf0556 protein C19ORF10	C19orf10	18795
IPI:IPI00641181.5	2	2	Marcks-related protein	MARCKSL1	19529
IPI:IPI00465431.8	2	2	Galectin-3	LGALS3	26152
IPI:IPI00011253.3	2	2	40S ribosomal protein S3	RPS3	26688

Table S1 (*continued*)

Table S1 (*continued*)

Protein name	Unique peptides	Spectral count	Protein description	Gene symbol	MW
IPI:IPI00032830.2	2	2	Isoform 1 of oligoribonuclease, mitochondrial (fragment)	REXO2	26833
IPI:IPI00017726.1	2	2	Isoform 1 of 3-hydroxyacyl-COA dehydrogenase type-2	HSD17B10	26923
IPI:IPI00056334.5	2	2	Protein kinase C delta-binding protein	PRKCDBP	27626
IPI:IPI00018146.1	2	2	14-3-3 protein theta	YWHAQ	27764
IPI:IPI00027569.1	2	2	Heterogeneous nuclear ribonucleoprotein C-like 1	HNRNPCL1	32142
IPI:IPI00072377.1	2	2	Isoform 1 of protein set	SET	33489
IPI:IPI00073772.5	2	2	Fructose-1,6-bisphosphatase 1	FBP1	36814
IPI:IPI00045396.1	2	2	Isoform 2 of calumenin	CALU	37135
IPI:IPI00007940.6	2	2	ER lipid raft associated 1	ERLIN1	39171
IPI:IPI00169383.3	2	2	Phosphoglycerate kinase 1	PGK1	44615
IPI:IPI00009030.1	2	2	Isoform lamp-2A of lysosome-associated membrane glycoprotein 2	LAMP2	44961
IPI:IPI00003881.5	2	2	Heterogeneous nuclear ribonucleoprotein F	HNRNPF	45672
IPI:IPI00401264.5	2	2	Thioredoxin domain-containing protein 4	TXNDC4	46971
IPI:IPI00027438.2	2	2	Flotillin-1	FLOT1	47355
IPI:IPI00025366.4	2	2	Citrate synthase, mitochondrial	CS	51712
IPI:IPI00008274.7	2	2	Isoform 1 of adenylyl cyclase-associated protein 1	CAP1	51855
IPI:IPI00031556.7	2	2	Isoform 1 of splicing factor U2AF 65 kDa subunit	U2AF2	53501
IPI:IPI00414320.1	2	2	Annexin A11	ANXA11	54390
IPI:IPI00026663.2	2	2	Aldehyde dehydrogenase family 1 member A3	ALDH1A3	56108
IPI:IPI00026530.4	2	2	Protein ergic-53	LMAN1	57549
IPI:IPI00301277.1	2	2	Heat shock 70 kDa protein 1L	HSPA1L	70375
IPI:IPI00009960.6	2	2	Isoform 1 of mitochondrial inner membrane protein	IMMT	83678
IPI:IPI00783982.1	2	2	Coatomer subunit gamma	COPG	97718
IPI:IPI00025846.2	2	2	CDNA FLJ55716, highly similar to desmocollin-2	DSC2	101083
IPI:IPI00028931.2	2	2	Desmoglein-2	DSG2	122294
IPI:IPI00455473.2	2	2	Isoform 1 of melanoma inhibitory activity protein 3	MIA3	213702